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Biological Characterization and *In Vitro* Effects of Human Concentrated Growth Factor Preparation: An Innovative Approach to Tissue Regeneration

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Summary

Scientific background: Platelet concentrates are nowadays widely applied in different clinical fields to improve soft tissue and bone regeneration. "Concentrated Growth Factors" (CGF) is a new generation of platelet concentrate products, which exhibits an interesting clinical and biotechnological application potential.

Aim of the study: The aim of this study is to assess the biological rationale for the use of CGF, by evaluating blood cell localization, the *in vitro* cumulative release of seven growth factors (PDGF-AB, VEGF, TNF-α, TGF-β1, BDNF, BMP-2 and IGF-1), its *in vitro* effects on cell proliferation and its mechanical behavior.

Methods: CGFs were obtained from volunteer donors. Blood cell localization was evaluated after properly morphological staining and immunohistochemistry. The amount of growth factors release was measured at 5 hours, 1, 3, 6, 7 and 8 days, using ELISA assay. Cells were cultured with and without CGF and their proliferation were evaluated after 72 hours, performing the quantification of Ki-67, using flow cytometry (FACS). The mechanical response of CGF under compression was also attempted.

Results: The results showed that platelets and leukocytes were found in a very thin space called "buffy coat", localized between the white and red part of CGF. Each growth factor evaluated, had a specific kinetic release with a great variability among subjects. The *in vitro* cell proliferation was stimulated. CGF showed an "apparent plasticity" and its mechanical response was influenced by fibrin network structure.

Conclusion: These findings support the CGF's clinical use and will allow us to better understand and improve the clinical outcomes.

Keywords: Concentrated Growth Factor (CGF); Human; Regeneration; Blood; Platelets

Abbreviations: A_0: Cross-Section; BDNF: Brain Derived Growth Factor; BMP: Bone Morphogenetic Proteins: CGF: Concentrated Growth Factor: EGM: Endothelial Growth Medium:EBM-2: Endothelial Basal Medium 2; F: Load; FGM: Fibroblast Growth Medium; FBM: Fibroblast Basal Medium; H: Height; H_0: Initial Height; HE: Hematoxylin And Eosin Staining; HOB: Human Osteoblasts; HRP: Horseradish Peroxidase; HUVEC: Human Umbilical Vein Endothelial Cells; IGF: Insulin-Like Growth Factor; MGG: May-Grünwald-Giemsa Stain; NHDF: Normal Human Dermal Fibroblasts; OBM: Osteoblast Basal Medium; OGM: Osteoblast Growth Medium; PDGF: Platelet Derived Growth Factor; PPP: Platelet Poor Plasma; PRF: Choukroun's Platelet Rich Fibrin; PRP: Platelet-Rich Plasma Preparations; RBC: Red Blood Cells; RP: Red Part; WP: White Part; BC: Buffy Coat; SEM: Scanning Electron Microscopy; TGF: Transforming Growth Factor; TNF: Tumor Necrosis Factor; V: Volunteer; VEGF: Vascular Endothelial Growth Factor; WB: Whole Blood; Σ: Nominal Stress; E: Nominal Strain.

Introduction

"Concentrated Growth Factors" (CGF) is one of the several types of platelet-rich plasma preparations (PRP) developed to date [1] and represents a new generation of PRP, which exhibits an interesting clinical and biotechnological potential. PRPs are defined as preparations with a high concentration of platelets in a small volume of plasma [2], containing also growth factors, leukocytes and fibrin matrix [3-7]. The strength of PRP is in the autologous technology to obtain growth factors [8,9] released from platelets in order to promote tissue regeneration [10]. These 100% autologous preparations not only enhance tissue healing, but also improve the clinical outcomes of various surgical procedures, reducing complications such as pain, inflammation and morbidity [11].

In regenerative medicine, three factors are important to optimize the regenerative process: scaffold (biological, natural or synthetic), growth factors and autologous cells. All of these are present in CGF developed by Sacco in 2006. CGF, in its solid form, is obtained by centrifuging of blood samples collected in vacuum tubes, using a special centrifuge device (Medifuge, Silfradent srl, Italy), similar to Choukroun's platelet rich fibrin (PRF) [12-17]. CGF technology has an interesting

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characteristic: i.e. the easy and speedy one-step preparation of a larger, denser and richer in growth factors fibrin matrix than the other solid PRPs. Using Scanning Electron Microscopy (SEM) analysis, Rodella and colleagues [18] showed the presence of a fibrin network constituted by thin and thick fibrillar elements with multiple platelets trapped among the fibrin network. We can therefore say that it represents an optimal autologous scaffold. Nevertheless, even if mechanical characterization of some PRPs has been reported in Literature to better understand their potential applications [19,20], no data are available about CGF.

The growth factors, are a class of natural biological mediators that regulate key cellular events in tissue repair, including cell proliferation, differentiation and extracellular matrix synthesis. Platelet activation and degranulation causes the release of a large number of biological factors, including Platelet Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF), Insulin-like Growth Factor (IGF), Transforming Growth Factor (TGF), Tumor Necrosis Factor (TNF), Brain Derived Growth Factor (BDNF) and Bone Morphogenetic Proteins (BMP) [21-24]. The presence in CGF of TGF-β1and VEGF has been reported by our research group [18]. Previous studies have demonstrated that local application of growth factors alone or mixed with bone allograft increased bone growth, by accelerating healing of soft tissues and facilitating periodontal ligament repair in both animal and human studies [25].

The presence of autologous cells such as platelets and leukocytes, including the CD34 positive cells, have been described [18]. Increasing evidences point to the role of circulating CD34 positive cells [26,27] in vascular maintenance, neovascularization and angiogenesis [28,29]. The presence of these cells in PRP preparations, promotes tissue regrowth [30].

CGF seems to possess a good regenerative capacity and versatility. For example, it has been reported that CGF has a positive effect for the following: sinus and alveolar ridge augmentation [31]; pre-implant augmentation procedures [32]; promotion of *in vitro* proliferation, osteogenic maturation and mineralization of mesenchymal stem cells and healing of critical-size bone defects *in vivo* [33]; promotion of *in vitro* periodontal ligament stem cells proliferation [34], management of chronic venous ulcers [35]. Considering the small amount of data on morphology, biological properties and regenerative potentiality of CGF, the aim of this study was to evaluate the blood cell localization, the *in vitro* cumulative release of growth factors and the *in vitro* power of growth on three different types of human cell lines: the Normal Human Dermal Fibroblasts (NHDF), the human umbilical vein endothelial cells (HUVEC) and the Human Osteoblast (HOB). Furthermore, a mechanical characterization of CGF was also performed by means of compression tests.

Materials and Methods

All experiments were conducted at the Laboratory of Anatomy and Physiopathology, Department of Clinical and Experimental Sciences, of the University of Brescia, between June 2014 and April 2015.

Blood collection

For the experiments, the venous blood was collected by piercing a superficial vein with a 21-gauge needle from 3 healthy adult volunteers of Caucasian ethnicity consisting of 1 men (V1) and 2 women (V2,V3), aged 28 to 39 years and with platelets, red blood cells and leukocytes levels within the normal range. Exclusion criteria included: systemic disorders, smoking, infections, non-steroidal anti-inflammatory drug use, an hemoglobin level <11 g/dl for females and <13.5 g/dl for males. The collected samples were always processed immediately after blood sampling. The research was conducted according to the principles of the Declaration of Helsinki.

Blood analysis

The subjects underwent to a hematologic blood test (erythrocytes, leukocytes and platelets) performed by laboratory S.T.E.M. analysis (Brescia, Italy) to recruit patients with normal hemochrome values, specified in inclusion criteria.

CGF preparation

The CGF was produced as follows: 9 mL of blood was drawn into each sterile Vacuette tube (Greiner Bio-One, GmbH, Kremsmunster, Austria) silicon coated as a serum clot activator. These tubes were then immediately centrifuged in a special machine (Medifuge MF200, Silfradent srl, Forlì, Italy) using a program with the following characteristics: 30 seconds acceleration, 2 minutes at 2,700 rpm, 4 minutes at 2,400 rpm, 4 minutes 2,700 rpm, 3 minutes at 3,000 rpm and 36 seconds deceleration and stopped. At the end of the process, three blood fractions were identified: (1) the upper layer, representing the liquid phase of plasma named platelet poor plasma (PPP), (2) the lower layer, at the bottom of the tube, consisting in free red blood cells (RBC); (3) the middle layer, representing the solid CGF, consisting in three parts: the upper white part (WP), the downer red part (RP) and the middle "buffy coat" (BC), interface between white and red part (Figure 1).

After centrifugation, CGF was removed from each tube, using sterile tweezers and placed on the surface of sterile petri dish, under a laminar flow cabinet. The solid CGF was obtained by cutting and discarding the lower fraction of the red part of CGF, 0.5 cm under the white part. Subsequently, each CGF was processed in relation to the experimental protocols.

The biological experiments were performed in triplicate to ensure repeatability of results, so each volunteer underwent a blood collection at different time-periods, for a total of 3 tubes for morphological staining and immunohistochemistry, 18 tubes for cumulative growth factor release and 18 tubes for each cell line cultures. The mechanical tests were carried out on 1 sample for each volunteer.

Histomorphological analysis

Immediately after centrifugation the CGF was collected and fixed in

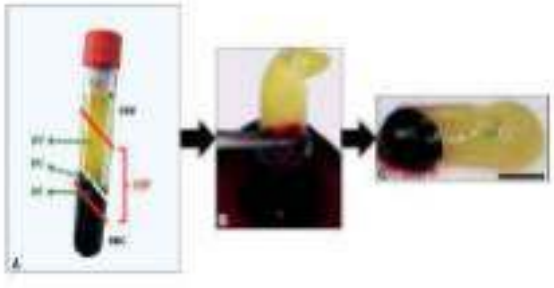

Figure 1: Blood sample after CGF protocol of centrifugation. Three layers are obtained: PPP, upper layer; CGF, middle layer; RBC, lower layer (A). CGF was removed from the tube, using sterile tweezers and cut the red part (RP) at 0,5 cm under buffy coat (BC) (B). The obtained CGF consists in three parts: the upper white part (WP), the downer red part (RP) and the middle BC part (C). Bar = 1 cm.

10% buffered formalin for 24 hours, embedded in paraffin according to standard procedures and cut at 8 µm by a microtome (Microm HM 325). Histomorphological assessment of CGF was made with two histological stain: the May-Grünwald-Giemsa (MGG; Bio-Optica, Milan, Italy) and the Hematoxylin and Eosin staining (HE; Bio-Optica, Milan, Italy). The stains were performed according to the manufacturer's protocol. However, with these stainings, the platelets were not clearly identifiable and for this reason CD61 immunohistochemistry, a platelet marker was also performed.

Platelet immunohistochemistry

Alternate paraffin sections were processed by immunohistochemistry. The sections were deparaffinised, rehydrated and subjected to antigen retrieval in 0.05M sodium citrate buffer (pH 6.0) in hot water bath (98°C for 20'). Endogenous peroxidase activity was blocked by incubation with a solution of 3% hydrogen peroxide. Sections were immunestained with the monoclonal antibody anti-human CD61 (platelet membrane glycoprotein IIIa, dilution 1:50, Diagnostic BioSystems, Pleasanton, CA). All sections were processed using UltraVision Quanto Detection System Horseradish Peroxidase (HRP; ThermoScientific, Bio-Optica, Milan, Italy), followed by development with diaminobenzidine (Amresco, Prodotti Gianni, Milan, Italy). Finally, they were counterstained with hematoxylin, dehydrated and mounted. The immunohistochemical control was performed by omitting the primary antibody, in presence of isotype-matched IgGs and performing pre-adsorption assay using the related peptide and gave negative results.

Platelet count

The indirect analysis of cellular component of CGF was performed on liquid re-mixed blood samples obtained after centrifugation with the same program to obtain CGF, using sterile Vacuette tubes with heparin (Greiner Bio-One, GmbH, Kremsmunster, Austria) to avoid coagulation. The hematologic blood test (leukocytes formula, platelets and erythrocytes) was performed by laboratory S.T.E.M. analysis (Brescia, Italy).

Scanning Electron Microscopy (SEM) Analysis

The samples of the CGF layers were fixed in 2% glutaraldehyde for 1 hour and then they were rinsed in cacodylate buffer solution and fixed for 1 hour with 1% osmium tetraoxide (OsO_4). Subsequently, the samples were dehydrated serially in 30, 50, 70, 90, and 100% ethanol solutions. The SEM procedures were completed by critical drying point of the material. Finally the CGF samples were observed at 20 kV using SEM LEO 1430 scanning electron microscope.

Cumulative growth factor release

The kinetics of PDGF-AB, VEGF, TNF-α, TGF-β1, IGF-I, BDNF and BMP-2 released from CGF clots were evaluated in triplicate by incubation of the CGF with RPMI 1640 cell medium (Lonza, Verviers, Belgium) without growth supplements for 5 hours, 1, 3, 6, 7 and 8 days (34). The CGF were placed in a 12-well plates (one in each well) with the addition of 1.6 ml of cell culture medium and then incubated at 37°C. After each incubation period, the medium was collected and centrifuged at 400 g for 10 min at room temperature. The supernatant was stored at −80°C until analysis [36]. The quantification of growth factors was performed using ELISA kits according to the manufacturer's protocol (R&D Systems Inc, Minneapolis, Minnesota, USA). The total quantity of growth factors present in the medium recovered at all time points was checked and reported both as mean value of all volunteers and as mean value of each volunteer at each time point.

In vitro cell proliferation

The CGF was prepared at the right time point of the experiment. Each CGF was placed into a sterile transwell insert (ThinCert™ cell culture inserts, Greiner Bio-One, Austria) with a semi-permeable membrane at the bottom and inserted into the 6-well plates (an insert in each well) for 72 hours. At the end of each treatment, the *in vitro* effect of CGF on cell proliferation was evaluated. Each experiment was performed in triplicate to ensure reproducibility of results and also to ensure a sufficient cell number for FACS analysis. At the end of the experiments, cell proliferation and morphology were evaluated in the three different cell lines used.

NHDF (Normal human dermal fibroblasts)

NHDF (cell derived from skin of adult donor; Lonza, USA) were cultured in Fibroblast Growth Medium (FGM; Lonza, Walkersville MD, USA) constituting by Fibroblast Basal medium (FBM; Lonza, Walkersville MD, USA) supplemented with gentamicin/amphotericin B (antibiotic/antifungal) and growth factors (rhFGF-B, insulin, fetal bovine serum - all from BulletKits®, Lonza, Walkersville MD, USA), at 37°C, 5% CO_2, in a humidified atmosphere until they reached about 80% confluence. The medium was changed every 2 days. NHDF from the third and sixth passage were used in the experiments. At confluence, NHDF were passaged and seeded, at a final density of 10000cell/cm^2, in 6-well culture plates (Sarstedt, Nuembrecht, Germany) and starved in FBM for 24 hours, before stimulation. The medium was then removed and four different treatments were tested for 72 hours: 1) only FBM, 2) only FGM, 3) FBM with whole solid CGF and 4) FGM with whole solid CGF.

HUVEC (Human Umbilical Vein Endothelial Cells)

HUVEC (pooled cells; Lonza, USA) were cultured in Endothelial Growth Medium (EGM; Lonza, Walkersville MD, USA) which consisted of Endothelial Basal Medium 2 (EBM2; Lonza, Walkersville MD, USA) supplemented with gentamicin/amphotericin B (antibiotic/antifungal) and growth factors (hFGF, VEGF, IGF-1, hEGF, fetal bovine serum - all from EGM-2 Single Quot®; Lonza, Walkersville MD, USA) at 37°C, 5% CO_2, in a humidified atmosphere until they reached about 80% confluence, with the medium changed every 2 days. Experiments were performed using HUVEC between the third and sixth passage. At confluence, HUVEC were passaged and seeded, at a final density of 10000cell/cm^2, in 6-well culture plates (Sarstedt, Nuembrecht, Germany) and starved in EBM2 for 24 hours, before stimulation. Subsequently, the medium was removed and four different treatments were tested for 72 hours: 1) only EBM2, 2) only EGM, 3) EBM2 with whole solid CGF and 4) EGM with whole solid CGF.

HOB (Human Osteoblasts)

HOB (cryopreserved cells; Promocell, Germany), were cultured in Osteoblast Growth Medium (OGM; Promocell, Heidelberg, Germany) which consisted of Osteoblast Basal Medium (OBM; Promocell, Heidelberg, Germany) supplemented with gentamicin/amphotericin B (antibiotic/antifungal) and SupplementMix (OGM Supplement Mix; Promocell, Heidelberg, Germany) containing growth factors (not specified by the manufacturer) at 37°C, 5% CO_2, in a humidified atmosphere until they reached about 80% confluence. The medium was changed every 2 days. Experiments were performed using cells between third and sixth passage. At confluence, HOB were passaged and seeded, at a final density of 5000cell/cm^2, in 6-well culture plates

(Sarstedt, Nuembrecht, Germany) and starved in OBM for 24 hours, before stimulation. The medium was then removed and four different treatments were tested for 72 hours: 1) only OBM, 2) only OGM, 3) OBM with whole solid CGF and 4) OBM with whole solid CGF.

FACS analysis

Cells were detached with the Trypsin (0.025%)/EDTA (0.01%) solution (Promocell, Heidelberg, Germany) and centrifuged at 1000 rpm for 5 minutes. After removing the supernatant, pellet was re-suspended in the appropriate culture medium. Cell suspension (100-200 µl), was transferred into each fresh tube (100000cells/tube) and permeabilized with Saponin (1 ml/tube), on ice for 10 minutes, preserving Ki-67 antigen. At the end of the incubation period with Saponin, cells were centrifuged at 1200 rpm for 5 minutes and the supernatant was removed. Cells were stained with the mouse monoclonal antibody Ki-67 FITC-conjugated (BD Bioscience, San Diego, CA). 20 µl of Ki-67 antibody were added to each tube and incubated in the dark for 30 minutes, at 4°C. As a positive control the Ki-67 isotype control (BD Bioscience, San Diego, CA) was used and as negative control the primary antibody was omitted and only a secondary FITC antibody was used. Cells were then washed with FACS buffer (PBS with 2% FBS- 2 ml/tube), centrifuged at 1200 rpm for 5 minutes and re-suspended in 0.5ml of FACS buffer (PBS with 2% FBS). Finally, the cell samples were analyzed with FACS (BD FACSCanto™- BD Bioscience, San Jose, CA) and the data were analyzed using the BD FACSDiva™ software version 8.8.7 (BD Bioscience, San Jose, CA).

Mechanical characterization

Compression tests were carried out on three CGFs, one for each donor. Each CGF, after being removed from the glass tube used for centrifugation, was cut with a steel scalpel into two pseudo-cylindrical specimens: one obtained from the white part and the other obtained from the buffy coat part. For each specimen, the initial height (h_0) and cross-section (A_0), were indirectly measured on the photograph of each specimen, captured with a high magnification photo-camera (Nikon D7000). The software ImageJ (v. 1.47) was used for the image analysis. A_0 values were typically between 100 and 150 mm^2 and h_0 values between 3 and 6 mm. The compression tests were performed at room temperature, using an Instron test system (model 3366) equipped with a 50 N load cell. A crosshead speed of 2 mm/min was used. To prevent the samples drying, they were wetted with some drops of saline solution (0.9% NaCl), before the beginning of each test.

For each specimen, starting from the load vs crosshead displacement curve, the nominal stress (σ) vs nominal strain (ε) curve was constructed. The nominal strain (ε) was evaluated as:

$$\varepsilon = 1 - \frac{h}{h_0} \quad (1)$$

where h and h_0 are the actual height (measured directly from the crosshead displacement) and the initial height of the specimen, respectively. The nominal stress (σ) was evaluated as:

$$\sigma = \frac{F}{A_0} \quad (2)$$

where F is the recorded load and A_0 the initial cross-section of the specimen. A reference test, carried out without the specimen in place, was also performed in order to verify the value of h_0, evaluated from the photographic analysis.

Statistical Analysis

One-way ANOVA test corrected by Bonferroni was used for statistical analysis. A P-value less than 0.05 was considered statistically significant. Results were expressed as mean ± standard error (SE).

Results

Blood analysis

The blood analysis laboratory confirmed that the tested subjects presented normal blood values and could be included in the experimental protocols.

Morphological characterization

Blood cells: The fibrin matrix appeared homogeneous in a light brown colour in MGG (Figure 2A) and a light pink colour in HE (Figure 2B). The leukocyte nuclei were stained in dark blue in MGG (Figure 2A) and violet in HE (Figure 2B). The erythrocytes were stained in light brown in MGG (Figure 2A) and pink/red in HE (Figure 2B). So, leukocytes and erythrocytes were clearly detected by both MGG and HE staining. The leucocytes were localized principally in the buffy coat but also scattered in the white and red part of CGF, close to buffy coat. The erythrocytes were present only in the red part of CGF. The platelets, because of their small size, were difficult to be visualized using these classic stains in a context where the fibrin network created a troubled background.

The immunohistochemistry for CD61, a platelet marker, showed a clear positivity in the buffy coat of CGF in all specimens analysed (Figure 2C). These data were supported by SEM analysis (Figure 2C inset). Platelets were also scattered in the white part of CGF, where formed aggregates, trapped in the fibrin network.

Fibrin network: Images of the CGF fractions by close to and far from the buffy coat were clearly detected by light microscopy, using EE staining (Figure 3). The images showed that the fibrin network and architecture changed moving from the buffy coat to the white part. In particular, close to the buffy coat, the fibrin network was strictly compact (Figure 3A) while those far from the buffy coat became a larger mesh (Figure 3B).

Platelet count: The blood subjected to CGF centrifugation program

Figure 2: Micrographs of CGF representing (1) red part, (2) buffy coat, (3) white part. A) HE staining, B) MGG staining, C) CD-61 immunostaining for platelets; the inset represent platelets in the buffy coat obtained by SEM analysis. The black arrows (white in inset) indicate platelets, the gray arrows indicate leukocytes.

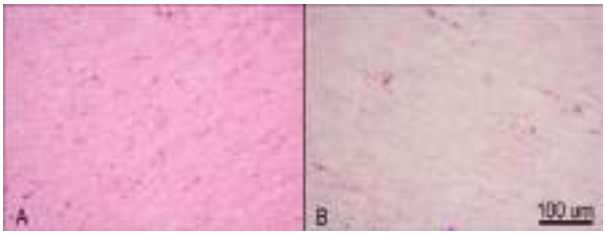

Figure 3: Micrographs of CGF fibrin network in white part (A) near the buffy coat and (B) far from the buffy coat stained with HE.

had a loss of platelets but the number of erythrocytes and leukocytes did not change. The recovered platelets represented the 50.04% ± 7.62. Therefore, we could suppose that the missing platelets were destroyed during CGF centrifugation.

Evaluation of cumulative growth factor release

The results varied greatly among volunteers.

Considering the mean value of the three volunteers, we can say that:

- PDGF-AB has a constant accumulation during the experimental time (Figure 4a),

- VEGF increases during all the experimental period, reaching the maximum accumulation at 8th day (Figure 4b),

- TNF-α reaches the maximum accumulation at the 1st day and then progressively decreases until the 8th day (Figure 4c),

- TGF-ß1 has a constant accumulation during all the experimental period (Figure 4d),

- BDNF reaches the maximum accumulation at the 1st day and after this it maintains a plateau during the experimental time (Figure4e),

-BMP-2 reaches the maximum accumulation at 8th day (Figure 4f), so its release is maximum in the last part of the experimental period,

- IGF-1 reaches the maximum accumulation at 6th day and then it decreases (Figure 4g).

Cell proliferation assay and morphology

The proliferative effect of CGF on NHDF, HUVEC and HOB was assessed using FACS analysis, by measuring the expression of intracellular proliferation marker Ki-67. The results are described in detail below.

NHDF

In basal medium, free of serum and growth factors, the cells showed a low percentage of Ki-67 positivity (18.68% ± 1.8) (Figure 5a). The cells did not show a clear spindle-like morphology, appearing short and not well spread over the plate surface (Figure 5b). In the complete medium, the percentage of Ki-67 positive cells increased (61.98% ± 6.35) compared with the basal medium (Figure 5a). The cells showed a clear characteristic spindle-like morphology, appearing elongated and well spread over the plate surface (Figure 5c). In the basal medium with CGF, the percentage of Ki-67 positive cells also increased (51.51% ± 7.12) (Figure 5a). The cells showed a clear characteristic spindle-like morphology, appearing elongated but not well spread over the plate surface (Figure 5d). In the complete medium with CGF, the percentage of Ki-67 positive cells markedly increased (75.9% ± 4.13) compared with basal medium alone and with CGF (Figure 5a). The cells showed a clear characteristic spindle-like morphology, appearing well elongated, larger and well spread over the plate surface compared with all the other culture conditions (Figure 5).

HUVEC

In the basal medium, free of serum and growth factors, the cells showed a very low percentage of Ki-67 (5.05% ± 0.19) positivity, being cell growth very influenced by culture conditions (Figure 6a). The cells showed a round shape morphology and appeared not well attached over the plate surface (Figure 6b). In the complete medium, the percentage of Ki-67 positive cells markedly increased (26.03% ± 2.79) compared with the basal medium (Figure 6a). The cells showed the typical polygonal shape morphology appearing well attached over the plate surface (Figure 6c).

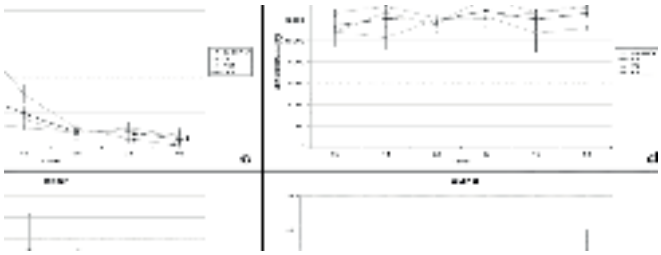

Figure 4: Cumulative growth factor released by CGF at 5 hours (5h), 1 day (1d), 3 days (3d), 6 days (6d), 7 days (7d) and 8 days (8d) of culture. Each volunteer's values are plotted (V1,V2,V3) as well as their mean value (mean V).The values are reported as total amount of growth factor released ± SE. °P<0.05 vs 5h; *P<0.05 vs 1d; ^P<0,05 vs 3d; #P<0.05 vs 6d; §P<0.05 vs 7d. (a) PDGF-AB, (b) VEGF, (c) TNF-α, (d) TGF-β1, (e) BDNF, (f) BMP-2 and (g) IGF-1.

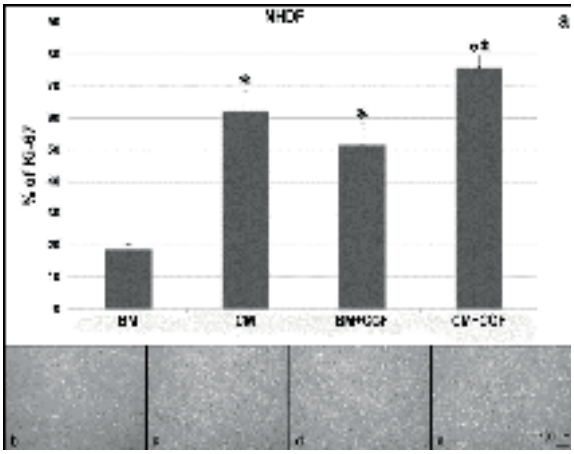

Figure 5: Effect of CGF on NHDF. (a) Ki-67 expression in NHDF grown in basal serum free medium (BM), complete medium (CM), basal medium with CGF (BM+CGF) and complete medium with CGF (CM+CGF). The Ki-67 positive cells are reported as % ± SE. *P<0.05 vs BM; °P<0.05 vs BM+CGF; ^P<0.05 vs CM.(b-e) Micrographs of NHDF after 72h of culture conditions; (b) BM, (c) CM, (d) BM+CGF, (e) CM+CGF; bar 100 um.

In the basal medium with CGF, the percentage of Ki-67 positive cells markedly increased (26.94% ± 1.96) (Figure 6a). The cells showed a more defined polygonal shape morphology appearing larger and well attached over the plate surface compared with basal and complete medium alone (Figure 6d).

In the complete medium with CGF, the percentage of Ki-67 positive cells markedly increased (38.62% ± 4.03) compared with all the other culture conditions (Figure 6a). The cells showed a more defined polygonal shape morphology appearing larger and well attached over the plate surface, compared with all the other culture conditions (Figure 6e).

HOB

In the basal medium, free of serum and growth factors, the cells showed a low percentage of Ki-67 positivity (15.07% ± 0.39) (Figure 7a). The cells showed their typical polygonal and flattened shape morphology and appeared well attached over the plate surface (Figure 7b). In the complete medium the percentage of Ki-67 positive cells

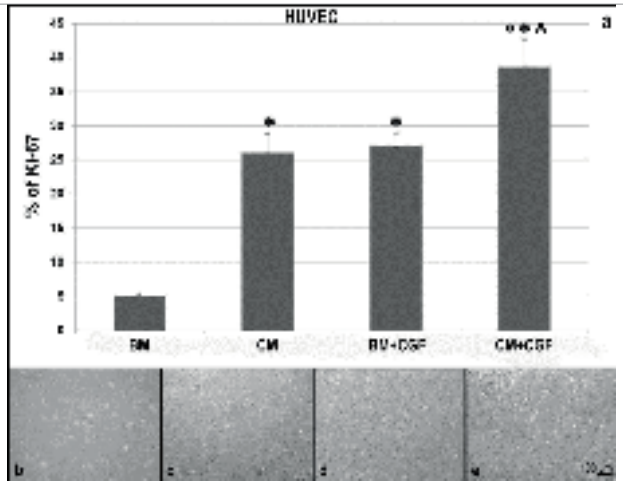

Figure 6: Effect of CGF on HUVEC. (a) Ki-67 expression in HUVEC grown in basal serum free medium (BM), complete medium (CM), basal medium with CGF (BM+CGF) and complete medium with CGF (CM+CGF). The Ki-67 positive cells are reported as % ± SE. *P<0.05 vs BM; °P<0,05 vs BM+CGF; ^P<0.05 vs CM. (b-e) Micrographs of Huvec after 72h of culture conditions; (b) BM, (c) CM, (d) BM+CGF, (e) CM+CGF; bar 100 um.

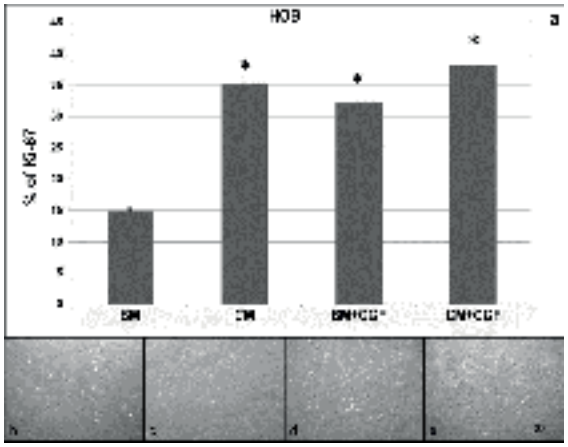

Figure 7: Effect of CGF on HOB. (a) Ki-67 expression in HOB grown in basal serum free medium (BM), complete medium (CM), basal medium with CGF (BM+CGF) and complete medium with CGF (CM+CGF). The Ki-67 positive cells are reported as % ± SE. *P<0.05 vs BM; °P<0.05 vs BM+CGF; ^P<0.05 vs CM.(b-e) Micrographs of HOB after 72h of culture conditions; (b) BM, (c) CM, (d) BM+CGF, (e) CM+CGF; bar 100 um.

markedly increased (35.31% ± 1. 21). compared with the basal medium (Figure 7a). The cells showed a more elongated polygonal and flattened shape morphology with the presence of extensions or very thin filopodia compared with the basal medium (Figure 7c). In the basal medium with CGF, the percentage of Ki-67 positive cells markedly increased (32.3% ± 2.46) compared with the basal medium alone (Figure 7a). The cells showed a more elongated, larger polygonal and flattened shape morphology with the presence of more extensions or very thin filopodia compared with the basal medium and complete medium (Figure 7d). In the complete medium with CGF, the percentage of Ki-67 positive cells markedly increased (38.13% ± 2.72) compared with all the other culture conditions (Figure 7a). The cells had a more elongated polygonal and flattened shape morphology with the presence of more extensions or very thin filopodia (Figure 7e).

Mechanical test

The nominal stress (σ) vs nominal strain (ε) curves obtained from the compression tests, are reported in Figure 8. Each test was interrupted at a large strain level, close to 0.9 mm/mm, and, once the load was removed, the specimen did not recover the deformation they had undergone during the compression test, but remained largely deformed. The mechanical response of the material appeared only slightly dependent on the part of the CGF examined (whether the white or the buffy coat part). Irrespective of the CGF part considered, the material exhibited a non-linear nominal σ-ε behavior, and became apparently stiffer as the strain level increases. From the nominal σ-ε curve of each specimen, two characteristic moduli, M_{small} and M_{large}, representative of the material stiffness at small and large deformations, respectively, were evaluated. M_{small} was evaluated as the slope of the line secant to the σ-ε curve at two fixed levels of strain (0.2 and 0.4 mm/mm) in the small strain region, whereas M_{large} as the slope of the secant at two fixed levels of stress (4 and 5 kPa) in the large strain region of the curve (at ε generally higher than 0.8 mm/mm). For each of the two parts of the CGF sample examined (white and buffy coat, respectively), average values of M_{small} and M_{large} were evaluated from the data obtained for the three different samples, and they are reported in Table 1. The results showed that, at small strains, the material from the white part

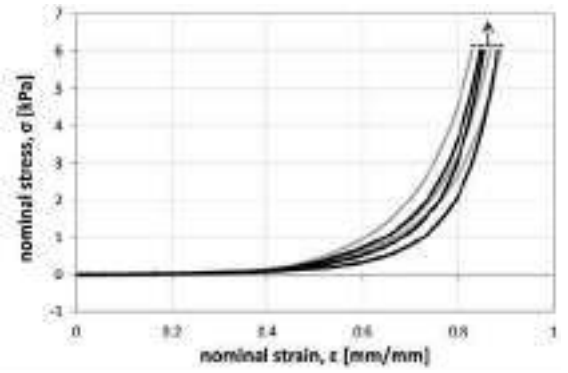

Figure 8: Compression tests.Nominal stress, σ, *vs* nominal strain, ε, curves from the compression tests on the specimens obtained from the white part (grey lines) and the buffy coat part (black lines) of the three CGF examined. The arrow indicates that final points of the curves and are not representative of specimen breakage.

	M_{small} [kPa]	M_{large} [kPa]
white part	0.70 ± 0.23	41 ± 1.9
buffy coat part	0.45 ± 0.13	54 ± 6.8

Table 1: Average values of the moduli M_{small} and M_{large}, evaluated at small and large deformations, respectively, for the white and the buffy coat parts of the CGF samples.

appears, on average, to be slightly stiffer than that from the buffy coat, whereas, at large strains, the buffy coat part appeared to be the stiffest.

Discussion

The first aim of this study was to evaluate the blood cell localization in CGF. Our results have shown that platelets are localized in the buffy coat, in a very thin space. Considering the platelets localization, the volume of the white part of CGF could be reduced, if necessary, in clinical practice. In the literature, the morphology of PRF, at the light microscopy, was performed [14]. The blood cells were localized in the buffy coat using two stains (Hematoxylin/Eosin and Masson's

trichrome modified by Goldner) but without obtaining a totally clear identification of the platelets. Moreover, an immunohistochemical localization of platelets in PRF membranes corroborated by a scanning electron microscopy analysis was also performed [19]. Platelets were concentrated on the surface of the region adjacent to the red thrombus and, considering two different types of compression, or either on one side of the surface or inside the membrane.

With regard to the quantity of recovered platelets, it is important to consider that, in our experiments, about the half of them are destroyed during centrifugation. This data support the hypothesis that CGF guarantees an immediate available free quantity of growth factors. In PRF [14], the count of blood cells was done on the clot exudates after compression. There was a decrease of platelets and leucocytes of approximately 97% and >50% respectively. In other liquid PRP preparations, the count of blood cells was easily performed using a routine haematology analyser. Some authors, using newly optimized PRP preparation method, obtained a final platelet recovery of 46.9 to 69.5% [37]. Bertrand-Duchesne et al. [38], using a platelet concentrate collection system (PCCS), reported a platelet recovery rate of 63% (range, 44.1-75.0%); Leitner et al. [39] compared different PRP systems with a collection efficiency ranging from about 13 and 78%. All these works support our data, nevertheless other articles report the number of recovered platelet as concentrations (number of platelets/volume) and usually do not mention any possible loss of cells and so cannot easily be compared with our data [40-44].

The second aim of this study was to evaluate the *in vitro* cumulative release of growth factors. Our data show that each growth factor has a specific kinetic of accumulation, probably due to a different accumulated quantity in α-granules and a different accumulation of mRNA in platelets useful to synthesize growth factors up to over 7 days after activation [45]. The different kinetics suggests a precise and programmed release to sustain regeneration. Furthermore, the growth factor concentrations varied greatly among the volunteers' samples, suggesting that the results could be influenced by the individual biological characteristics. In fact, Weibrich et al. [46], suggested that different individuals may require different platelet concentration ratios to achieve a comparable biological effect. Marx [2] did suggest however that 1×10^6 platelet/μL should be set as the threshold concentration of therapeutic PRP in order to ensure a therapeutically effective amount of growth factors in PRPs.

We therefore decided to report the quantity of growth factors as an absolute value and not as concentration as it sometimes referred to in the literature. This decision was made considering the solid nature of CGF and the necessity to clearly understand in clinical practice the real amount of bioactive molecules at disposable for patients at a specific time point.

PDGF and TGF-β1 are the majority growth factors and the most important in PRPs and many reports focus especially on PDGF, TGF-β1 and IGF [47-50]. In our experiment we observed a mean constant accumulation of PDGF-AB and TGF- β1. PDGF-AB is involved in all three phases of wound healing, including angiogenesis, the formation of fibrous tissue and re-epithelialization [51]. Our results suggest a clear constant PDGF-AB release, with a mean range between 10000 and 20000 pg, assuring a steady contribution to wound healing repair. A similar kinetic was found for TGF- β1, a multi-functional cytokine. In our experiment, TGF- β1 has a mean value of about 12000 pg. even if it seems that the ideal PRP concentration is when the TGF-β1 is between 50 and 100 ng/ml [42]. Furthermore, in a rat tibial fracture model, injections of TGF-β (4 and 40 ng) every other day for 40 days caused a dose-dependent increase in bone thickness. As regards IGF-1, it reaches the maximum accumulation at 6th day and then it decreases. IGF-1 has an anabolic effects and in our experiment, it has a mean value of around 200-300ng. This data is supported by another study where the content in IGF-1 remained close to 250 ng along the time course of the study. This agrees with other previous observations that IGF-1 is in majority present in plasma [52,53] and therefore was not expected to increase as a consequence of platelet activation and release from PRF [54].

On the other hand, VEGF and BMP-2 have only a slow kinetic release. VEGF, which increases angiogenesis and vascular permeability [55], has a linear kinetic release, reaching the maximum accumulation and so therefore its plateau, at the end of the experimental period with about 4000 pg. BMP-2, which induces osteoblast differentiation with osteo-inductive properties, has a slow kinetic with a linear increase from the 6th day and so reaching the maximum accumulation at the end of the experimental period with only about 10 pg. Only a few studies have analysed the contribution of BMP-2 from PRP during the healing process. One of these evaluated BMP-2 quantity in PRP which was the lowest compared with the other growth factors analysed [56]. The other one demonstrated that PRP accelerated bone fracture healing of rat femurs via modulation of BMP-2 and also TGF-β1 and growth factor expression [57].

Nevertheless, TNF-α and BDNF have a fast kinetic release, considering the mean value. TNF-α reaches the maximum accumulation at 1 day and after this decreases. It has controversial effects and elicits a variety of responses, depending on the cellular context [58]. In fact, it is able also to induce inflammatory response as it is also produced by leukocytes. TNF-α is also present in PRP [37] and in activated PRP. Bendinelli et al. [59] observed increases in TNF-α, hepatocyte growth factor (HGF) and IL-4. TNF- α and HGF, by disrupting NF-kB-transactivating activity, are important for the anti-inflammatory function in this context. BDNF is known to be an important member of the neurotrophic family with neural growth and differentiation properties and the large amounts of circulating BDNF proteins are stored in platelets [60,61]. It reaches the maximum accumulation at 1day (about 30000 pg) and after this maintains a plateau. This is a growth factor that is not usually investigated in PRPs but, considering some interesting results on its application in neurodegenerative pathologies, such as Alzheimer's disease [62], we have included it in our investigation.

In a recent study [54], PDGF-AB, TGF-β1 and VEGF content released from PRF releasate increased dramatically and gradually over the time course of the study. Content in the releasate reached mean values of 52.37, 72.21, 1.04 ng at 300 minutes for PDGF-AB, TGF-β1, EGF, and VEGF respectively. The values were greater compared with ours but this was probably due to different methods of evaluation. By contrast, the IGF-1 content remained stable at about 250 ng, as in our experiment. Another study on growth factor release was performed by Anitua et al. [36] showing that the growth factor delivery is diffusion controlled with a rapid initial release by 30% of the bioactive content and a steady state release when almost 70% of the growth factor content is delivered. In particular, the kinetics of PDGF-AB and VEGF release from PRGF clot is linear reaching a plateau after the 3rd experimental day. In our experiments, we did not observe a linear accumulation in all the growth factors analyzed probably due to short half-life, proteolitic processes and decrease of growth factor release [63-65]. In particular, PDGF-AB was linear up to 3rd day, while VEGF was linear up to 6th day when it reached its plateau. These data are supported by a recent paper where the growth factor release of L-PRF from 8 hours to 28 days was

evaluated, showing a non-linear kinetic release [47]. As shown above, there are different results in the literature concerning the quantitative measurement of the growth factors contained in PRPs [48,49]. Such differences seem to be multifactorial and include inter-patient variability of the proteins amount contained in the platelets, different degrees of platelets concentration during PRP preparation, activation or inactivation, as well as different degrees of platelet membrane breakage and the specific degree of platelet activation at the time of measurement [66].

The third aim of our study was to assess the *in vitro* CGF growth potentiality, on three different human cell lines: NHDF, HUVEC and HOB. The activity of growth factors released by CGF clearly influences the cell growth.

The proliferative effect of CGF was evident in all three cell lines. However the mitogenic potential of CGF was more evident when added to the basal medium, especially in HUVEC and HOB cells, where the percentage of Ki-67 positive cells was similar with both the basal and complete medium. Moreover, except than in fibroblasts, CGF addition to complete medium, did not enhance the growth effect of medium standard supplements, most probably because the cells were already fully stimulated.

NHDF grow in presence of bFGF (basic Fibroblast Growth Factor), IGF-1, EGF (Epidermal Growth Factor), PDGF and TGF-β. Some data are particularly interesting regarding the role of specific growth factors on NHDF proliferation. Indeed the results of Gasparri et al. [67] and Okuda et al. [68] confirm that PDGF, bFGF and TGF-β potently induce NHDF to proliferate, as previously reported [69,70]. On the other hand, EGF and IGF-1 also stimulate proliferation [71,72] but less potently than PDGF or bFGF. Furthermore, consistent with our results, a recent work [73] reports that PRP markedly increases fibroblast proliferation compared with the serum-treated control group.

Also in HUVEC, the CGF supplementation positively influences cell growth, but less so than in fibroblasts. HUVEC proliferate in presence of specific growth factors such as EGF, VEGF, bFGF and IGF-1. Indeed it has been shown that these growth factors are, at least in part, responsible of the PRP [38] and PRGF [74] mitogenic and angiogenetic effect for endothelial cells.

As regards HOB, here CGF was able to significantly stimulate their *in vitro* proliferation. The mechanism responsible for the cell proliferation by CGF may be explained as follows: CGF is rich in a variety of growth factors, such as TGF-β, PDGF-AB, VEGF and IGF-I [18]. It has been demonstrated [75] that these growth factors seem to be highly relevant for osteoblast proliferation and differentiation, suggesting a beneficial effect of platelet preparations on HOB growth and viability [76]. In particular, PDGF and TGF-β1 seem to be the most dominant factors which significantly contribute to HOB proliferation [77]. As regards IGF-1, Ogino et al. [77] suggest that this growth factor is able to enhance osteoblast proliferation when it is combined with other growth factors such as PDGF and TGF-β1. Moreover, unlike PRP, CGF does not dissolve rapidly after application, instead, the strong fibrin gel in the matrix addition is slowly remodeled in a similar manner to a natural blood clot. Thus, CGF prolongs the duration of growth factor action and enhances cell proliferation and osteogenic differentiation.

Furthermore, due to its higher strength and viscosity, CGF could better protect the growth factors from proteolysis, compared with PRP and PRF [63]. This seems to agree with our *in vitro* results, where the CGF addition enhances cell proliferation in all the three different cell lines (fibroblasts, endothelial cells and osteoblasts) involved in angiogenesis, tissue remodeling and regeneration. The results concerning other PRP preparations support this data. The capacity of two human blood fractions (the supernatant serum and the releasate PRF) were evaluated monitoring the cell proliferation in two human cell lines, human embryonic kidney fibroblasts HEK293 and human MG-63 osteoblastic cell line. The supernatant serum and the releasate PRF at 1-10%, stimulated cell growth more significantly than FBS-free medium [78]. It is interesting that the addition of platelet lysate-plasma, obtained from pooled PRP, to human mesenchymal stromal cell (hMSCs) culture showed a consistently greater proliferation rate than with FBS at the same percentage [79].

The fourth aim of the study was to analyze the mechanical behaviour of CGF. Specimens did not recover from deformation once the compression load was removed. This "apparent plasticity" seems to indicate that, under compression at high strain levels (similar to the deformational state experienced by CGF during positioning in the patient), the material microstructure undergoes modifications that can have permanent character. Regarding its non-linear nominal σ-ε behavior, it is interesting to note that literature works report that a strain-stiffening effect is observed for fibrin gels under shear deformation [80]. For the CGF materials here examined, the volume variations which the specimen underwent during the compression test did not allow the construction of reliable true stress vs true strain curves. Consequently, at this stage of the work, it is not possible to confirm that the apparent strain-stiffening effect observed here is intrinsic to the material. Further experiments are needed to better investigate this aspect.

In relation to the moduli M_{small} and M_{large}, it can be reasonably thought that, at large strains, the mechanical response of the material under analysis is governed by the architecture of the fibrin network. With this in mind, the higher M_{large} exhibited by the buffy coat part would reflect the tighter structure of the fibrin network observed in this zone of the CGF sample by histomorphological analysis, compared to that observed in the white part.

It seems clear that CGF represents a plasma preparation with great potentiality, whose clinical use seems to be promising. Indeed, our data underline that the growth factors present in CGF influence in a specific way the different cell types. CGF has a solid-like nature, it is high compliant, can be easily handled and could be useful for specialized application in regenerative medicine, dentistry, oral implantology and cell therapy.

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Case Report Open Access

C3 Glomerulonephritis and Plasma Cell Dyscrasia: Expanding the Etiologic Spectrum

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Abstract

Background: C3 glomerulopathy (C3GP) including dense deposit disease (DDD) is mediated by abnormal activation of the alternative complement pathway (ACP). In children and young adults, mutations of complement or complement regulatory proteins are the major causative factors but in adults there appears to be an increased incidence of monoclonal gammopathy and it has been proposed that the paraprotein is functioning as a C3 nephritic factor or through other unknown mechanisms resulting in abnormal ACP activity.

We describe five patients with C3GP and plasma cell dyscrasias including two patients with symptomatic multiple myeloma and three patients with monoclonal gammopathy of renal significance one of whom progressed to symptomatic myeloma. One patient with DDD and elevated C3 nephritic factor responded to myeloma therapy with cyclophosphamide plus bortezomib and dexamethasone while another patient seemed to rapidly worsen both times after receiving lenalidomide, a drug with potent immunomodulatory activity. In two patients, the effect of myeloma therapy was indeterminate secondary to advanced disease. Two patients with renal transplant had recurrence C3GP in the transplanted kidney at 2 months and four years, respectively.

Conclusion: Adult patients with C3GP should be screened for plasma cell dyscrasias. Further studies are required to assess the value of myeloma-directed treatment and/or ACP inhibition.

Keywords: Monoclonal; Plasma cell; C3 Glomerulonephritis; C3 Convertase; Eculizemab

Abbreviations

C3GP: C3 Glomerulopathy; DDD: Dense Deposit Disease; ACP: Alternative Complement Pathway; MPGN: Membranoproliferative Glomerulonephritis; CRP: Complement Regulatory Proteins; MGRS: Monoclonal Gammopathy Of Renal Significance; Smac: Soluble Membrane Attack Complex

Background

Membranoproliferative glomerulonephritis (MPGN) is a descriptive term for abnormal glomerular changes characterized by distinct histological findings. Although originally categorized by the location of abnormal immune complex deposits on electron microscopy (EM), recently a more pathogenetic classification is used that relies on immunohistochemistry for the presence or absence of abnormal immunoglobulin and C3 [1, 2]. The presence of significant immunoglobulin staining is associated with an immune complex etiology whereas dominant staining for C3 with little or no immunoglobulin is more indicative of abnormal activation of the alternative complement pathway (ACP). In the latter situation, the location and appearance of the deposits by EM can distinguish dense deposit disease (DDD, formerly type II MPGN) from other types of C3 glomerulonephritis (C3GP). Particularly, as C3GP and DDD may be amenable to emerging treatments that interrupt the ACP such as eculizumab [3] and other agents in development [4], the newer classification has potential therapeutic implications apart from the assignment of pathophysiology.

Monoclonal gammopathy has been associated with both immune complex type MPGN and C3GP, including DDD. The incidence of monoclonal gammopathy in patients with MPGN may be as high as 41% [5]. In patients with immune complex type MPGN, the paraprotein likely forms part of the immune complex causing activation of the classic complement pathway and resulting in renal injury with both abnormal immunoglobulin and complement on immunohistochemistry.

Similarly, while DDD [6,7] and C3GP [8] are much more common in children and young adults and are often associated with mutations in complement components or complement regulatory proteins (CRP), in adult patients with C3GP, 31% of patients were found to have a monoclonal gammopathy [9]. This number increased to 71% in adults with DDD [10]. Most of these patients did not have symptomatic multiple myeloma and would now be considered to have monoclonal gammopathy of renal significance (MGRS) [11]. With respect to pathophysiology of paraproteinemia-associated C3GP and DDD, it has been suspected but not proved that the paraprotein is functioning as an autoantibody that stabilizes C3 convertase (C3 nephritic factor) or possibly inhibits one of the CRPs, such as Factor H or I [9,10]. In this report, we confirm and expand the association of C3G and plasma cell dyscrasias, including symptomatic multiple myeloma. We also show an apparent response to treatment with cyclophosphamide and bortezomib in a patient with DDD.

Case Series

We report five patients with plasma cell dyscrasia, four had C3GP and one had DDD proven by kidney biopsies. Clinical history and lab values

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]Patent #	Age/Sex	Serum Cr	SPEP/IFE	Spot urine P/C (nl<0.1)	C3/C4	C3Nef nl < 0.3	sMAC nl < 244
1	79/F	2.7	1.5 gm/dl IgG-kappa	0.7	Nl/Nl	NA	NA
2	67/F	7.2	1.9 gm/dl IgG -kappa	NA	L/L	0.42	H 606
3	67/M	4.4	1.0 gm/dl IgG-lambda	5.2	NA	0.33	H 460
4	81/F	6.2	1.0 gm/dl IgG/IgG lambda	6.8	Nl/Nl	0.66	NA
5	57/F	1.4	3.2 gm/dl IgG lambda	1.7	NA	NA	H 1200

Table 1: Patient demographics and initial laboratory results. Cr:creatinine; SPEP: serum protein electrophoresis; IFE: immunofixation electrophoresis; P/C: protein: creatinine ratio; C3Nef: C3 nephritic factor; sMAC: soluble membrane attack complex; NA: not assessed; Nl: normal

are summarized in Table 1. Patient 1 had a 2-yr history of hematuria, proteinuria and renal insufficiency and a bone marrow biopsy showed 15% clonal plasmacytosis. A renal biopsy showed MPGN and she was followed without treatment but eventually developed both ESRD and symptomatic myeloma with a large lytic bony lesion. She was treated with a modified cyclophosphamide, bortezomib, dexamethasone (CyBorD) regimen [12] and had a near complete serologic response. She has remained on hemodialysis.

Patient 2 was diagnosed with symptomatic multiple myeloma with lytic bony lesions and presented with acute renal failure two weeks after starting lenalidomide/dexamethasone. A renal biopsy showed ATN and C3GP. She had a partial serologic response and improvement in her renal function after treatment with modified CyBord but then had therapy changed to lenalidomide and dexamethasone secondary to worsening neuropathy. She quickly developed worsening renal insufficiency and hematuria. Lenalidomide was stopped and her renal function stabilized.

Patient 3 had a history of MPGN with gradually worsening renal function with a stable paraprotein and normal free light chain ratio. A review of a previous kidney biopsy established the diagnosis of C3GP. He was treated with modified CyBorD but did not show a serologic response. He developed ESRD and underwent renal transplantation. Because of increasing creatinine, hematuria and proteinuria, he underwent a renal biopsy two months after transplant that showed recurrent C3GP. He has been started on eculizumab.

Patient 4 presented with hematuria, renal failure and nephrotic range proteinuria. A renal biopsy showed DDD. Bone marrow biopsy showed 5% clonal plasmacytosis. She did not respond to high dose steroids and was started on a modified CyBorD regimen. She achieved a partial serologic response and improvement in her renal function and proteinuria. She remains on bortezomib/dexamethasone maintenance nearly 18 months after the diagnosis of DDD.

Patient 5 was status post-renal transplant for MPGN. Although she had a diagnosis of smoldering multiple myeloma for 2-3 years, she did not receive treatment until being considered for a renal transplant. Prior to a living-related donor transplant, she was treated with bortezomib plus dexamethasone and achieved a serologic complete remission. About four years after kidney transplant, she had evidence of an increasing paraprotein and a bone marrow showed 20% plasmacytosis. She was then evaluated for recurrent hematuria and worsening renal function. A biopsy of the renal allograft showed C3GP and she was started back on modified CyBorD and continued tacrolimus immunoprophylaxis for the kidney transplant. She has not been followed long enough to assess her response.

Discussion

Our study confirms and expands previous series showing a relationship between C3GP (including DDD) and plasma cell dyscrasia [9,10]. In contrast to the latter reports that showed most patients had monoclonal gammopathy of undetermined significance (MGUS), one of our patients was diagnosed with C3GP just after the diagnosis of symptomatic MM and a second patient evolved from smoldering to symptomatic myeloma a few years after the diagnosis of MPGN (retrospectively reviewed and confirmed as C3GP). In addition, patient 5 developed recurrence of C3GP in the setting of a rising paraprotein and 20% plasmacytosis. Thus far, only the patient with DDD has had a meaningful response to myeloma-based treatment (improved creatinine and decrease in proteinuria) but two others probably had disease that was too far advanced to show renal recovery.

The relationship between paraproteinemia and ACP dysregulation is unclear. However, patient 4 with DDD had a significantly increased C3 nephritic factor that normalized after treatment. Three patients (2,3 and 5) showed high levels of soluble membrane attack complex (sMAC) two of whom also had borderline high C3 nephritic factor levels. High sMAC is consistent with increased C5 convertase activity and may serve as a biomarker predicting favorable response to the C5 convertase inhibitor, eculizumab [3].

As it has been recognized that C3GP and DDD are due to dysregulation of the ACP, there has been growing interest in the use of eculizumab, particularly given its effectiveness in treating paroxysmal nocturnal hemoglobinuria [13] and atypical hemolytic anemia [14,15], two other diseases mediated by abnormal control of ACP. Thus far, there have been few patients with C3GP that have been treated with eculizumab, recently summarized by Bomback [3]. In eight case reports, 7 of 8 patients responded [16-23]. In an open label series of six C3GP patients (3 patients had DDD), clinical and/or pathologic improvement was seen in 4 patients, but two patients experienced worsening disease. These findings indicate that eculizumab might not be appropriate for all patients with C3GP [24,25]. Importantly, the sMAC normalized in all patients who had elevations before eculizumab therapy and the two patients who had worsening renal function had normal sMAC levels before treatment (Figure 1).

A recent report has advocated treatment of the underlying disorder in patients with MGRS [26]. However, there have been very few reports of improvement in renal function/proteinuria following treatment. In patients with symptomatic and smoldering multiple myeloma it is certainly reasonable to treat the underlying plasma cell dyscrasia. However, it should be noted that complications such as MGUS-associated neuropathy inconsistently respond to treatment of the underlying disorder [27]. Our patient 2 had dramatic worsening of her renal function on two occasions shortly after treatment with

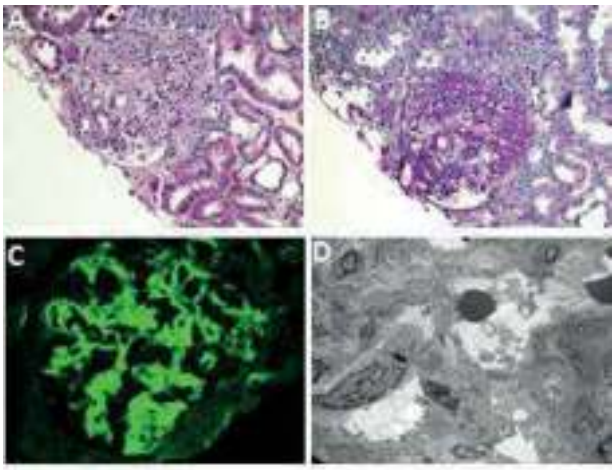

Figure 1: Representative light, immnofluorescence, and electron microscopy in patient 3 with C3 glomerulopathy and monoclonal gammopathy. (A, B) Light microscopy demonstrates a membranoproliferative pattern of injury. [A] Hematoxylin & Eosin stain, 20X; [B] Periodic acid-Schiff stain, 20X. (C) Immunofluorescence shows bright C3 in the mesangium and capillary loops, 400x. (D) Electron microscopy shows numerous subendothelial deposits and occasional subepithelial deposits.

the immunomodulatory drug (IMiD) lenalidomide suggesting that activation of the immune system by an IMiD may have worsened her disease [28,29].

It seems likely that C3GP is under-diagnosed. Indeed, three of the five patients in the current series were initially diagnosed as MPGN. The diagnosis of C3GP was only established after either repeat biopsies or review of older ones. Since awareness of the diagnostic criteria has recently increased, there will likely be an increase in patients diagnosed with C3GP.

Conclusion

Our study strengthens the evidence that paraproteinemia is an underlying etiology of C3GP. In the future it will be important to determine whether patients like these should receive treatment for symptomatic MM, complement inhibitors alone or both.

Competing Interests

The authors declare that they have no competing interests

Authors Contribution

DC and GM conceived of the study, WM and DC compiled the clinical data, all authors have read the manuscript.

Consent

"Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review by the Editor of this journal."

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Research Article Open Access

Clinico Hematological Profile and Phase Distribution of Chronic Myeloid Leukemia

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Summary

Objectives: To evaluate the Clinico hematological profile based on the age, sex and Clinico hematological presentations and frequencies of three phases of chronic myeloid leukemia (CML), This study highlight the Ph positively by real time polymerase chain reaction (RT-PCR) technique contribute towards understanding the disease biology, and have important implications for diagnosis and management of CML patients.

Study design: This is an experimental and observational study.

Place and duration: This study was conducted in medical ward and pathology department of Peoples University of Medical and Health Sciences for women (PUMHS-W) Nawabshah from June 2013 to June 2014.

Materials and methods: Total 83 patients including 52 male, 31 female at their age ranges between 23 and 57 years admitted in medical ward of PUMHS hospital were selected for study. The clinical history and physical examination of these patients were noted. All the blood samples and bone marrow biopsy sent to the pathology department of PUMHS for the analysis of complete blood count, peripheral blood and bone marrow examination for the diagnosis of three phases of chronic myeloid leukemia.

Results: Out of 83 patients, 52 were male and 31 were female with male to female ratio of 1.6:1, the mean age of these subjects was 39.5 ± 16.5 years. The mean total leukocyte counts, platelet counts, hemoglobin levels and marrow blast frequencies were 121,000 ± 35,000/cmm, 285,000 ± 122,000/cmm, 7.5 ± 4.9 and 15 ± 9 respectively. The majority of patients 62 (74.6%) were classified in the chronic phase (CP), 17 (20.4%) in the accelerated phase (AP) and 3 (5.0%) in blast crisis (BC). The most frequent patient age ranges were 21-30 years for CP, 41-50 years for AP and 41-50 years for BC.

Conclusion: This study concluded that most CML patients are from a younger age group (33-47 years). Males were more commonly affected than the females. The detection of ph chromosome positively by resent and advanced RT-PCR technique is mandatory for the diagnosis and treatment of CML patients.

Keywords: Chronic myeloid leukemia; Phase distribution; Response to therapy; RT-PCR; Philadelphia chromosome

Introduction

Chronic myeloid leukemia (CML) is a clonal malignant neoplasms of pleuripotent hematopoietic stem cell characterized by the excessive proliferation of mature granulocytes and their precursors in the bone marrow and peripheral blood caused by in 90% of cases due to the presence of Philadelphia chromosome and rarely by Hyperdiploidy of >50 chromosomes [1]. The translocation between chromosome 9 and 22 t (9,22) (q34;q11) leads to the formation of break point cluster region and Abelson's (BCR-ABL) a new hybrid fusion genes that encodes for an oncoprotein (P210) located in the cytoplasm that has a strong, capacity to activate tyrosine kinas resulting in the activation of several downstream signals that transform hematopoietic stem cells in to the leukemic cells, thus increased tyrosine kinas activity is currently thought to play a central role in the pathogenesis of CML [2]. In spite of leukemia induced factors, there are risk factors that enhance the CML and these factors include lower socio-economic status, occupational exposure to benzene, formaldehyde, high doses of ionizing radiation among the atomic bomb survivors, other risk factor such as alcohol abuse, obesity, weight gain during adulthood and effects of preservatives or pesticides used in the food industry causes CML [3,4].

Clinically in 50% of cases patients with CML are asymptomatic and remaining were present with anemia, spleenomegally, fever, bleeding tendency, hepatomegally, lymphadenopathy and complications such as renal failure, hearing loss and priapism, and laboratory findings include complete blood count, peripheral blood and bone marrow examinations showing low hemoglobin, total WBC count between 287×10^9/L and 535.7×10^9/L, thrombocytopenia or normal platelet count or thrombosytosis and peripheral blood smear showing increase number of mature and immature granulocytes including predominantly [5,6]. The Bone marrow pictures in CML without treatment showing hypercellularity due to excessive proliferation of the granulocytes with myelocytes predominantly and presence of blast cells from <10% to >20% in the bone marrow and peripheral blood according to the world health organization criteria that divide the CML in to chronic, accelerated phases and blast crisis, there is decreased or normal or increased megekayrioposis as well as moderate to marked reticulin fibrosis with presence of small megakaryocyte containing hypolobulated nuclei, sea-blue histiocytes and gaucher cell and

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these changes are return to the normal state after treatment and the immunohisto-chemistry is used for differentiating the myeloblastic and lymphoblastic crisis of CML [7,8]. The recent developments in the confirmation of diagnosis of CML by sensitive tests such as qualitative real time-polymerase chain reaction (RT-PCR) to identify transcript variants of BCR-ABL fusion genes and quantitative droplet digital PCR as well as RT-PCR tests are used for ratio of BCR- ABL transcripts levels with normal genes on the international scale (>1016 and monitoring the response to therapy of patients with chronic myeloid leukemia [7,8]. The Cytogenetic must be performed by chrosome banding analysis (CBA) of marrow cell metaphases for the detection of BCR-ABL+ nuclei and additional chromosome abnormalities among patient with CML, if marrow cell cannot be obtained, CBA can be substituted by inter-phase fluorescence in site hybridization (I-FISH) of blood cell using dual color dual fusion probes [9]. The marked improvements in the management of CML with first line gold slandered therapy of imatinib mesylate (IM), the first tyrosine kinase inhibitor (TKI) targeting the BCR-ABL1 oncoprotein that causes leukemia, the second line therapy of TKI such as nilotinib and desatinib and allogenic bone marrow transplantation are used in case of failure of three TKI in CP as a 3rd line therapy while treatment of AP and blast phase is required prolong use of TKI and allogenic bone marrow transplant [10] BCR- ABL – positive cells are genetically unstable and are prone to develop multiple and heterogeneous genomic abnormalities such as point mutations >90 in the kinas domain (KD) resulting in the transformation of the luekemic phenotype from chronic to acute hence leading to resistance to the tyrosine kinase inhibitors [11].

Material and Methods

Inclusion criteria

The study was conducted in the Pathology Department of the Peoples University of Medical and Health Science (PUMHS) at Shaheed Benazirabad between June 2013 and May 2014. Samples were collected from outpatient clinics and inpatients suspected to be suffering from blood cancer. In this study, all newly diagnosed CML patients (based on hematological profile) older than 17 years of age were included. A detailed history was obtained for each patient, and questionnaires and physical examinations were also administered Additional file 1. Investigations included complete blood counts with differential and bone marrow aspirations. After completion of the investigation, patients were categorized into various CML phases based on the World Health Organization (WHO) criteria. The chronic phase (CP) was defined as myeloid blasts less than 10% in the peripheral blood or bone marrow. The accelerated phase (AP) was defined as blasts 10-19% of white blood cells in peripheral and/or nucleated bone marrow cells; persistent thrombocytopenia (<100×109/L) unrelated to therapy or persistent thrombocytosis (>1000×109/L) unresponsive to therapy; increasing white blood cells and spleen size unresponsive to therapy and or cytogenetic evidence of clonal evolution. Blast crisis (BC) phase was defined as peripheral blood blasts ≥20% of peripheral blood white blood cells or nucleated bone marrow cells; extra medullary blast proliferation; and large foci or clusters of blasts on bone marrow biopsy.

Exclusion criteria

Ph chromosome - negative or BCR-ABL - negative CML, Pregnant or breastfeeding woman, and patients taking imatinib for treatment of CML were excluded.

Results

In our study total 83 patients, including 52 male and 31 female with male to female ratio of 1.6:1 and their mean ages was 39.5 ± 16.5 years were selected. The major clinical features in these subjects were anemia, massive splenomegally, Hepatomegally, history of fever with cough and bleeding. The Mean hemoglobin levels, 9.5 ± 2.9 g/dl, total leukocyte counts /cumm, 121000 ± 35000 /cumm differential leukocyte count % including mature leucocytes 43% such as neotrophils 21 ± 7, lymphocyte 8 ± 2, eosinophils 9 ± 2, monocytes 5 ± 3 and immature cells 57% composed of Blast 18 ± 12, promylocytes 4 ± 1, Myelocytes 25 ± 5, Metamyelocytes 9 ± 2, band cells 13 ± 3 platelet counts / cumm, 285000 ± 220000 / cumm respectively were noted in the present study (Table 1). The examination of peripheral blood smear in these patients was showing the nomocytic normochronic red blood cells with variability in size and shapes [12,13]. Plenty nucleated red blood cells and many mature and immature leucocytes including predominantly myelocytes with number of the blast cells form 6% to 30% were present in the chronic, accelerated phases and blast crises were seen while bone marrow smear were showing hypercellularity due to excessive proliferation of myeloid cell line predominantly of myelocytes hypolobated megakaryocyts with few blue histocytes and pseudogaucher cell. Total 83 patients with CML were divided in to chronic phase (CP) 62 (74.6%), 17 (20.4%) in the accelerated phase (AP) and 3 (5.0%) in blast crisis (BC). The parameters included in this study are present Table 2 show frequency of three phases of CML based on age, sex, spleenic size and number of blast cells in peripheral blood and bone marrow smears (Table 2).

Discussion

The Chronic myeloid leukemia(CML) being a commonest leukemia in Asia, needed Clinico hematological profile and frequency of three phases of CML with early diagnosis and treatment among the Asian populations to improve survival rate in CML reported by Altekruse et al. [7].

Hence Ahmed et al. [14] Reported that frequency of chronic phase (CP), accelerated phases (AP) and blast crisis (BC) were 77.8%, 15.5% and 6.7% respectively were observed in among the 45 patients suffering from CML with their mean age 37.9 yrs, and male: female ratio of 2.2:1 while Clinico hematological features were Anemia and massive splenomegally, hemoglobin 9.94 g/dl. The mean total leukocyte count 214.3x109/L, platelet count 551.4×109/L, and marrow blasts were 9.3% respectively. Buchner-Daley L, Brady-West D were reported the presenting features of 70 patients diagnosed with chronic myeloid leukemia, with male to female ratio of 2.4:1, had age incidence of 37 years while Weight loss and splenomegally were the most frequently seen and frequencies of three phases of CML were similar with above study. Bhatti et al. [13] studied the 335 patients with CML had mean age of 35.5 yrs, with male to female ratio of 2:1 while similar Clinico hematological features and frequency of three phases of CML were recorded. Mutaleb et al. [8] were detected Philadelphia (PH) chromosome positive CML cases by Real Time-Polymerase Chain Reaction RT-PCR in 58 (90.1%) out of 63 patients with male to female ratio of 2.0:1.0 and the mean values of age, hemoglobin levels, total leukocyte counts, platelet counts, and marrow blast frequencies were 37.4 years, 12.2 g/dl, 101×10^9/L, 409×10^9/L, and 2.8% in (CP),while 45.4 years, 8.7 g/dl, 121×10^9/L, 418×10^9/L, and 15% in (AP) and 45.5, 9.2 g/dl years, 311×10^9/L, 396×10^9/L, and 26% in (BC) respectively, They recorded the frequency of CP (81.2), AP (14.5), BC (4.1) respectively and the risk factors contributing to the early onset of CML were

`Mean age in years	Sex	Socioeconomic status
40 ± 17 years	Male 52 (63.8%) Female 31(37.4%) Male to female ratio 1.6:1.0	Poor 63 (75.9%) Lower middle class 15 (18.0%) Upper middle class 4 (4.8%)
Symptoms / clinical history		Physical examination
Asymptomatic; 10(12.03%) Symptoms due to Anemia 79 (95.1%) Pallor Fatigue, lethargy, Body aches, dizziness, nausea & vomiting Difficulty in breathing, Symptoms due to splenomegally 70 (84.3%) Abdominal distension Abdominal discomfort Pain left side of abdomen History of bleeding 12 (14.4) Symptoms due to infection; fever with cough 18(21.6%) Hypermetabolic state; loss of weight night sweat 10(12.0%)		Anemia Mild 19 (22.8) Moderate 39 (46.9%) Severe 25 (30.1%) Splenomegally Massive (≥ 10cm) 60 (70.2%) Moderate(4-9 cm) 55(66.2%) Mild (1-3 cm) 14 (16.8%) Hepatomegally 15(18.0%) Lymphadenopathy 8 (9.6%)
Hematological parameters		Result & Value
Hemoglobin g/dl, Total leucocyte count /cumm Differential leucocyte count; The % of mature and immature cells calculated out of 100 leucocytes / HPF Platelet count /cumm		9.5 ± 2.9 g/dl 121000 ± 35000 /cumm Mature cells 43% (neotrophils 21±7, lymphocyte 8± 2, eosinophils 9± 2, monocytes 5 ±3) Immature cells57% (Blast 18±12, promylocytes 4± 1, Myelocytes 25± 5, Metamyelocytes 9±2, band cells 13±3) 285000 ± 220000 / cumm
Examination of PBS		Examination of bone marrow
The red blood cells are nomocytic normochronic with variable in size and shapes. Plenty nucleated red blood cells are seen and many mature and immature leucocytes are seen, the majority of cells are myelocytes.		The bone marrow is hypercelurar due to excessive proliferation of myeloid cell line predominantly of myelocytes with few blue histocytes and pseudogaucher cell. The megakaryocytes or hypolobated.

N= Number of Patient PBS=peripheral blood smear
HPF= High Power Field %= percentage

Table 1: The evaluation of chronic myeloid leukemia based on age in year, sex, socioeconomic status and Clinico laboratory findings. (N=83)

Phases of CML	Chronic Phase	Accelerated Phases	Myeloid Blast Crisis
Frequency	62 (74.6%)	17 (20.4%)	3 (5.0%)
Age	30 ± 7	45 ± 6	45.5 ± 9.5
Sex	Male 42	Male 11	Male 2
	Female 20	Female 6	Female 1
	Ratio 2.1.1	Ratio 1.8.1	Ratio 2.0;1.0
Spleenic Size	< 10 / cm	>10 / cm	> 15 / cm
Number of blast cells in peripheral blood and bone marrow smears	6 ± 2	16 ± 4	26 ± 5

Table 2: The frequency of three phases of CML based on age, sex, spleenic size and number of blast cells in peripheral blood and bone marrow smears N=83

Formalin applied on fish for preservation, calcium carbide on fruits to ripen, brick dust in chili powder, urea to whiten rice and puffed rice, sawdust in loose tea, soap in Ghee, artificial sweetener, coal tar, textile dyes in sweetmeats and occupationally exposure of benzene, ionizing radiation in x-ray department, any form of formaldehyde used in industries including formalin. Studies detected Abelsons and break point cluster region (ABL-BCR) positive cases of CML in 40 patients, out of 48 patients by RT-PCR test with the mean age of 37.6 ± 14.1 years, male to female ratio 1.8:1 spleenic size 9.8 ± 5.8 cm, TLC 284.5 $\times 10^9 \pm 267.5 \times 10^9$. That indicated 92% specificity sensitivity and reliability of this test. Yaghmaie et al. [14] detected expression of one of the P120BCR-ABL transcripts including b3a2 (62%) and b2a2 (21%) among the 83% out of 75 Iranian patients, while the remaining showed one of the transcript of b3a3 and b2a2 while the similar two types of transcripts and additional cytogenetic abnormalities such as double PH chromosome, +8, +19 among the Indian 208 patients with CML ph chromosome positive had male to female ratio of 1.8:1 and mean age of 38 years of all the three phases of CML were observed by Anand et al. [15].

Conclusion

From the above discussions, following conclusion and recommendation were made. In our study, total 83 patients including 52 male and 31 female with male to female ratio of 1.6:1 and their mean ages was 39.5 ± 16.5 years and frequency of three phase of CML was 62.(76.4%) were in the chronic phase (CP), 17 (14.58%) respectively. The male are affected more than the female and chronic phase of CML was common in younger age group. The Philadelphia chromosome detection by RT-PCR in CML patients due to the limited sources, we can't perform this advanced test for the molecular analysis of CML.

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Case Report Open Access

Correlations between Risky Sexual Behavior and Parental Communication among Youth in Dilla Town, Gedeo Zone, South Ethiopia

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Abstract

Background: The youth is vulnerable to risky sexual behaviors that could lead to unfavorable health outcomes. Youth communication can be one of the most effective strategies in reducing risky sexual behaviors. Only a little has been explored about the role of a parent influence in protecting youths from risky sexual behaviors. Thus, this study tried to assess risky sexual behaviors and the influence of parents on risky sexual behavior among youths in Dilla Ethiopia.

Methods: A community based cross-sectional study design, supplemented by a qualitative study was employed. The data were collected in January, 2012 by using interview administered questionnaire for the quantitative part, while focus group discussion was employed for qualitative part of the study. Statistical Package for Social Sciences version 20 was used to analyze the data.

Results: From sexually active youths, nearly half (48.3%) of youths reported unprotected sex. In the recent sex, 23.9 % of youths had two or more lifetime sexual partners and 12.6% of youths had sex with non-regular partners. Males had two times more sexual partners than females (AOR: 2.02, 95% CI: 1.02, 4.21), on the other hand, females had three times more sex with non-regular partners than males (AOR: 2.67, 95% CI: 1.10, 6.51). Parental communication showed a significant relation to risky sexual behavior. The odds of having had multiple sexual partners were three fold higher among youths who don't discuss about sexual issues than who discussed (AOR: 3.12, 95% CI: (1.37,7.08). About one-fifth of youth had a discussion about sexual issues with their parents and they preferred the same sex to discuss on sexual issues with their parents and peers.

Conclusion: A substantial proportion of youths engaged in risky sexual behaviors in both sexes. Parents play a greater role in shaping the behavior of youths. Therefore, Behavior change communication should consider family environment and other factors which predict risk sexual behaviors has to be strengthened.

Keywords: Risky sexual behavior; Sexual communication; Parental communication; Dilla Ethiopia

Background

According to the World Health Organization (WHO), youths cover the age of 15 to 24 years, while young people cover the age of 10 to 24 years [1]. During this year, the challenges that youth face and the decisions they can make have a great impact on the quality of their lives [2]. In this stage, youth begin thinking about the future and places more emphasis on goal-setting and self-esteem. However, youth may begin to exhibit more risky sexual behaviors during these ages [3]. It may result from being easily influenced by peers, cultural taboos, inadequate sexual communication, limited support from parents and inappropriate parenting roles [4-6]. Risky sexual behaviors are any behavior that could lead to unfavorable health outcomes, including HIV/AIDS or other sexually transmitted diseases (STD), unplanned pregnancy and unsafe abortions [7,8]. It also includes behaviors like, having multiple partners, having risky casual or unknown sexual partners, early sexual initiation, having sex under the influence of stimulant substances, or having sex immediately after watching pornographic media and failure to take protective actions, such as use of condoms and birth control [4-6,9].

Worldwide, risky behaviors related to sexual practices among young people were one of the great challenges [10]. Young people's involvement in risky sexual activities remains a concern in sub-Saharan Africa [11]. Against the prevailing cultural norms in Sub-Saharan Africa, young people tend to engage in having multiple sexual partners, concurrent sexual partners and unprotected sexual intercourse [12,13]. Risky sexual behavior is not an exception in Ethiopia [14]. In Ethiopia, 60% of adolescent pregnancies are unwanted resulting from unprotected sexual intercourse [15]. Among youth, around 1.1% of women are infected with HIV [16]. Published researches also indicate that young adults are at high risk of practicing risky sexual behaviors [17,18].

Sexual communication is a crucial aspect of sexual socialization and plays a key role in influencing risky sexual behaviors [19]. The expectation is that frequent and positive parent–child communication on such matters will lower the probability of sexual risk taking [20]. Available evidence suggests that frequent, open and positive sexual communication between youth with their parents, teachers as well as peers decreases sexual-risk taking behaviors and promotes positive sexual behavioral outcomes, including delaying sexual debut, having fewer sex partners and promoting contraceptive use [21-24]. According to published research work, lack of communication of youth with their

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parents was highly associated with risky sexual behavior [25-28].

Youth believed that their sexual health and sexual decision making is influenced by their parents, and says parental communication would help them make healthy choices [22]. But due to cultural taboos, being ashamed and lack of communication skill makes them not to discuss openly with their parent [29,30]. In addition to these, parent's lack of confidence, talk perceived as unnecessary, and talk perceived as encouraging sex are considered as barriers to communication about sex [31]. Due to different barriers only 1 in 5 Ethiopian youth had discussed on sexual issues with their parents [27]. Another study conducted in Nekemte Town, West Ethiopia, only 23.4% of the respondents had ever communicated with their parents, i.e., mother or father [32].

The low levels of parent-youth sexual communication shown by previous studies might raise concerns regarding the efficiency of programs aiming to promote safer sexual practices among young people by targeting parent-child communication as a complementary source of information on reproductive health. Hence, parent-child sexual communication, when it starts during early adolescence and youth is quite important. Yet the dynamic nature of the problem of youth's risky sexual behavior is exacerbated by lack of studies about the influence of parents on the risky sexual behavior and communication in Ethiopia. Thus, this study tried to assess risky sexual behaviors and the influence of parents on risky sexual behavior among youth in Dilla Ethiopia.

Methods

Study settings and participants

The study was conducted in Dilla town, Gedeo Zone, which is located about 365 Kms south of Addis Ababa, the capital city of Ethiopia, and 85 Kms south of Awassa, the regional capital of Southern Nations Nationalities and Peoples' Regional State. A community based cross-sectional study design, supplemented by a qualitative study was employed in this study. This study included youth in the age group of 15-24 years residing in four randomly selected Kebeles of Dilla town.

Sample and sampling procedure

The sample size was calculated with a single population formula by considering, proportion of unprotected sexual intercourse among youth (P= 65%) in Dessie town [19], 95% confidence level and a 4 percent margin of error. After adding 10 % for the non - response rate, the total sample size was 603. Study subjects were sampled with systematic sampling technique from four randomly selected kebeles. The selection of study participants was based on probability proportional to size for randomly selected kebeles. Systematic sampling technique was used to select the study participants by considering the intervals. If there were more than one eligible youth in a household, one youth was selected randomly by using a lottery method during the data collection period; however, if eligible youth was not found in a household, the next immediate household was considered. A total of thirty youth was included in focus group discussion session with an average of seven youth per group within similar age groups. Among the total discussant sixteen males and fourth females were attended. Selection study subjects purposely sampling techniques which were living in a similar residential area.

Data collection method and measurement of variables

A quantitative data was collected using a standardized pre-tested interviewer questionnaire adapted from Sexual and Reproductive Health (SRH) questionnaires of the World Health Organization (WHO) [33] and Ethiopia demographic health survey questionnaire. Data collectors were given three days intensive training. Five trained data collectors were collecting the data through a face to face interview. Qualitative data were collected by using semi-structured, open-ended questionnaires.

Measurement of study variables

Risky sexual behavior was defined as a behavior that includes the number of sexual partners or sex with non-regular sexual partner or unprotected sex in the last 12 months. Risky sexual behavior was measured by using three yes / no item questions. We asked whether the participants used condom in their every instance of sexual intercourse and the frequency of condom use, number of sexual partners and the tendency of having sex with non-regular sexual partners in the last 12 months. The responses to these questions were dichotomous; "Yes" and "No" and these were used as the dependent variables in this study. Four dichotomous variables were created for the analysis: 1) sexually active in the last 12 months, 2) multiple partnerships in the last 12 months, 3) condom use with sexual partners in the last 12 months, and 4) having sex with non – regular sexual partners in the last 12 months.

Parent-youth communication was defined as the exchange of ideas or information about sexual issues between parent and youth. Parent–youth communication was assessed by four "yes or no" questions. We asked if respondents ever discussed with their parents at each key moment about sexual intercourse or sexual education, condom use during sexual intercourse or safe sex, number of sexual partners and sex with causal partner or unknown partner in last 12 months. These four items were summed and the scores ranged from 0 to 4.

Data processing and analysis

Data were entered and cleaned by using EpI INFO vision 3.5.1 and was transported to SPSS V- 20 for analysis. Descriptive statistics were run to see the overall distribution of the study subjects with regard to the variables under study. Bivariate logistic regression analysis was used to test the possible association of the independent variables with the dependent one. Furthermore, multivariate logistic regression analysis was used to see the net effects of each of the independent variables in explaining variation in the outcome variables. A level of significance at $\alpha \leq 0.05$ was determined for statistical tests. A qualitative study was analyzed using a thematic approach. Themes arising from the summary were used to write the text and were used complement the quantitative results and discussions.

Ethical considerations

Ethical clearance was obtained from Research and Ethics Committee (REC) of the school of public health, Addis Ababa University. After getting ethical clearance, permission letter was obtained from the Dilla town Administration office for data collection process. More importantly informed consent was obtained from all participants as well as confidentiality of the data was ensured.

Results

Socio-demographic characteristics of participants

Out of 603 youths, a total of 598 youths participated in the study making the response rate of 99.2 %. Nearly half (48.5%) of females and 51.5% males were participating in the study. Among total (60.2%) of youths were between the age ranges of 20 to 24 years, 49.8% of them were between 15 to 19 years. The mean age of the respondents was 20.23 (± 2.56 SD) years. The majority of respondents were Gedeo 150 (25.1%) followed by Wolayita 103 (17.2). Two hundred sixty five, 44.3% of the

respondents were protestant. Four hundred ninety seven respondents (83.1%) were living with their both parents, whereas 62 (10.4%) of them were living only with their mother (Table 1).

Initiation of sex and recent sexual behavior

Of the 598 participants, 273 (45.7%) had initiated sex, with the mean age of sexual initiation18.6 (± 1. 57 SD) years. More than half, 63.7% of the sexual initiations occurred from age 15-19 years. Among those who initiated sex, only 58 (21.2%) had used condoms, while 215 (78.8%) reported that they did not use condoms during their first sexual intercourse. The leading reasons (multiple responses) for their first sexual intercourse were partner trust pressure 111 (32.6%), maintaining relationship with their partner 81 (23.8%) followed by alcohol/ substances influence 26 (7.6%). Risky sexual behavior was noted among the study subjects, 19 (6.9%) and 58 (21.3%) had sex with commercial sex workers and non-regular sexual partners respectively. Moreover, a significant proportion of first sexual practice was unplanned (42.9%) and unprotected 215 (78.8%). The proportion of female youth who had unprotected sexual intercourse was higher than males (42.2% Vs 36.6%) in their first sex. The commonest reason for unprotected sex was reported to be partner trust 114 (49.4%) and accidental sex 85 (36.8%) (Tables 2 and 3).

Recent (in the last 12 months) sexual behavior indicated that 230(84.3%) had had sexual intercourse during that time. When asked about the type of recent sex partner/s, 207 (79.4%) reported regular partners and 29 (12.6 %) indicated non-regular partners. Regarding the number of recent sex partners, 175 (76.1%) had one partner, while 55 (23.9 %) had two or more partners. Among those who had multiple sexual partner, 31 (13.5%) had more than two sexual partners, while 24 (10.4%) had two sexual partners**.** Only 119 (51.7%) reported using condoms in their most recent sexual encounter, while 111(48.3%) had not used condoms. Of those who used condoms, only 106 (89.1%) used them consistently. The leading reasons (multiple responses) for not using condom during the recent sex were partner trust 99 (87.6%), accidental sex 6 (5.4%) and It reduces sexual feeling 4 (3.6%) (Tables 2 and 3).

This finding was also supported by focus group discussion results. Discussants mentioned that risky sexual behaviors were very common in this age group. "A twenty two year old girl said that "having multiple sexual partners, unprotected sex, and sex with non-regular partners were very common. Even when they had condoms, they practiced unprotected sex just by trusting the partners; some partners were still not open to discuss on the use of condom".

Substance use and sexual behaviors

Of 150 alcohol drinker, 129 (86%) had sex in the last 12 months under the influence of alcohol. Among sexually active alcohol drinker, 23 (14.7%) had sex with non-regular sexual partners and about (30%) of youth practiced unprotected sex in last 12 months. Twenty-one (13.5%) of male respondents had sex with commercial sex workers. Of 87 khat chewer, 77 (88.5%) had sex in the last 12 months under the influence of khat. Among sexually active khat chewer, 14 (14.6%) had sex with non-regular sexual partners and about (21%) of sexual practice was unprotected. Moreover, twenty (25.9%) of male respondents had sex with commercial sex workers after consuming khat in the past 12 months**.**

Parent-youth communication about sexual issues

Among the total respondents, almost all (98.7%) of respondents answered youth-parent sexual communication was important for future life. Generally, 137 (22.9%) youths reported that they had ever

Variable	Number	Percent
Sex (n=598)		
Male	308	51.5
Female	290	48.5
Age (n=598)		
15-19	238	
20-24	360	60.2
Educational status of respondents (n=598)		
High school (grade 9-10)	290	48.5
Primary and junior (grade 1-8)	146	24.4
preparatory (grade 11-12)	115	19.2
Diploma	46	7.7
Illiterate	1	0.2
Religion of respondents(n=598)		
Protestant	265	44.3
Orthodox	257	43
Islam	52	8.7
Catholic and no religion	24	4
Ethnic group(n=598)		
Gedeo	150	25.1
Wolayita	103	17.2
Gurage	101	16.9
Amhara	90	15.1
Oromo	82	13.7
Sidama	69	11.5
Others specify (burge, kembata, tigray)		
Youth living with (n=598)		
Father and Mother	497	83.1
Mother only	62	10.4
Father only	39	6
Educational status of parents (n=598)		
Both parents literate	409	68.4
At least one parent literate	154	25.8
Both illiterate	35	5.9
Fathers' Occupation (n=598)		
Government employee	180	30.1
Merchant	157	26.3
Daily Labour	73	12.2
Driver	71	11.9
private employee	57	9.5
Farmer	37	6.2
Has no job	16	2.7
Others Specify	7	1.2
Mothers' Occupation(n=598)		
House wife	251	42
Merchant	112	18.7
Private employee	89	14.9
Government employee	69	11.5
Daily lobar	58	9.7
Farmer	11	1.8
Others Specify	8	1.3
Family's monthly income (n= 598)		
< 500	79	13.5
500-1000	136	23.2
1000-1500	185	31.6
>1500	183	30.6
Don't known	15	2.5

Table 1: Socio-demographic characteristics of youths and youths' parent in Dilla town, Gedeo zone, January 2012/13.

Variable	Male n (%)	Female n (%)	Total n (%)
Ever had sex (n=598)			
Yes	144 (24.1%)	12(21.6%)	273 (45.7%)
No	164 (27.4%)	161(26.9%)	325 (54.3%)
Age at first sex (n=273)			
15-19	82 (30%)	92 (33.7%)	174 (63.7%)
20-24	40 (14.7%)	28 (10.2%)	68 (24.9%)
I do not remember	22 (8.1%)	9 (3.3%)	31(11.4%)
Reasons to start sex (n=341)			
Peer influence	60 (17.6%)	51 (15%)	111 (32.6%)
Maintain relationship	42 (12.3%)	39 (11.5%)	81 (23.8%)
Love of boy/girl friend	41 (12%)	33 (9.7%)	74 (21.7%)
Influence of alcohol	21 (6.2%)	5 (1.4%)	26 (7.6%)
Sex film influence	19 (5.6%)	-	19 (5.6%))
Rape/sexual coercion sex	-	17 (5%)	17 (5.0 %)
To gain money	-	9 (2.9%)	9 (2.9%)
Influence of Khat	4 (1.2%)	2 (0.5%)	6 (1.7%)
Other	3 (0.9%)	1 (0.3%)	4 (1.2)
(I don't know, personal interest)			
Relation of the first sex partner (n=273)			
Boy/girl friend	104 (38.1%)	91(33.3%)	195 (71.4%)
Non-regular partner	20 (7.3%)	38 (14%)	58 (21.3%)
Commercial sex workers	19 (6.9%)	-	19 (6.9%)
Other (classmate, friend)	1 (0.4%)	-	1 (0.4%)
Condom used at first sex (n = 273)			
Yes	44 (16.1%)	14 (5.1%)	58 (21.2%)
No	100 (36.6%)	115 (42.2%)	215 (78.8%)
Ever had sex in last 12 month (n=273)			
Yes	120 (44%)	110 (40.3%)	230 (84.3%)
N	24 (8.7%)	19 (7%)	43 (15.7%)
Relation of the last 12 months sex (n = 261)			
Boy/girl friend	102 (44.3%)	105 (45.7%)	207 (79.4%)
Commercial sex workers	24 (10.4%)	-	24 (10. 4%)
Non-regular partner	12 (5.4 %)	17(7.2%)	29 (12. 6%)
Condom use in last 12 months sex (n=230)			
Yes	63 (27.4%)	56 (24.3%)	119 (51.7%)
No	57 (24.8%)	54 (23.5%)	111 (48.3%)
Frequency of Condom use in last 12 months (n=119)			
Every time	57 (47.9%)	49 (41.2%)	106 (89.1%)
Some times	6 (5%)	7 (5.9%)	13 (10.9%)
Number of sexual partner (n=230)			
Only one	80 (34.8%)	95 (41.3%)	175 (76.1%)
Two	16 (7%)	8 (3.4%)	24 (10.4%)
More than two	24 (10.4%)	7 (3.1%)	31(13.5%)
Condom used when have sex more than one partner in the last 12 month (n=55)			
Yes	40 (72.7%)	14 (25.5%)	54 (98.2%)
No	-	1(1.8%)	1(1.8%)
Frequency of Condom use when sex more than one partner in last 12 months (n=54)			
Every time	37 (68.5%)	13 (24.1%)	50 (92.6%)
Some times	3 (5.6%)	1 (1.9%)	4 (7.4%)

Table 2: Prevalence of risky sexual behavior among sexually active youths in Dilla town, Gedeo zone, January 2012/13.

discussed on sexual issues with their parents, while 461 (77.1%) had never discussed on sexual issues with their parents. Female respondents reported significantly more sexual communication with their parents than males (12.4 Vs 10.5%) (Figure 1). The proportion of youth who have multiple sexual partners was higher among youths who don't discuss about sexual matters compare to their counterparts (19.6% Vs 4.3%). Parental communication showed a significant relation to risky sexual behavior. The odds of having had multiple sexual partners were three fold higher among youths who don't discuss about sexual matters than who discussed *(AOR: 3.12, 95% CI: (1.37, 7.08)* (Table 4). Youth who reported low rates of parental discussion on sex with non-regular

Variable	Male n (%)	Female n (%)	Total n (%)
Reasons for not using a condom in first sex (n 231)			
I trust my sexual partner	61(26.4%)	53(23.0%)	114 (49.4%)
I had accidental sex	35(15.2%)	50 (21.6%)	85 (36.8%)
I was drunk	10 (4.3%)	6 (2.6%)	16 (6.9%)
It reduces sexual feeling	2(0.8%)	5(2.2%)	7 (3 %)
I did not have a condom	1(0.4%)	3 (1.3%)	4 (1.7%)
Not like condom	2(0.9)	-	2 (0.9)
Partners are not like	-	3 (1.3)	3 (1.3)
Reasons not using a condom at last 12-month sex (n=113)			
I trust my sexual partner	53 (46.9%)	46 (40.7%)	99 (87.6%)
I had accidental sex	2(1.8%)	4 (3.6%)	6 (5.4%)
It reduces sexual feeling	2 (1.8%)	2 (1.8%)	4 (3.6%)
Not have a condom	2 (1.8%)	-	2 (1.8%)
My partner do not like condoms	1 (0.9)	-	1 (0. 9%)
Embarrassing to buy	1 (0.9)	-	1 (0. 9%)

Table 3: Reasons for not using condom among sexually active youths in Dilla town, Gedeo zone, January 2012.

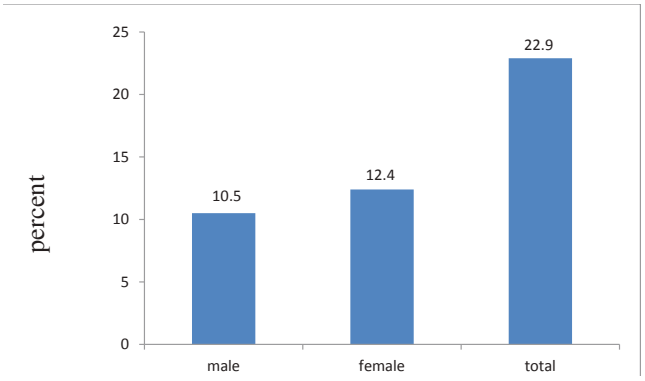

Figure 1: Proportion of youths who had communicated on sexual issues with their parents by gender in Dilla town, January 2012 (N=598

Variables	Had sex with multiple sexual partners in the last 12 months			
Sex	**Yes**	**No**	**COR (95% CI)**	**AOR (95% CI)**
Male	40 (57.4)	80 (34.8)	**3.17 (1.63, 6.15)***	**2.02 (1.02, 4.21)***
Female	15 (6.5)	95 (41.3)	**1**	**1**
Sexual communication				
Yes	10 (4.3)	60 (26.1)	1	**1**
No	45 (19.6)	115 (50)	**2.35 (1.11, 4.99)***	**3.12 (1.37, 7.08)***
khat Chewing				
Yes	34 (14.8)	43 (18.7)	**4.97(2.61, 9.46)***	**2.66 (1.25, 5.67)***
No	21 (9.1)	132 (57.4)	**1**	**1**
Alcohol drink				
Yes	47 (20.4)	82 (35.7)	**6.66 (2.98, 14.92)***	**4.16 (1.70, 10.17)***
No	8 (3.5)	93 (40.4)	1	**1**

Key* = (P-Value < 0.05, P-Value < 0.001), Parental communication includes at least one sexual issue

Table 4: Bivariate and multiva riate analysis of factors associated with risky sexual behavior of having multiple sexual partners among (230)sexually active youths in Dilla town, January, 2012/13.

partner reported higher rates of sexual behaviors with non-regular partner compare to their counterparts(11.7% Vs 0.8%).

With regard to youth-parent communication on different sexual issues, 135 (56.2%), 41 (17.1%), 34 (14.2%) and 30 (12.5%) of youth discussed on sexual intercourse, multiple sexual partners, condom use and non-regular sexual partner with their parents respectively (Figure 2).

On preference of the parents, males discussed on sexual intercourse with both their father and mother (18.4%% Vs 28.6%) respectively, while females preferred to discuss with their mother 67 (45.6%). Male respondents preferred to discuss on condom use with their father (22.2%), female youths preferred to discuss on condom use with their mother (47.1%). With regard to sex with non-regular sexual partners, males preferred to discuss with their mother than father (33.3% Vs 16.7%), whereas female chose their mother (47.7% (Table 5). Regarding youth discussion with their peer friends, they discussed with the same sex from their peer friends (Table 6).

Hindrances to youth, parent communication on sexual issues

Quite a number of factors were identified as hindering youth, parent communication on sexual issues. It was noted that youth blamed the Ethiopian culture which makes it a taboo to talk about sexuality issues with their parents. The most commonly mentioned reasons were shame to discuss followed by unacceptability with cultural taboo (Table 7).

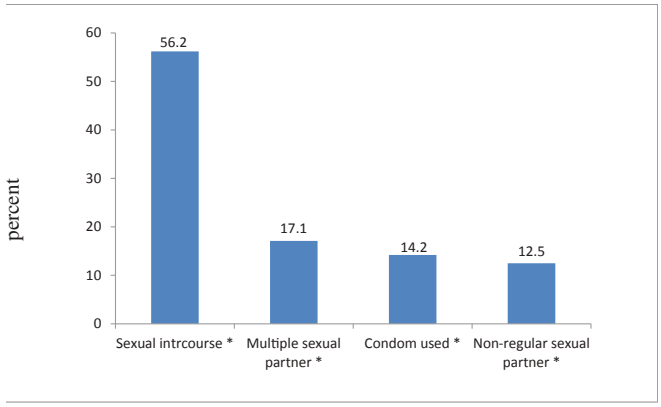

Figure 2: Youths' response to communication on different sexual issues with their parents in Dilla town, Gedeo zone, January 2012, (* = multiple responses were possible).

Topics of discussion	With whom they had discussed	
	Mother * (n= %)	Father * (n = %)
Sexual intercourse (n=147)		
Male	42 (28.6)	27 (18.4)
Female	67 (45.6)	11 (7.5)
Condom used (n=36)		
Male	10(27.8)	8(22.2)
Female	16(44.4)	2(5.6)
Sex with multiple sexual partner (n= 44)		
Male	11(25)	8(18.2)
Female	21(47.7)	4(9.1)
Sex with non-regular sexual partner (n=33)		
Male	10(30.3)	5(15.2)
Female	14(42.4)	4(12.1)

***= multiple responses were possible**

Table 5: Youths' response to the preference of parent's to communicate on different sexual issues in Dilla town, Gedeo zone, January 2012.

Topics of discussion	With whom they had discussed other than parents (n= %)				
Yes	Male friend*	Female Friend *	Boy/girl friend*	Brother*	Sister*
Sexual intercourse (n=998)					
Male	253 (25.4)	111(11.1)	94 (9.4)	44 (4.5)	24 (2.4)
Female	84 (8.4)	238 (23.8)	85 (8.2)	15 (1.5)	50 (5)
Condom use (n=558)					
Male	249(44.6)	80 (14.3)	43 (7.7)	56 (10)	9 (1.6)
Female	72 (12.9)	226 (40.5)	45 (8.1)	24 (4.3)	34 (6.1)
Multiple sexual partner(n=714)					
Male	66 (9.2)	248 (34.7)	16 (2.2)	19 (2.7)	9 (1.3)
Female	239 (33.5)	43 (6)	17 (2.3)	20 (2.8)	26 (3.6)
Non-regular partner(n=668)					
Male	248 (37.1)	57 (8.5)	16 (2.4)	32 (4.8)	9 (1.3)
Female	43 (6.3)	236 (35.3)	17 (2.5)	5 (0.7)	26 (3.9)

* Multiple responses were possible

Table 6: Youths' response to preference of other than parents to communicate on different sexual issues in Dilla town, Gedeo zone, January 2012.

Topics of discussed	Reasons for not communicate			
	Shame	**Culturally unacceptable**	**Parents are not good Listener**	**Don't know the reason**
Sexual intercourse (n= 474)	323(47.9%)	217(45.8%)	123(25.9%)	11(1.6))
Condom use (n= 711)	404(56.8%)	221(31.1%)	61(8.6%)	22(3.1%)
Sex with multiple sexual partner (n=694)	400(57.6%)	213 (30.7%)	59(8.5%)	22(31.7%)
Sex with non-regular sexual partner(n=714)	422(59.1%)	213 (29.8%)	58(8.1%)	21(29.4%)

Nb. Multiple responses were possible

Table 7: The major reasons for not communicating on different sexual issues with their parents among youths in Dilla town, Gdeo zone, January 2012/13.

This finding was supported by focus group discussion results.

A twenty three years old young man said that "youth realize the importance of discussing with their parents on sexual issues, but it is considered as a taboo topic and culturally unacceptable. Even it creates discomfort with families and feels embarrassed."

Chi-square test analysis for factor associated with risky sexual behavior

Socio-demographic variables were cross-tabulated to identify factors associated with risky sexual behavior. As presented in Table 8, Alcohol drinking showed a significant relation with sex with non-regular sexual partners ($p = 0.005$). Sex of respondents showed a significant relation to risky sexual behavior of having multiple sexual partners ($p = 0.001$). Parental communication was an important variable to influence youth's risky sexual behavior. It showed a significant relation to risky sexual behavior of having multiple sexual partners ($p = 0.024$). Similarly, alcohol drinking and Khat chewing showed a significant relation of having multiple sexual partners ($p = 0.001$) (Table 9).

"Focus group discussant said that, the use of substance, particularly alcohol, chat, and hashish led them to risky sexual behavior. Majority youth remarked that nightclubbing might facilitate for casual sex, commercial sex worker, and having multiple sexual partners. Peer pressure was also motioned as an important predisposing factor for inducing youth to risky sexual behavior".

Multivariate analysis of factors associated with risky sexual behavior

In multivariate analysis, sex of the respondent, parental communication, Khat chewing and alcohol drinking showed significant association with risky sexual behavior. Besides, female youth had three times more sex with non-regular partner than male youth, *(AOR: 2.67, 95% CI: 1.10, 6.6)*. Youth who drank alcohol had nearly four times more sexual intercourse with non-regular sexual partner than those who were not drinking alcohol (*AOR: 3.65, 95% CI: 1.26–10.42)* (Table 10).

On the other hand, male youth had two times more sexual partners than females, *(AOR: 2.02, 95% CI: 1.02, 4.21)*. Parental communication showed a significant relation to sexual practice of multiple sexual partners before and after adjusting for other variables. Youth who had never discussed on sexual issues with their parents had three times more sexual partners than those who discussed on sexual issues with their parents *(AOR: 3.12, 95% CI: (1.37, 7.08)*.

By adjusting for other variables, it was found that khat chewers were nearly three times more likely to have multiple sexual partners than those who did not chewing of khat *(AOR: 2.66, 95% CI: (1.25, 5.67)*. In addition, youth who drank alcohol were four times more likely to have multiple sexual partners than those who were not *(AOR: 4.16, 95% CI: (1.70, 10.17)* (Table 4).

Variables	Had sex with non-regular partners in the last 12 months		
	Yes (n %)	No (n %)	X2 (p-value)
Sex			
Male	12 (5.2)	106 (46.1)	3.32 (0.06)
Female	17 (7.4)	95 (41.3)	
Age			
15-19	6 (2.6)	25 (10.9)	1.48 (0.22)
20-24	23 (10)	176 (76,5)	
Educational status of youth			
Primary and High school	17 (7.4)	96 (41.7)	1.20 (0.23)
Preparatory and above	12 (5.2)	105 (45.6)	
Parent educational status			
Both literate	18 (7.8)	149 (64.8)	1.85 (0.17)
Other(++)	11 (4.8)	52 (22.2)	
Youth live with			
Both parents	23 (10)	165 (71.7)	0.13 (0.72)
Single parent	6 (2.6)	36 (15.7)	
Religion			
Orthodox	14 (6.1)	105 (45,7)	1.16 (0.69)
Other(+++)	15 (6.5)	96 (41.7)	
Ethnicity			
Gedeo	7 (3.0)	43 (18.7)	1.64 (0.83)
Non-Gedeo	22 (9.6)	158 (68.7)	
Family income			
< 1000	15 (6.5)	89 (38.7)	2.03 (0.25)
>1000	14 (6.1)	109 (47.4)	
Father's occupation			
Private and government Employer	17 (7.4)	122 (53)	2.75 (0.27)
Other (++V)	12 (5.2)	79 (34.3)	
Mother's occupation			
Housewife	14 (6.1)	79 (34.3)	0.85 (0.36)
Other (+++v)	15 (6.5)	122 (53)	
Sexual communication			
Yes	5 (2.2)	67 (29.)	3.05 (0.06)
No	24 (10.4)	134 (58.3)	
Chew khat			
Yes	14 (6.1)	61 (26.5)	3.71 (0.06)
No	15 (6.5)	140 (60.9)	
Alcohol drink			
Yes	23 (10)	104 (45.2)	7.79 (0.005)*
No	6 (2.6)	97 (42.2)	

Key: * showed significant, Other (++) includes (illiterate and one parent literate), Other (+++) includes (Protestant, Muslim, Catholic---) non Gedeo includes (Amahara, sidama, gurage --), Other (++V) includes (merchant, driver, farmer---) Other (+++V)includes(merchant, gov't and private employer -------)

Table 8: Chi-square test analysis offactors associated with risky sexual behavior of non-regular sexual practice among (230) sexually active youths in Dilla town, January, 2012/13

Variables	Had sex with multiple sexual partners in the last 12 months		
	Yes (n %)	No (n %)	X2 (p-value)
Sex			
Male	40 (57.4)	80 (34.8)	12.24 (< 0.001)*
Female	15 (6.5)	95 (41.3)	
Educational status of youths			
Primary and high school	32 (13.9)	105 (45.7)	0.06 (0.81)
Preparatory & above	23 (10)	70 (30.4)	
Parent education			
Both literate	37 (16.1)	130 (56.5)	1.04 (0.31)
Others (+)	18 (7.8)	45 (19.6)	
Living with			
Both parents	43 (18.7)	145 (63)	0.61 (0.43)
Single parent	12 (5.2)	30 (13.1)	
Religion			
Orthodox	33 (14.3)	87 (37.8)	1.77 (0.18)
Other(++)	22 (9.6)	88 (38.3)	
Ethnicity			
Gedeo	14 (6.1)	36 (15.7)	0.59 (0.44)
Non-Gedeo	41 (17.8)	139 (60.4)	
Family income			
< 1000	21(9.1)	58 (25.2)	0.48 (0.49)
>1000	34 (14.8)	169 (73.5)	
Father's occupation			
Private and government Employer	24 (10.4)	107 (46.5)	1.05 (0.45)
Other (+V)	31(13.5)	68 (29.6)	
Mother's occupation			
Housewife	22 (9.6)	72 (31.3)	0.02 (0.88)
Other (++V)	33 (14.3	103 (44.8)	
Sexual communication			
Yes	10 (4.3)	60 (26.1)	5.13 (0.02)*
No	45 (19.6)	115 (50)	
Chew khat			
Yes	34 (14.8)	43 (18.7)	26.07 (< 0.001)*
No	21 (9.1)	132 (57.4)	
Alcohol drink			
Yes	47 (20.4)	82 (35.7)	25.31 (< 0.001)*
No	8 (3.5)	93 (40.4)	

Key: Other (+) includes (illiterate and one parent literate), Other (++) includes (Protestant, Muslim, Catholic) non-Gedeo includes (Amahara, sidama, gurage --), Other (+V) includes (merchant, driver, farmer---) Other (++V) includes (merchant, gov't and private employer) Parental communication includes at least one sexual issue

Table 9: Chi-square test analysis of factors associated with risky sexual behavior of having multiple sexual partners among (230) sexually active youths in Dilla town, January, 2012/13.

Variables	Had sexual intercourse with non-regular partners in the last 12 months			
	Yes	No	COR (95% CI)	AOR (95% CI)
Sex				
Male	12 (5.2)	106 (46.1)	1	1
Female	17 (7.4)	95 (41.3)	1.58(0.72, 3.48)	2.67 (1.10, 6.51)*
Sexual communication				
Yes	5 (2.2)	67 (29.1)	1	1
No	24 (10.4)	134 (58.3)	2.40(0.88, 6.57)	2.76 (0.98, 7.77)
Chew khat				
Yes	14 (6.1)	61(26.5)	2.14 (0.97, 4.71)	1.88 (0.74, 4.83)
No	15 (6.5)	140 (60.9)	1	1
Alcohol drink				
Yes	23 (10)	104 (45.2)	3.58(1.40, 9.15)*	3.65 (1.26, 10.42)*
No	6 (2.6)	97 (42.2)	1	1

Key: * (P-Value < 0.05, P-Value < 0.001), Parental communication includes at least one sexual issue

Table 10: Bivariate and multivariate analysis of factors associated with risky sexual behavior of non-regular sexual practice among (230)sexually active youths in Dilla town, January, 2012/13.

Discussions

This study attempted to provide some insights on risky sexual behaviors and parent–youth communication on sexual issues. In addition, the study tried to see the influences of parent on risky sexual behavior of youths. This current study illustrates that as 84.3% youths had sex in the last 12 months. This finding is slightly higher than the study done in Dessie town (51.3%), in Bahir Dar town (64.8%), in Hawossa town (51.1%) and in the Gedeo zone (52.9%) [25,26,34,35]. This inconsistency may be due to the sample size and geographical variation. In this study, 6.9% and 21.3% of youth initiated sex with commercial sex workers and non-regular sexual partner respectively. In their first sex, about 42.9% and 78.8% of sexual practices were unplanned and unprotected respectively. This calls for a well-organized information, education and communication through peer educators to bring about behavioral change

In this study, 12.6% of youth had sex with non-regular sexual partner. This finding was lower than the studies done in Hawassa (43.3%), Bahir Dar (33%) and Dessie towns (14.2%) in Ethiopia and abroad in Nigeria (55.1%), [25,26,35,36]. Even though there is a lower proportion of this study, still it needs extraordinary attention to change their sexual behavior. Comparatively, in this study, 7.2 % of female youths and 5.4% of male youths had sex with non- sexual partners.

In this study, female youth had three times more sex with non-regular partner than male youth. Youths who drink alcohol were nearly four times more likely to engage in sexual activity with a non-sexual partner than those who didn't drink. This may be individuals who believe that alcohol promotes sexual behavior should be more likely to engage in risky behaviors when they drink than those who do not hold these beliefs. Others also indicated that alcohol users are almost two times more likely to have non-regular sex partner than non-users [37,38]. Yet sexual communication has an insignificant association with sex with non-regular sexual partner in this study.

In the current study, 23.9 % of youth had two or more sexual partners in the last 12 months, of which 10.4% had two and 13.5% had more than two sexual partners**.** This finding was slightly higher than the studies conducted in Assedabo town (21.5%) and Gedeo Zone-Ethiopia (8.9%) [26,33]. However, it was lower than the studies done in Bahir Dar 26.1%, Nekemet 34.5%, and Dessie towns 36% in Ethiopia and abroad in Nigeria (59.2%) [25,29,36,39]. Comparatively, in this study, 57.4 % of male youths and 6.5% of female youth had two or more sexual partners. Indeed, male youth had two times more sexual partner than female youth.

Parents are a powerful influence in the lives of their children. Youths believe that parents are the ones who have the most influence on their children's decisions about sex and were less likely to have risky sexual behavior. In this current study, the proportion of youth who have multiple sexual partners was higher among youth who don't discuss about sexual issues compare to their counterparts. Parental communication showed a significant relation to risky sexual behavior in this study. The odds of having had multiple sexual partners were three fold higher among youths who don't discuss about sexual issues than who discussed. Different research elsewhere showed youth who has more perceived parental connectedness have reduced the level of risky sexual behavior [40].

Alcohol consumption was significant predictors of risky sexual behavior and it showed that alcohol users are four times more likely to have multiple sexual partners than those who didn't drink. Regarding khat chewing, the odds of having had multiple sexual partners were three fold higher among youth who chewing of kaht than who didn't it. For the successful behavior change, individual should pay more attention about the linkage and possible consequences of such exposure to risky sexual behavior. Joining of knowledge on the linkage of exposure is helping youth to make lifestyle changes and offer the support to achieve optimal health.

In this study, 48.3% of youth practiced unprotected sex. This finding was higher than other study conducted in Ethiopia [33,34]. However, this finding was lower than the studies done in Dessie and Assendabo towns [25,41]. The finding was consistent with focus group discussion results; *one female participant said that "even though we knew about the importance of condom, due to lack of social support, we are not using it with confidence at all time. We need always to be encouraged to 'think out of the box'."*

Hence the availability of scientific knowledge and attitude concerning condom utilization is an important issue.

This study finding showed that less than one third (22.9%) of youth had communication at least one sexual issue topics with their parent. This finding was much lower than studies done in the USA (50%) and China (46%) [26,42]. This finding was also lower than studies done in Bullen woreda (29.8%) and Bahir Dar special Zone (60%) in Ethiopia [28,43]. The difference might be due to variation in the content of topics and cultural factors between these countries. Another possible reason may be due to difference in accessing information and the background of the parents. Researchers' interpretations of the low levels of parent–child communication emphasize the following main aspects. First, the transmission of information on the cultural norms of sexual conduct by parents is not a traditional practice. Second, low levels of parent–child communication about sexual issues may be explained by the sharing of child-rearing responsibilities between the parents and other family members from the nuclear or the extended family unit [44]. This finding is consistent with the FGD result, which may indicate that there is a gap in discussing the positive aspect of youth sexuality related issues. This shows us, it is important to establish and strengthen reproductive health club in and out-of-school youth centers; so that they can provide adequate information and services they need for in and out-of-school youths on reproductive and sexual issues.

In this study female youths had more sexual communication with

their parents than male youths. One explanation is that parents may have perceived that their unmarried youth were more vulnerable to social and health consequences of sexual activity, and engaged early discussions on sexual matters with them. The preference of youth to discuss on sexual issues depends on same sex. This finding was consistent with a study done in Bulleln woreda, Bahir Dar special zone region of Ethiopia [28,44]. The focus group discussion finding of this study also suggests mothers are more comfortable to talk with their daughter and father to son. Youth also prefers the same sex from their peers' friends to discuss sexual issues. This implies discussion with friends rather than parents may have a negative impact on youth' sexual behavior if their peer friends were not equipped with appropriate information on sexual issues. Therefore, there is a need to equip friends on sexual issues to avoid on such negative impact of youth' sexual behavior. But, why youth preferred non family member to discuss on the sexual issue could be another important research question that needs further investigation.

Cultural taboos, being ashamed and parent failure to give time to listen makes them not to discuss openly with their parent about sexual issue. This finding was in line with studies done in Bulleln woreda, Bahir Dar special in Ethiopia and abroad in Nigeria and Tanzania stated that the reason for not discussing about sexual issues with their parent is fear of parents, embarrassment, taboo attached to sex, parent fails to give time to listen, and parents lack of interest to discuss [28,43,45]. This is due to the fact that sexual conversations are deemed a taboo subject in many African communities. Hence sexual communication program on different sexual issues should be considered at the program level.

Strength and Limitation of the Study

The strength of this study is using quantitative and qualitative data. The limitations that sexual behavior was assessed based on self-reporting and it might be affected by social desirability bias because of sensitive nature and cultural barrier. Again, communication on sexual behaviors and attitude outcomes are sensitive and based on self-reported information, therefore some information may not be reported honestly. Longitudinal research is needed to examine what triggers, quality and timing of parent- youth communication on sexual related issues and the effect of communication on safer sexual behaviors.

Conclusions

This study has shown that a considerable proportion of youth engage in risky sexual behaviors in both sexes. Khat chewing, alcohol consumption and lack of parental communication were significantly associated with risky sexual behavior. There was low communication about sexual and reproductive health issues between parent and youth. Communications about sexual issues depend on the same sex basis and held more with peers than parents. Cultural taboo, feel ashamed and parent failure to give time to listen affect youth-parent communication about sexual issues.

Understanding the sexual experience of youth about the risks associated with sexual activities must be the fundamental element of interventions that are working in the area of risky sexual behaviors. Family environment, mostly family communication and positive relationships between parents and youth are linked to prevent or minimize risky sexual behaviors and also associated with avoidance or lower use of substance and less likely to initiate sex or be sexually active. Interventions that emphasize different domains of the risk factors and protective factors (family connection and support) in an integrated manner may be the most effective strategies. Consequently, programs and policies focused on reducing youth's sexual activity and the negative results should encourage the parents' presence and involvement in the lives of their children.

Competing Interests

All authors declare that they have no conflict of interest associated with the publication of this manuscript.

Authors' Contributions

AE conceived and designed the study and collected data in the field, performed analysis, interpretation of data, and draft the manuscript. AZ assisted with the design, analysis, and interpretation of data and the critical review of the manuscript. HT assisted with the design, interpretation of data and the critical review of the manuscript. YA participated in preparing the draft manuscript and critically reviewed the manuscript. All authors read and approved the final manuscript. All authors participated in critical appraisal and revision of the manuscript.

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Short Communication Open Access

Effects of Monacolin K of Red Rice and Glucomannan, Combined with a Low Calorie Diet, in Treatment of Dyslipidemia and Hypertension

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Abstract

Alarming increase in incidence of cardiovascular disease is result of a nutrition pattern characterized by an increase in consumption of fats, cholesterol, sugar and other refined carbohydrates, concomitant with low consumption of polyunsaturated fatty acids and fibers. The purpose of this study was to evaluate the efficacy of the monacolin K of red rice and glucomannan, associated with low-calorie diet, on improvement of lipid profile and hypertension, symptoms of metabolic syndrome. In 180 Neapolitan patients with metabolic syndrome, we evaluated effects of monacolin K of red rice and glucomannan, associated with a low-calorie diet, on total cholesterol (CT), LDL cholesterol (LDL), HDL cholesterol (HDL), triglycerides (TG) and blood pressure (BP). The results showed a significant decrease in average value of CT (-20%), LDL (-25%), TG (-20%), with an increase in HDL (+15%) and a decrease in BP (-20%). These findings corroborate evidences showing a therapeutic effects of these nutraceuticals in the treatment of dyslipidemia and hypertension, when a low-calorie diet alone is ineffective.

Keywords: Hypertension; Dyslipidemia; Cholesterol; Blood pressure

Introduction

Metabolic syndrome is a medical condition deserving of special attention because of its prevalence and impact [1]. This term does not indicate a single disease, but a group of predisposing factors which, joined together, place the subject in a band of high risk for diseases such as diabetes, cardiovascular problems and hepatic steatosis [2-5].

Numerous epidemiological and experimental studies have demonstrated that metabolic syndrome affects almost half of adults over 50-60 years [4,6-12]. Probably, this incidence will grow in the future, considering spreading childhood obesity. An excess of body fat, especially when it is localized in the abdominal region, leads to an imbalance of the metabolism of fats and sugars that induces hyperinsulinemia (high insulin level in the blood, an indicator of increased resistance to this hormone) [9,13]. In addition to strategies based on changing lifestyle, it is frequent utilization of natural substances, with beneficial properties [14-18]. Monacolin K of red rice (10 mg, 90% by policosanol) is able to inhibit the HMG-CoA reductase, which is a key enzyme in the biosynthesis of cholesterol. Glucomannan (3 g per day) is particularly useful in the reduction of body weight and cholesterol [19]. It is also useful in constipation to regulate bowel function alternately in irritable bowel syndrome. The use of these products called "nutraceuticals" would appear useful in patients with poor tolerance to traditional drugs or those who reject traditional drugs [20-23]. The purpose of the study was to evaluate efficacy of the monacolin K of red rice and glucomannan, associated with low-calorie diet, on improvement of lipid profile and hypertension, symptoms of metabolic syndrome.

Materials and Methods

180 subjects (83 females and 97 males) with mild to moderate hypercholesterolemia, (range of total cholesterol between 200 and 290 mg/dl), mild hypertension (no antihypertensive drug therapy; 140-159/90-99 mm Hg systolic/diastolic blood pressure), in which low-fat diet for three months did not achieve the treatment goal, were treated also with monacolin K of red rice (10 mg, 90% in policosanol per day) and fiber glucomannan (3 g per day) for 24 weeks. Blood pressure (BP), lipid profile [total cholesterol (CT), LDL cholesterol (LDL), HDL cholesterol (HDL), triglycerides (TG)], biochemical parameters related to tolerability (GOT, GPT, CPK) and body mass index were determined at baseline (T0), to 12th week (T1) and 24th week (T2). All biochemical parameters were performed by a single laboratory accredited to ISO parameters accuracy. All subjects expressed informed consent. The data were expressed as mean ± standard deviation (M ± DS) and percentage (%) values. Significance of differences between groups was determined by Student's test for paired data, for linear data and the X2 for nonparametric data (Table 1).

Results

The results are reported in table. Monacolin K of red rice and fiber glucomannan, associated with a low-calorie diet, for 24 weeks showed a reduction in CT, LDL and systolic-diastolic BP. At T1 COL was reduced by 12.8% in males and 13% females, LDL was reduced by 15% in males and 16.2% females. HDL was increased by 6.1% in males and 6.6% females, TG were reduced by 14.9% in males and 13% females. BP was decreased by 20% for both diastolic and systolic values. At T2 COL was reduced by 19.3% in males and 19.5% in females, LDL was reduced by 25.2% in males and 27% females; HDL was increased by 11.2% in males and 15.4% females. TG were reduced by 22% in males and 21% females. Body weight decreased in association to decrease in lipid parameters.

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	Basal	12th week		24th week		Statistical significance
	M ± DS	M ± DS	D % vs Basale	M ± DS	D % vs Basale	p
Total cholesterol (mg/dl)	262 ± 35	228 ± 32	-12.7	213 ± 27	-19.5	≥ 0.01
LDL cholesterol (mg/dl)	179 ± 29	149 ± 28	-15.6	135 ± 24	-25.9	≥ 0.01
HDL cholesterol (mg/dl)	52 ± 12	54 ± 14	6.7	55 ± 13	13.4	n.s
Triglycerides (mg/dl)	175 ± 13	135 ± 46	-14	136 ± 35	- 20.2	n.s
Systolic blood pressure (mmHg)	150 ± 9	141 ± 10	-9	117 ± 5	-33	≥ 0.01
Diastolic blood pressure (mmHg)	94 ± 4	87 ± 5	-7	77 ± 6	-17	≥ 0.01
Body mass index	28.1 ± 2.2	26.1 ± 2.5	-6.4	24.9 ± 2.3	-11,3	≥ 0.01

Table 1: Average values (SD) at baseline at 12th week and 24th week in 180 patients.

Discussion

High COL is one of many factors that predispose to cardiovascular diseases. Some of these factors are modifiable (smoking cigarette, blood pressure, diabetes mellitus), while others are called non-modifiable (age, sex, family history and genetic factors). The influence of diet on COL is on average equal to 15%, although significant modifications of the contribution dietary can cause variations up to a ± 30%. Possible failure of the diet requires use of lipid-lowering agents. Medicines used in presence of hypercholesterolemia are statins (HMG-CoA reductase) and fibrates (most useful in presence of high triglycerides).

An alternative strategy to classical pharmacological intervention is use of nutraceuticals with a good tolerability profile and activity on lipid profile [24]. A good effect on cholesterol synthesis is obtained by policosanol. These depress expression of HMG-CoA-reductase in concentration-dependent manner, probably by receptor-mediated mechanisms that inhibit transcription for this enzyme [25,26]. Some studies showed that policosanol induces other effects on cardiovascular risk, similar to pleiotropic effects attributed to statins [27]. Red rice to 5% in monacolin K is a natural substance that is used for thousands of years in China to achieve desirable values of cholesterol. Glucomannan is a polysaccharide with high molecular weight, is extracted from tuber of Amorphophallus konjac, a plant used in Japanese cuisine as an agent gelatificator. This fiber has the ability to attract a lot of water, increasing its volume up to 60-100 times and giving rise to a soft gelatinous mass. This feature gives glucomannan a dual effect: it reduces sense of hunger and absorption of fats and sugars, which are trapped in soft, viscous mass that forms in the intestine [27-34].

The present study showed that monacolin K of red rice and glucomannan is useful in therapy of dyslipidemia and hypertension, when a low-calorie diet alone is ineffective, and it confirm effectiveness of these nutraceuticals. Since low-fat diet alone for three months (before integration of monocolin K and glicomannan) did not achieve effects on metabolic parameters, the improvement in the lipid results could be attributed to a direct effect of monacolin K and glucomannan. In perspective, this experiment should be extended to younger subjects before appearance of metabolic syndrome. In this way, we could assess possible positive effects in prevention of metabolic syndrome.

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Case Report Open Access

Epidemiological Study on H. pylori in Cattle and Its Milk with Special Reference to its Zoonotic Importance

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Abstract

This study was carried out to investigate some epidemiological aspects on the occurrence of Helicobacter pylori in cattle, milk and humans at Dakahlia province, Egypt during the period from February 2014 to June 2015. A total of 304 samples including 117 rectal swabs (53 cows and 64 buffaloes) and 85 milk samples (36 cows and 49 buffaloes) sand 102 humans stools were collected and subjected to bacteriological examination by culturing on Columbia Blood Agar (CBA) and biochemically identified. The results showed that the overall occurrence of *H. pylori* were 21.7% in collected samples. The occurrence in cattle faeces was 18.8% (11.9% in cows and 6.9% in buffaloes). However, the isolation rate from cattle milk was 28.2% (10.5% in cows and 17.7% in buffaloes). Moreover, the frequency distribution of *H. pylori* from human stool was 19.6%. Concerning animal breed, native breed of cattle and their milk showed higher occurrence (5.2% in cow's faeces, 11.9% in buffalo's faeces, 7% of cow's milk and 17.7% of buffalo's milk). Regarding animal age, the occurrence of *H. pylori* was increased with increasing age. On the other hand, the frequency distribution of *H. pylori* was more prevalent in the samples (faeces and milk) collected from Mansoura center. In relation to human samples, with respect to gender, males showed higher isolation rate (11.7%) than females (7.8%).Whereas, frequency of *H. pylori* in adults (4.9%) was higher than young (1.96%).On the other hand, *H. pylori* was more frequently isolated from patients (8.8%) with gastrointestinal disorders. Moreover, the occurrence of *H. pylori* was higher in human samples collected from persons of occupations related to animals such as veterinarians (6.8%), dairy workers, and farmers (3.92% each) than others. It could be concluded that *H. pylori* could be isolated from cattle, milk and humans with recognizable percentages, suggesting its zoonotic significance and role played by cattle especially buffaloes and its milk as potential reservoir and source of human infection. The zoonotic significance for *H. pylori* as well as the recommended preventive measures which should be taken to avoid the risk of contamination of milk and human infection were fully discussed.

Keywords: Epidemiology; Zoonosis; *H. pylori*

Introduction

The Helicobacter genus consists of a group of microaerophilic, none sporulating, Gram-negative rods that colonize on the mucus layer covering the epithelial surface of the gastrointestinal tract of humans and a variety of animal species. There are currently 6 validated Helicobacter species isolated from gastric tissue and 16 validated entero hepatic species. Some Helicobacter species may be commonly (*H. aurati*) or occasionally (*H. bilis and H. muridarum*) isolated from both gastric and entero hepatic sites [1].

Although *H. pylori* is present in the stomachs of about half of world's population, the routes of transmission are still unclear and non-human reservoirs have not been identified. The prevalence of *H. pylori* infection increases with age and is inversely related to socio-economic and hygiene status, suggesting person-to person transmission. Several studies have shown high prevalence of antibodies against *H. pylori* in abattoir workers, such as veterinarians, butchers and slaughterers, suggesting that *H. pylori* might be transmitted from animals to man. Dogs and sheep have also been implicated in the transmission of Helicobacter infection [2].

From the zoonotic point of view and public health importance of H. pylori, recently recovery of *H. pylori* from cows by Dore et al. [3] supported this idea. Milk (especially raw milk) and its products are indicated as an important vehicle for transmission of pathogenic microorganisms, where, milk is considered as cultural and growth media for such organism [4].

Little information about the epidemiology of *H. pylori* in dairy animals in Egypt are known , also the reports dealing with its zoonotic importance and role of cattle and its milk which act as reservoir and source of human infection in Egypt are scarce or absent. So, this work was conducted to carry out some epidemiological studies on the occurrence of *H. pylori* in cattle and their milk. Also, the epidemiological aspects of *H. pylori* in man were investigated.

Materials and Methods

A total of 304 cattle, milk and human samples were collected from Mansoura and Aga centers, at Dakahlia province, Egypt. From cattle, 117 faecal samples including (53 cows faeces, 64 buffaloes faeces) of different ages and breeds were taken directly from the rectum of animals using sterile swabs, The swabs then directly immersed in tubes containing Tryptone soya broth and immediately transported to the laboratory in ice box under complete aseptic conditions. The detailed data concerning locality, age, sex, breed, housing, health status, number of parturition, stage of lactation, water supply, feeding pattern and hygienic disposal of animal wastes. From raw milk, 85 milk samples consisting of (36 cows and 49 buffaloes) were

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collected in sterile cups. From human stool specimens, 102 represented (45 apparently healthy, 35 with gastrointestinal disorders, and 22 diarrheal stool specimens) were obtained .All samples were collected in sterile cups and transported to laboratory in ice box under complete aseptic conditions. The data including age, gender, health status, socio-economic level, hygienic practices, occupations and previous suffering from dyspepsia. All collected samples were cultured within six hours from collection.

Isolation and Identification of *H. pylori*

All collected animal rectal swabs were directly streaked on CBA supplemented with antibiotic (Vancomycin, Trimethoprim and Cefsulodin). A loopful from each collected milk sample was plated on CBA. A sterile swab was taken from each human stool sample and directly cultured on CBA. All cultured plates were incubated under microaerophilic conditions (5% O_2, 15% Co_2 and 80% N_2) using generating kits (Campygen TM 2.5 L) (Oxoid CN0025A) for creation of microaerophilic atmosphere at 37°C for one week (5-7 days) After the incubation, the suspected growing colonies (tiny, small and translucent) were picked up and inoculated on CBA slope, incubated microaerophilically at 37°C for 5-7 days for further identifications [5].

The tests used for identification of *H. pylori* isolates were Gram staining, oxidase, catalase, urease, TSI, growth at 1% glycine and resistance to Nalidixic acid and cephalothin [6].

Results and Discussion

Helicobacter pylori, is well established as a major cause of gastritis, peptic ulcer, duodenal ulcer and chronic gastritis. It is also implicated in the development of gastric cancer. Consequently, the detection of *H. pylori* infection has become important, due to fastidious and slow growing nature of *H. pylori,* great care was needed in the collection, transport and culture [7]. However, the origin and transmission of this bacterium has not been obviously explained. One of the suggested modes of transmission is cattle milk consumed by human beings [8].

H. pylori is one of the most common bacterial infectious agents inhabits the stomach of more than half of world's population. The presence of *H. pylori* antigens in faeces of cows which contaminates the cow's milk and might be a transmission route of *H. pylori* infection to man. Moreover, faecal contamination to milk due to improper hygienic practice during production and management processing could transfer *H. pylori* to milk consumers. From the zoonotic point of view, it was utmost of importance to study the role of cattle and its milk as reservoir and source of human infection with *H. pylori* .According to available data and literatures, there was no previous studies in Egypt dealing with zoonotic importance of H. pylori, moreover, the articles investigated and discussed the epidemiology of *H. pylori* in cattle in Egypt are scarce or absent.

In the present study, the frequency distribution of *H. pylori* from Holstein cows was 1.7% (Table 1). In a previous study carried out on 92 lactating Holstein cows in Shahrekord, Iran found that the prevalence of *H. pylori* antigens in faeces of cows were 10.8 %. The recorded results in Table 1 showed that the native or Baladi breeds of cows had higher frequency distribution of *H. pylori* (5.2%) While the buffaloes native breed were showed a percentage of (11.9%). From the results recorded in Table 1, it could be notice that the examined native breeds of cows and buffaloes showed higher isolation rates of *H. pylori* than the examined Holstein breeds. This might be suggested that either native or Baladi breeds are more susceptible to exposed to carry *H. pylori* in their gut and shedding in their faeces or the native breeds might be reared under low level of hygienic practices (Table 2).

The frequency distribution of *H. pylori* in animal faecal samples in relation to age was illustrated in Table 2. There were various frequency distribution of H.pylori with different age group in cattle (from 0-6 months, from 6-12 month, from 12-18 month, from 18-24 month and over 2 years), the frequency distribution of *H. pylori* were 1.7%, 0.85%, 0.85%, 0.85%, and 2.65%, respectively, whereas their respective frequency distribution of *H. pylori* in buffaloes were 0.85%, 0.85%, 1.7%, 1.7% and 6.8% (Table 3).

It was obvious from recorded results in Table 2 that the highest Incidence (6.8% and 2.65%) of *H. pylori* was in buffaloes and cows over than 2 years, respectively. The most examined over than 2 years .Females are kept for lactation and breeding for old ages which they were more exposed to *H. pylori* infection. However, the present results showed that small or young ages were with low frequency rates (1.7%) in cows and (0.85%) in buffaloes. This explained that the age may be considered as a risk factor for *H. pylori* infection. This result suggesting the old age of cows and buffaloes are more exposed to harbor *H. pylori* infection (Table 4).

The results showed that the examination of the faecal samples collected from various localities showed variable frequency distribution rates from one area to another (Table 3). In cows the samples collected from Aga district, Nawasa Al-Bahr village, Mitishna village, Mansoura district, Awish Al-Hagar village were showed occurrence of 0.85%, 1.7%, 0.85%, 2.65% and 0.85%, respectively, while the respective occurrence frequency distribution in buffaloes were 1.7%, 0.85%, 2.6%, 5.9% and 0.85%. The recorded results revealed that higher frequencies (5.9% and 2.65%) were found in buffaloes and cows in Mansoura district (Table 5).

This might be due to the examined farms in Mansoura district were mixed herds of cows and buffaloes of low hygienic conditions in farms, which facilitate the contact between buffaloes and cows and give the opportunities to harbor the *H. pylori* microorganism.

In the present study, the frequency distribution of *H. pylori* from Holstein cow's milk was 3.5 %, while from the native breed or Baladi breed was 7%. On the other hand the frequency distribution of buffalo's milk from the examined native breed (which is the only buffalo breed in Egypt) was 17.7% which was higher than Holstein cows and native breed cows. Lower frequency distribution from raw samples from Holstein cows in different geographic areas of Japan was previously detected by Fujimura et al. [4] who found that 72.2% were positive for *H. pylori* from raw milk samples and 55% commercial pasteurized milk.

It was found that the frequency distribution of *H. pylori* in cow's milk from different localities of Aga district, Nawasa Al-Bahr village,

	Breed of the animal	Number of examined samples	Number of Positive Samples	Percentage of Positive samples
Cows	Holstein	12	2	1.7
	Native breed cow (Baladi)	41	6	5.2
	Total	53	8	6.9
Buffaloes	Native breed buffaloes	64	14	11.9
Total		117	22	18.8

Table 1: Frequency distribution of *H. Pylori* in animal faecal samples in relation to breed.

	Age of the animal	Number of examined samples	Number of Positive Samples	Percentage of Positive samples
Cows	0-6 month	4	2	1.7
	6-12 month	7	1	0.85
	12-18 month	2	1	0.85
	18-24month	4	1	0.85
	Over 2 years	36	3	2.65
	Total	53	8	6.9
Buffaloes	0-6 month	2	1	0.85
	6-12 month	4	1	0.85
	12-18 month	6	2	1.7
	18-24month	3	2	1.7
	Over 2 years	49	8	6.8
	Total	64	14	11.9
Total		117	22	18.8

Table 2: Frequency distribution of *H. Pylori* in animal faecal samples in relation to age.

		Locality of collected animal faecal samples	Number of examined samples	Number of Positive Samples	Percentage of Positive samples
Cows	Aga Center	Aga district	8	1	0.85
		Nawasa Al-Bahr village	14	2	1.7
		Mitishna village	9	1	0.85
	Mansoura Center	Mansoura district	12	3	2.65
		Awish Al-Hagar village	10	1	0.85
		Total	53	8	6.9
Buffaloes	Aga Center	Aga district	13	2	1.7
		Nawasa Al-Bahr village	6	1	0.85
		Mitishna village	21	3	2.6
	Mansoura Center	Mansoura district	16	7	5.9
		Awish Al-Hagar village	8	1	0.85
		Total	64	14	11.9
	Total		117	22	18.8

Table 3: Frequency distribution of H. pylori in animal faecal samples in relation to locality.

	Breed of the animal	Number of examined samples	Number of Positive Samples	Percentage of Positive samples
Cows	Holstein	13	3	3.5
	Native breed cow(Baladi)	23	6	7
		36	9	10.5
Buffaloes	Native breed buffalo	49	15	17.7
Total		85	24	28.2

Table 4: Frequency distribution of H. pylori in raw milk samples in relation to breed.

Mitishna village, Mansoura district, Awish Al-Hagar village were 2.3%, 3.55%, 1.2%, 4.7% and 2.3%, respectively, while their respective frequency distribution of buffaloes milk were 3.55%, 3.55%, 1.2%, 8.2% and 1.2%. The highest frequency distribution of *H. pylori* (8.2% and 4.7%) was from cow's milk and buffalo milk in relation to locality was found in Mansoura district. The lowest frequency distribution was found in Mitishna village which was 1.2% in both cows and buffaloes (Table 5).

		Locality of collected raw milk samples	Number of examined samples	Number of Positive Samples	Percentage of Positive samples
Cows	Aga Center	Aga district	7	2	2.3
		Nawasa Al-Bahr village	7	3	3.55
		Mitishna village	2	1	1.2
	Mansoura Center	Mansoura district	13	4	4.7
		Awish Al-Hagar village	9	2	2.3
		Cows	36	9	10.5
Buffaloes	Aga Center	Aga district	11	3	3.55
		Nawasa Al-Bahr village	7	3	3.55
		Mitishna village	2	1	1.2
	Mansoura Center	Mansoura district	20	7	8.2
		Awish Al-Hagar District	9	1	1.2
		Buffaloes	49	15	17.7
Total			85	24	28.2

Table 5: Frequency distribution of H. pylori in raw milk Samples in relation to locality

The overall isolation rate of *H. pylori* from human stool samples was 19.6%. The isolation rates were 11.7 % and 7.8% in males and females, respectively. In a study higher isolation rates (48.27%) were recorded of patients with hydatid liver diseases were positive, of which were 32.7% and 15.5% were in males and females, respectively. in another study carried out by Uemura et al. [9] who detected *H. pylori* in 445 non ulcer dyspeptic patients with the percentage of 46.2% in males and 53.7% in females, moreover in patients with duodenal ulcers, the isolation rates in males were 72% and 28% in females. In patients with gastric ulcer, the isolation rates of *H. pylori* were76% and 24%in males and females, respectively. Furthermore, in patients with gastric polyps, the results were 36.6% and 64.4% in males and females. The higher isolation rates were also previously reported by Rasheed et al. [10] who found the overall percentage of *H. pylori* was 74.4%, moreover the incidence of *H. pylori* was 73.5% in males and 75.4% in females. The isolation rate of *H. pylori* from females (7.8%) was almost similar to the isolation rate of a study previously reported by Ahmed et al. [11] who found the isolation rates of the examined females percentage was 7.4%, while their isolation in males was 28%. The recorded results revealed that *H. pylori* was slightly higher in examined males than females, this could illustrate that males which are more susceptible than females to carrying and infection with *H. pylori* (Tables 6 and 7).

This conviction was fully supported by the idea of Klein [12] who stated that males which are more susceptible than females to infections caused by bacteria, viral, fungi and parasites due to males generally exhibit reduced immune responses fully compared to females. These differences are usually attributed to socio-ecological, physiological (hormonal in origin), and occupational (referred to animal contact), so, the females less susceptible to infection than males, not only because of the androgenic hormones which reduce the immunity but also sex steroid hormones affect disease resistant genes and behavior which make males more susceptible to infection.

Regarding frequency of *H. pylori* from human stool with regard to age, there were various frequency distribution of *H. pylori* with different age groups in humans, the frequency distribution of *H. pylori* in human were 2.95%, 1.96%, 3.92%, 3.92%, 1.96 % and 4.9%, respectively. The age

Gender of human	Number of examined samples	Number of positive samples	Percentage of Positive samples
Male	59	12	11.7
Female	43	8	7.8
Total	102	20	19.6

Table 6: Isolation rate of H. Pylori from Human stool concerning to gender.

Age of human	Number of examined samples	Number of positive samples	Percentage of Positive samples
1-10 years	15	3	2.95
10-20 years	17	2	1.96
20-30 years	22	4	3.92
30-40 years	20	4	3.92
40-50 years	15	2	1.96
50-60 years	13	5	4.9
Total	102	20	19.6

Table 7: Frequency of *H. pylori* from human stool with regard to age.

groups varied (From 1-10 years, from 10-20years, from 20-30, from 30-40, from 40-50, from 50-60). The highest rate (4.9%)was found in the age group from 50-60 years old, while the lowest frequency distribution rate (1.96%) was found in 2 age groups from 10-20 years and from 40-50 years .In a study performed by Windsor et al. [13] recorded that the males under age group less than 10 years showed highest prevalence (11%), whereas, females aged from 11 to 20 years showed that the highest prevalence (14%) in rural community. In urban community, the males aged from 11to 20 years was highest (9%), however in females, the highest prevalence was in age group from 31-40 years with (13%). Incidence rate of *H. pylori* detected in adults in the present study agreed with the previous reports of Malaty et al. [14] who carried out a study on a total of 413 person (161 adults and 252 children) adult age range from 20 to 75 years and the children from 1 to 19 years. The overall seropositivity rate of *H. pylori* was 75% among adults and 22% among children. Higher frequency of isolation of *H. pylori* in young age was also recorded in another study carried out by Naficy et al.[15] who reported that 42 % children aged from 6-17 months were positive for *H.pylori* infection.

It was obvious that adults showed higher prevalence of *H. pylori* than young. This indicated that the prevalence increases with increasing the age, this conviction was previously supported by Rasheed et al. [10] who mentioned that the prevalence of *H. pylori* in human population increased with increasing age and presence of household animals and size of family and members of family.

Concerning results of bacteriological examinations of human stool samples for *H. pylori* in relation to healthy state, the highest occurrence (8.8%) had been showed in the patients with gastrointestinal disorders, the lowest percentage (4.9%) was found in the diarrheal patients, while the apparently healthy persons showed a percentage of (5.8 %) (Table 8).

From the achieved results, it was obvious that *H. pylori* isolated was more frequently isolated from patients with gastrointestinal disorders; this conviction was fully supported by the results of Javed et al. [16] who reported that *H. pylori* isolated was more frequently isolated from patients with gastrointestinal disorders. *H.pylori* had been isolated from patients with upper gastrointestinal symptoms of peptic disease, in patients with gastritis and peptic ulcer [17] (Table 8).

Healthy state of human	Number of Examined samples	Number of positive samples	Percentage of Positive samples
			%
Apparently healthy	45	6	5.8
Patients with gastrointestinal disorders	35	9	8.8
Diarrheal stool samples	22	5	4.9
Total	102	20	19.6

Table 8: Results of bacteriological examination of human stool samples for *H. pylori* in relation to healthy state.

Source of samples	Occupation	Number of examined samples	Number of positive samples	Percentage of Positive samples
Human stool	Children less than 10 years	17	2	1.96
	Farmers	15	4	3.92
	Veterinarians	22	7	6.8
	Officers	15	1	0.98
	Housewives	13	2	1.96
	Dairy workers	20	4	3.92
	Total	102	20	19.6

Table 9: The occurrence of H. pylori in human stool samples with respect to occupation.

The occurrences of *H. pylori* in human stool samples with respect to occupation in Table 9 are illustrated. The occurrence of *H. pylori* in children, farmers, veterinarians, officers, Housewives and dairy workers were 1.96%, 3.92%, 6.8 %, 0.98%, 1.96% and 3.92%, respectively. The highest occurrence 6.8% was found in veterinarians and the lowest occurrence 0.98% were found in officers the dairy worker group showed occurrence of 3.92%. It could be concluded that *H. pylori* could be isolated from cattle faeces, their milk and humans in the examined area reflecting the important role of cattle especially buffaloes as new potential zoonotic reservoir. So, the recommended measures to reduce and avoid the risk of *H. pylori* are standard hygienic practices in animal management, feeding, hygienic disposal of animal wastes, preparation of silage, and periodical cleaning and disinfection must be applied to reduce *H. pylori* carriages in cattle. Avoid faecal contamination for milk. All raw milk and its products must be efficiently heat treated to avoid risk of *H. pylori* for milk consumers. Gastroenterology hospitals must be provided with a rapid urease test kits, all patients with peptic ulcers disease and other gastric disease must be monitored for detection of *H. pylori* by rapid tests then confirming with culturing or PCR assays. The positive *H. pylori* cases must be treated to avoid development of gastric cancers. Persons had occupations related to animals such as veterinarian, dairy worker and farmers must be healthy educated to avoid the risk of *H. pylori* from cattle by application strictly personal hygienic practices. Further research in needed to explain the role of other domestic animals such as horses, sheep, goats and pet animals as reservoir for *H. pylori*. Also, the effect of ecology, seasons and other risk factors on isolation rates of *H. pylori* must be studied in different geographic areas in Egypt to obtain clear picture on the epidemiology of *H. pylori* in animals and man.

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Research Article Open Access

Evaluation of Therapeutic Efficiency of Hilsha Fish Oil on Cardiovascular Disease and Hepatic Disease Marker in Hypercholesterolemic Mice

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Abstract

Hypercholesterolemia is a clinical situation characterized by the elevated serum cholesterol and associated with the higher risk of cardiovascular disease (CVD), hypertension and stroke. This study aimed to evaluate the therapeutic efficiency of hilsha fish (*Tenualosa ilisha*) oil on diet-induced hypercholesterolemic (HC) albino mice. Mice were divided in three groups consisting each of six mice : control group, HC control group (fed the basal diet containing 1.5% cholesterol and 0.5% cholic acid) and the other group of mice fed the same previous hypercholesterolemic diet supplemented with hilsha fish oil (HFO) 5%. Serum lipid profile (total cholesterol-TC, low density lipoprotein-LDL, high density lipoprotein-HDL, triglyceride-TG and very low density lipoprotein-VLDL) were determined using commercial kits. After treatment with HFO a potential antilipidemic effect was observed as TC, TG, LDL, VLDL showed significant ($p<0.001$) decrease whereas HDL showed significant increase ($p<0.001$) compared to the HC control group. The SGPT, SGOT and CRP were also significantly decrease ($p<0.001$). Therefore HFO might have hepatoprotective activity. Regarding liver tissue extract, the levels of total cholesterol and triglyceride were decreased significantly in treated mice. Gas chromatography (GC)-MS analysis of HFO showed that it contained a high amount of poly unsaturated fatty acids (PUFA) especially EPA and DHA. These Omega-3 fatty acids have an indicative effect to reduce the risk of CVD and other chronic diseases. From the above findings, it can be concluded that HFO has a potential benefit in the treatment of CVD and play a role in its management as well as in reducing the risk of CVD associated hepatic complications.

Keywords: Hypercholesterolemia; CVD; Lipid profile; CRP; EPA; DHA

Abbreviations: Hilsha Fish Oil (HFO); Hypercholesterolemic (HC); Atherogenic Index (AI); Cardiovascular Disease (CVD); Eicosapentaenoic Acid (EPA); Docosahexaenoic Acid (DHA); Serum Glutamic Pyruvic Transaminase (SGPT); Serum Glutamic Oxaloacetic Transaminase (SGOT); Coronary Heart Disease (CHD)

Introduction

Hypercholesterolemia, also called dyslipidemia is the presence of high levels of cholesterol in the blood. Hypercholesterolemia has been considered as a major risk factor for coronary heart disease (CHD) and atherosclerosis. Hyperlipidemia, particularly elevated serum cholesterol and Low-Density Lipoprotein (LDL) levels, responsible for the development of atherosclerotic heart disease [1]. Hypercholesterolemia is a major problem to many societies especially the health professionals because of the close correlation between cardiovascular diseases (CVD) and lipid abnormalities [2,3]. Dietary factors such as continuous ingestion of high amounts of saturated fats and cholesterol are believed to be directly related to hypercholesterolemia and susceptibility to atherosclerosis [4]. Clinical trials have demonstrated that intensive reduction of plasma low density lipoprotein (LDL) levels could reverse atherosclerosis and decrease the incidence of cardiovascular diseases [5]. It is believed that hypercholesterolemia is correlated to elevated sugar level and hepatic problems.

Tenualosa ilisha (Hilsha) belongs to subfamily Alosinae, family Clupeidae, order Clupeiformes, is one of the most important tropical fishes of the Indo-Pacific region. Hilsha fish contain a high amount of protein, minerals, vitamins as well as polyunsaturated fatty acids (PUFAs). Many studies have been conducted on support of various effects of fish oil. It is suggested that fish oil contain long chain polyunsaturated fatty acid [6] more specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which have a great benefit for cardiac health [7] ,controlling blood glucose [8] reduction of arterial disease [9]. Fish oil containing ω-3 PUFAs is more effective than the vegetable oil (ω-6 PUFAs) in reduction of lipid profile in human [10]. Docosahexaenoic acid (ω-6 PUFA) is more effective in lowering serum cholesterol level of experimentally induced hypercholesterolemic rats [11]. Epidemiological studies also show that eating fish or vegetable oil concurrently decreases blood cholesterol and LDL levels and increases HDL, and thus reduces the risk of coronary death [12,13]. Thus fish has medicinal and therapeutic value [14]. Many studies revealed that hilsha fish oil (HFO) can reduce blood glucose and insulin level in diabetes induced rats. Hilsha fish oil can also decrease non-esterified fatty acids (NEFA), platelet aggregation and increase total anti-oxidant status [15].

Previous studies showed that long chain n-3 polyunsaturated fatty acid in fish reduced CHD mortality [16] and cardiovascular risk factors like serum triglyceride (TG) concentration, blood pressure, arrhythmias and inflammation [17]. However there is no

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sufficient evidence of decreasing cholesterol level by consuming HFO in experimental animals. The aim of the present study is to investigate the effect of HFO on serum lipid profile and on hepatic disease marker of experimentally induced HC mice.

Material and Methods

Collection of hilsha fish

Tenualosa ilisha is essentially a marine water fish but it is abundantly found in the big and small rivers of Bangladesh. 3-5 fishes were collected from Padma River for this study. After collection fishes were cut into small pieces (about 15-20 g of weight) and sun dried at temperature 40-42°C for 6 hours to completely remove moisture from it.

Extraction of oil

Oil was extracted from the dried fish material with n-hexane by Soxhlet apparatus according to [18]. The extract was evaporated under reduced pressure in a rotary evaporator to obtain the oil.

GC mass analysis of fatty acids in hilsha fish oil

Fatty acid composition of extracted oil was determined by Gas-liquid chromatography according to [19]. Gas chromatography was conducted with a Gas Chromatograph GC-2025 series with AOC-20i Auto Injector (Shimadzu Co, Japan). GC- 2025 Gas chromatography status include temperature -280°C, pressure-175.4KPa, total flow-165ml/min, purge flow- 3 ml/min, column temperature-270°C. Hilsha fish oil was first saponified to produce the free fatty acid salts. The fatty acid salts then are derivatized to form the fatty acid methyl esters (FAME) according to the American Oil Chemists Society (AOCS). The FAME was extracted with a non-polar solvent (e.g., hexane) for analysis by GC.

Each FAME (Fatty Acid Methyl Ester) in extract was identified by comparing retention times with those of known standard FAME (Lipid Standard Sigma chemical Co, St Louis, MO, USA).The area of fatty acids was measured with GC solution 2011. The results were expressed as relative percentage of fatty acids. The relative percentage of fatty acids was calculated by the formula:

$$\text{Relative percentage of fatty acid} = \frac{\text{Area of fatty acid} \times 100}{\text{Total area of detected fatty acids}}$$

Experimental animals and treatment

Albino mice weighing ranged (24-26 g) were purchased from the Animal House of International Centre for Diarrheal Disease and Research, Bangladesh (ICDDRB), Dhaka, Bangladesh. They were adapted for one week before the experiment. All the animals were kept and maintained under laboratory conditions of temperature (22 ± 72°C), humidity (45.75%) and 12 h day: 12 h night cycle; and were allowed free access to food (standard pellet diet) and water *ad libitum*.

The animals were divided into three groups containing six mice (n=6) in each and divided into following groups:

Group 1 (G1): Control mice; fed basal diet according to [20].

Group 2 (G2): Hypercholesterolemic mice, fed the basal diet + 1.5% cholesterol + 0.5% cholic acid according to [21]

Group 3 (G3): Treated mice; fed hypercholesterolemic (HC) diet supplemented with 5% hilsha fish oil (HFO).

Body weight of mice was measured at the initial and final day of the experiment of 6 weeks. At the end of experimental period (6 weeks), mice were sacrificed after overnight fasting. Mice were anesthetized with diethyl ether (1.9%) and blood was collected from the heart. The blood samples were left for 15 minutes at room temperature and then centrifuged at 3000 rpm for 20 minutes to separate the serum, then kept in plastic vials at -20°C until analysis. Main organs such as liver, heart and kidney were carefully separated and washed with cold saline and dry on filter paper and the weight were recorded. Liver tissues were quickly dissected, rinsed in ice-chilled normal saline, blotted on filter paper. The tissues were cut into small portions and stored at -20°C until using for measuring cholesterol and triglyceride contents. Ethical guideline was maintained in animal handling during the study and permission was obtained from the concerned department.

Biochemical Analysis

Serum lipid profile such as triglyceride, total cholesterol, HDL-cholesterol, Very Low Density Lipoprotein were measured using quantification kits (Linear chemicals, Barcelona, Spain) by automatic Bioanalyzer (Hitachi 7180, Hitachi, Tokyo, Japan). Serum LDL was determined according to the Friedewald formula with use of HDL and total cholesterol value. Hepatic enzymes such as SGPT and SGOT as well as CRP were also measured using quantification kits. Atherogenic index (AI) and HTR ratio was calculated by an equations development by Friedewald [22].

Atherogenic index (AI) = (serum total cholesterol –HDL-c)/ HDL-c

HTR ratio= HDL-c/TC x100

Extraction and analysis liver total cholesterol and triglyceride

Estimation of total cholesterol and triglycerides from liver extract was carried out according to [23]. 1 g of liver portion from each animal was homogenized in 10 ml 2-propanol. The liver homogenate was allowed to stand for 48 h at 4°C. The mixture was centrifuged 15 min at 2500 rpm and the supernatant was used for lipid analysis. Total cholesterol and triacylglycerol were quantified enzymatically as described above.

Results

Fatty acid composition of hilsha fish oil (HFO) was illustrated in Table 1. These data revealed that HFO contain 34.14% total saturated fatty acid (TSFA), 22.81% mono unsaturated (MUFAs) and 15.19% polyunsaturated fatty acids (PUFAs). Among those fatty acids, palmitic acid (C16:0) 25.17%, oleic acid (C18:1) 22.81% and eicosapentaenoic acid (C20:5) 7.01% accounted the highest proportions of fatty acid.

Data in Table 2 showed the changes of lipids profile in different group of mice. These data viewed that in hypercholesterolemic mice (HC) group TC, TG,VLDL and LDL-c concentration increased significantly $P<0.001$ whereas level of good cholesterol HDL-c significantly decreased compared to healthy control group. Serum TC, TG, VLDL and LDL-c level of HFO treated HC mice decreased by 30.94%, 65.90%, 42.77% and 13.65% respectively whereas HDL-c increased 64.00%.

Data in Table 3 depicted the initial and final body weight as well as body weight gain after the treatment period. These data revealed that there is less increase in body weight in HC mice group. On the other hand by treatment with HFO body weight gain was decreased by 5.59%.

In Table 4, the results was indicated the effect of HFO on AI,

Items	Formula	Hilsha fish oil
Saturated fatty acids (SFA)	C14:1	2.45
	C16:0	25.17
	C16:1	1.65
	C18:0	4.19
	C22:0	0.68
Total saturated fatty acid (TSFA)		34.14
Monounsaturated fatty acid (MUFA)	C18:1	22.81
Omega-6	C18:2	1.43
	C20:4	0.40
Omega-3	C18:3	3.42
	C20:5	7.01
	C22:6	3.33
Total Polyunsaturated fatty acid (PUFA)	15.19	

Table 1: Relative percentage of fatty acids in hilsha fish (*Tenualosa ilisha*) oil by GC mass.

Groups	TC	HDL	LDL	TG	VLDL
G1 control	5.50 ± 0.08	0.30 ± 0.03	4.53 ± 0.11	1.48 ± 0.12	0.67 ± 0.02
G2 (HC)	9.05 ± 0.38^{a*}	0.17 ± 0.03^{a*}	6.74 ± 0.39^{a*}	3.52 ± 0.21^{a*}	1.59 ± 0.09^{a*}
G3 (HC)+ fish oil	6.25 ± 0.42^{a**}	0.28 ± 0.03^{a**}	5.82 ± 0.47^{a**}	1.20 ± 0.16^{a**}	0.91 ± 0.07^{a**}

Total Cholesterol (TC), High density lipoprotein (HDL), Low density lipoprotein (LDL), Triacylglycerol (TG), Very low density lipoprotein (VLDL). a: P<0.001; * vs control group, ** vs hypercholesterolemic control.

Table 2: Effect of hilsha fish oil on the lipid profiles of different group of mice (mmol/L) Mean ± SD.

Groups	IBW	FBW	BWG
G1 control	25.55 ± 0.77	40.06 ± 1.12	14.51 ± 0.86
G2 (HC)	25.40 ± 0.81^{a*}	42.03 ± 1.33^{b*}	16.63 ± 1.02^{b*}
G3 (HC)+ fish oil	25.30 ± 0.76^{a**}	41.00 ± 1.37^{a**}	15.70 ± 0.82^{a**}

Initial body weight (IBW), Final body weight (FBW), Body weight gain (BWG). a: P<0.001; b<0.05, * vs control group, ** vs hypercholesterolemic control.

Table 3: Effect of hilsha fish oil on body weight of different group of mice (g) Mean ± SD.

LDL-C/HDL-C Ratio and HTR in hypercholesterolemic mice. Atherogenic index and LDL-C/HDL-C ratio increased markedly while HTR% decreased in the HC group. But by treating HC mice with HFO supplementation AI and LDL-C/HDL-C ratio reduced to 21.32 and 20.78 respectively and HTR% elevated to 4.48.

Lipid level of liver tissue was viewed in Table 5. High cholesterol diet caused significant increase p<0.001 of hepatic cholesterol (50.00%) and triacylglycerol (52.55%) compared to the healthy control group. Administration hypercholesterolemic diet supplemented with HFO reduced hepatic TC and TG by 13.91% and 16.10% respectively.

Effect of HFO on the weight of main organs of HC mice was represented in (Table 6). Results revealed that liver weight was markedly increased in mice fed hypercholesterolemic diet compared to normal control group. Meanwhile, there was no change in the weight of other organs. Supplemented diet of HC mice with 5% HFO reduced liver weight compared to HC group.

There was a significant (P<0.001) increase of SGPT and SGOT level after induction of hypercholesterolemia which was decreased by HFO significantly (P<0.001). SGPT level increased by 70.25% in HC group and decreased by 23.77% with HFO supplementation. Moreover treatment with HFO decreased SGOT level by 30.87%. This result was illustrated in (Figure 1).

C - Reactive protein (CRP) level was also higher in hypercholesterolemic mice group than the normal control mice which was declined significantly (P<0.001) by 32.35% with HFO supplementation (Figure 2).

Discussion

Two important groups of PUFA in human nutrition are the omega-6 and omega-3 fatty acids. Many studies have shown EPA and DHA are beneficial to our heart system and have protective effects for different diseases. A study showed that the percentage of polyunsaturated fatty acids (PUFA) in halibut, mackerel, bloater and sprat were 31.9%, 45.4%, 40.8% and 37.0% respectively [24]. These marine fishes also have cholesterol lowering effect.Omega-3 fatty acids have TG-lowering effect that may be mediated by inhibition of the soluble phosphatidate phosphohydrolase and the effect on serum cholesterol may be partly due to inhibition of HMG-CoA reductase which is the rate-limiting enzyme in cholesterol biosynthesis [25]. Omega-3 fatty acids also improve hepatic steatosis in mice and may be used to increase the pool of potential live liver donors that are currently excluded because of the presence of macrovesicular steatosis [26]. Hilsha fish oil effectively reduced serum lipid profile level and this finding was found consistent as reported by others. Dietary marine fish oil reduced blood cholesterol in the experimentally induced hypercholesterolemic rats [27]. Anti cholesterolemic effects of Hilsha fish oil was also reported in streptozotocin-treated diabetic rats [15]. It has been suggested that the longer lifespan of Japanese and Nordic populations may be partially due to their higher consumption of fish and seafood. A study showed that after 10 months of eating 100g hilsa fish per day, serum total cholesterol level fell from 285.1 to 244.6 mg/dl (14.2% decrease) in the hypercholesterolemic subjects [28]. Induction of hypercholesterolemia significantly increased body weight of experimental mice. This result was supported by a finding which reported that rats fed high cholesterol diet showed significant increase in body weight gain [29]. But feeding diet containing HFO decreases body weight compared with HC mice. This occurs may be due to the catabolism of lipid accumulated in adipose tissue causing a decrease in body weight. HFO also showed significant effect on AI, LDL-C/HDL-C Ratio and HTR%. Reduction in HTR ratio is important

Groups	AI	LDL/HDL Ratio	HTR Ratio
G1 control	17.33 ± 0.87	15.1 ± 1.56	5.45 ± 0.34
G2 (HC)	52.23 ± 2.12*	39.64 ± 2.34*	1.88 ± 0.45*
G3 (HC)+ fish oil	21.32 ± 1.09**	20.78 ± 1.76**	4.48 ± 0.37**

Atherogenic index; * vs control group, ** vs hypercholesterolemic control.

Table 4: Levels of AI, LDL/HDL Ratio and HTR of different group of mice Mean ± SD.

Groups	TC (Mean ± SD)	TG (Mean ± SD)
G1 control	2.06 ± 0.25	11.97 ± 0.60
G2 (HC)	3.09 ± 0.53^{b*}	18.26 ± 0.68^{a*}
G3 (HC)+ fish oil	2.66 ± 0.76**	15.32 ± 0.63^{a**}

Total cholesterol (TC), Triacylglycerol (TG). a: P<0.001; b<0.05; * vs control group, ** vs hypercholesterolemic control

Table 5: Levels of total cholesterol, triglyceride in liver tissue in different group of mice liver (mg/g, wet liver).

Groups	Liver	Heart	Kidney
G1 control	2.15 ± 0.03	0.18 ± 0.01	0.195 ± 0.01
G2 (HC)	2.61 ± 0.04^{a*}	0.20 ± 0.004*	0.206 ± 0.01*
G3 (HC)+ fish oil	2.03 ± 0.07^{a**}	0.15 ± 0.002^{a**}	0.196 ± 0.01**

a: P<0.001; * vs control group, ** vs hypercholesterolemic control

Table 6: Changes of weight of organs different group of mice (g) Mean ± SD.

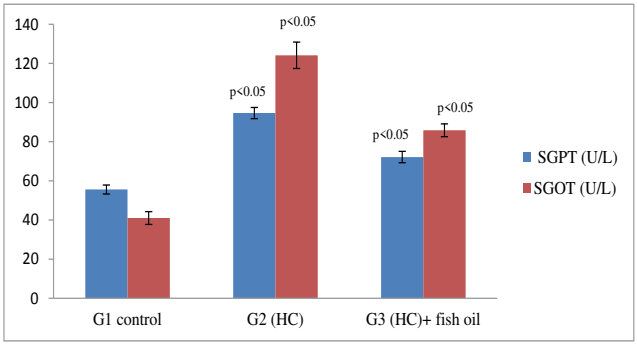

Figure 1: Effect of hilsha fish oil on serum SGPT and SGOT of experimental mice.

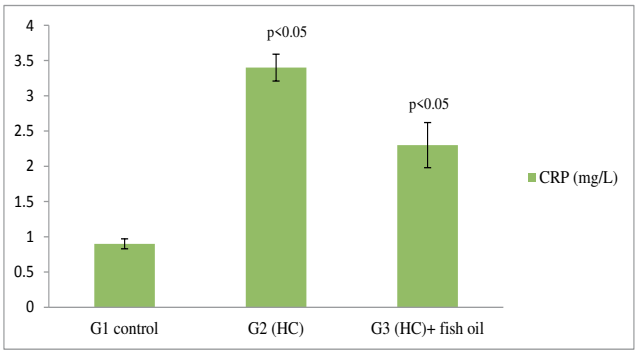

Figure 2: Effect of hilsha fish oil on serum CRP of experimental mice.

in predicting coronary heart disease in human being, an increase in this ratio is believed to furnish a beneficial effect. This result was supported by another experiment who stated that the increase in HDL-C or HTR ratio is one of the most important criteria of anti-hypercholesterolemic agent [30]. Supplementation of HFO also significantly decreased liver cholesterol and triglyceride level. Animal-derived Hilsha ilisha fish oil is more effective in reducing the serum and liver cholesterol than soybean and palm oil, though both soybean and palm oil are also effective in reducing serum and liver cholesterol [31]. Weight of liver in HC mice increased significantly ($p<0.001$) which was compensated by treatment with HFO. The increase in hepatic enzymes SGPT and SGOT in hypercholesterolemic mice indicate dysfunctions of liver. On the other hand supplementation of HFO significantly decreased the level of these enzymes indicate effective recovery of the hepatic function by improvement of lipid metabolism or delaying the hepatic disease.C- Reactive Protein (CRP) is a simple cost effective test, which can predict the cardiovascular risk. The addition of CRP-testing to standard lipid screening appears to provide an important method to determine Cardiovascular Disease (CVD) risk factor [32]. Dietary supplementation of HFO declined CRP level significantly and thus reduced the risk of cardiovascular diseases.

Statistical Analysis

The assays were carried out in triplicate, and the results were expressed as mean values and the standard deviation (SD). Results were analyzed by using Scientific Package of Social Science (SPSS) version 17.0. The descriptive statistic was used to analyze mean, standard deviation where by analytical statistics, one-way ANOVA was used to determine statistical significance ($p<0.05$) among the groups.

Conclusion

There is considerable evidence from experimental studies that hilsha fish oil has significant benefit in the management of hypercholesterolemia. This anti lipidemic effect is due to the presence of omega-3 fatty acids that inhibit enzymes in lipid biosynthetic pathway. Hilsha fish oil also reduced SGPT, SGOT and CRP level so it may have hepatoprotective activity. Therefore these results indicate that hilsha fish oil have important implication in the management of hypercholesterolemia as well as cardiovascular and hepatic complications.

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Research Article Open Access

Genetic Determinants of CYP2C19 Gene *2 and *3 Loss of Function Alleles and Response to Anti Platelet Therapy (Clopidogrel) and Cardiovascular Events. (A Study in Kashmir, North India)

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Summary

Background: Studies have demonstrated that the mutant *2 and*3 allele of the *CYP2C19* loss-of-function polymorphism is associated with diminished metabolization of clopidogrel and an attenuated platelet response to clopidogrel treatment. Since no such study has been conducted in this region, we examined *CYP2C19* polymorphism in Acute Coronary Syndrome (ACS) patients on clopidogrel treatment, and its effect on the cardiovascular outcomes.

Material and Methods: A total of 100 samples of ACS were included in this study and genotyping of *CYP2C19* *2 and *3 gene polymorphisms was performed by a Polymerase chain reaction-Restriction fragment length polymorphism (PCR-RFLP).

Results: The distribution of *CYP2C19**2 allele wild *1/*1, Heterozygous *1/*2 and homozygous mutant *2/*2 genotypes was 56%, 34% and 10% respectively while for *CYP2C19*3* wild*1/*1 and heterozygous *1/*3 genotypes was 84% and 16% respectively. The frequency of compound heterozygotes (*2/*3) was found in 9% (9 of 100 patients). *CYP2C19* *1/*2 allele was found in 03 of 34 (8.8%) patients who had CV events followed by 2 of 10 (20%) patients with mutant genotype *CYP2C19**2 (*2/*2) on follow up. In the *CYP2C19**3, 31.2% having heterozygous genotype (*1/*3) had CV events as compared to 11.9% with *1/*1 (31% v/s 11.9% p> 0 .05). In the poor-metabolizer group (*2/*2 or *2/*3), 20.1% of patients had CV events on follow up compared to 15.6% in the extensive metabolizer group (*1/*1), whereas in the intermediate group only 10% of patients had CV events (p>0.05).

Conclusion: We conclude that patients carrying *CYP2C19* loss-of-function alleles had a higher rate of subsequent cardiovascular events as against those with normal allele. Lack of significant events even in presence of variant alleles justifies us to some extent to continue clopidogrel in our patients.

Keywords: Acute Coronary syndrome; *CYP2C19*; Clopidogrel; Poor-metabolizer; Allele

Introduction

The hepatic *CYP2C19* enzyme contributes to the metabolism of many clinically relevant drugs such as antidepressants, benzodiazepines, mephenytoin, some proton pump inhibitors, and clopidogrel. Like many other *CYP450* superfamily members, the *CYP2C19* gene is highly polymorphic, having more than 25 known variant alleles (http://www.cypalleles. ki.se/cyp2c 19.htm). *CYP2C19**1 represents the wild-type allele. On the basis of their ability to metabolise (S)-mephenytoin or other probe drugs, individuals can be categorized as Extensive Metabolizer (EM), PM (Poor Metabolizer or Ultra rapid Metabolizer (UM) for *CYP2C19*. Heterozygous EMs are sometimes also referred to as Intermediate Metabolizer (IMs) [1]. The majority of the *CYP2C19* PMs are carriers of the variant alleles *2 and *3, which are loss of function alleles (LOF) whereas the *17 variant is a gain of function (GOF) allele associated with increased activity [2]. Studies have shown a marked interethnic variation in the distribution of variant alleles. The allelic frequency of *CYP2C19**2 has been shown to be 15% in Africans, 29–35 % in Asians, 12–15% in Caucasians and 61% in Oceanians. The *CYP2C19*3* is mainly found in Asians (5–9% in Asians, less than 0.5% in Caucasians). The allelic frequency of *CYP2C19**17 has been shown to be 16% in Africans, 3–6% in Asians and 16–21% in Caucasians [3].

Clopidogrel is a prodrug whose in vivo metabolite binds to the platelet P2Y12 receptor causing irreversible blockade. The pharmacodynamic response to clopidogrel has substantial inter patient variability [4-7] and patients with coronary disease with lesser degrees of platelet inhibition in response to clopidogrel appear to be at increased risk for cardiovascular events [7-9]. Approximately 85% of the parent drug is inactivated by human carboxylesterase 1, whereas the remainder is transformed to the intermediate, inactive oxo-clopidogrel by *CYP2C19*, CYP1A2 and CYP2B6 [10].

Clopidogrel is an antiplatelet drug used in atherothrombotic diseases, such as myocardial infarction and stroke, which is an inactive prodrug that needs to be bioactivated by a liver enzyme, *CYP2C19*. Several loss-of-function alleles have been previously identified [11]. *CY2C19*2* and *CYP2C19*3* are the two most frequent variants in Occidentals and Asians, respectively. Major cardiovascular events occur two- to three-times more frequently in patients treated with clopidogrel having decreased *CYP2C19* function compared with wild-type patients after myocardial infarction, stent thrombosis [12-14]. A gene-dose effect seems to occur with the patients heterozygous for one *CYP2C19* variant showing an intermediate clinical response between wild-type patients and patients homozygous for the loss-of-function

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#-Equally Contributed

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variants [12]. Identification *CYP2C19* alleles with reduced function has also been associated with reduced responsiveness to, and thus reduced therapeutic benefit of, clopidogrel in cardiovascular patients [12,15-19]. The presence of *CYP2C19* loss of function alleles *2 and *3 may result in lower exposure to Clopidogrel and thus decreased platelet inhibition in clopidogrel treated patients [20]. For this reason FDA issued a Boxed Warning to the clopidogrel label to stress upon the need to identify patients with *CYP2C19* alleles classified as PMs who are considered at an increased risk of adverse cardiovascular outcomes due to reduced effectiveness of clopidogrel [21]. A systematic review and meta-analysis was conducted to examine the association between *CYP2C19* genotype and the clinical efficacy of clopidogrel where it was shown that the presence of at least one loss of function allele was associated with an increased risk of stent thrombosis [22]. Owing to the established role of *CYP2C19* genotype and its relation in anti-platelet (Clopidogrel) therapy, the guiding principles for clopidogrel dosing is still a matter of debate. Besides, ethnic difference exists in both pharmacokinetics and pharmacodynamic and they play an important role in optimization of therapy for the individual patient and drug administration. Since no such study has been conducted in this part, so our investigation will examine the *CYP2C19* polymorphism in our patients on clopidogrel treatment, and its effect on the cardiovascular outcomes in patients with Acute Coronary Syndrome.

Methodology

This study was conducted in Sher-i-Kashmir Institute of Medical Sciences, (Jammu and Kashmir, India) in the Department of cardiology, Immunology and Molecular Medicine and Advanced Centre for Human Genetics for a period of 2 years. Clearance from local SKIMS ethical committee was taken prior to study.

Study subjects

A total of 100 samples were included in this study which comprised of confirmed cases of ACS which were selected from Department of Cardiology, SKIMS. Blood samples were taken from the patients after a written pre informed consent was obtained. Demographic and clinic-pathological characteristics of each patient were recorded in a Questionnaire. Patients were followed over a period of 2 years in Cardiology Out-patient Department (OPD) and also through telephonically to check the compliance of the drug and cardiovascular events like death from any cardiovascular cause, ACS, fatal and non-fatal MI. The patients were included on criteria like ACS with or without undergoing PCI, ST Elevation Myocardial Infarction (STEMI), Non ST Elevation Myocardial Infarction (NSTEMI) and those with Unstable Angina (USA). The patients who include history of bleeding diathesis, stroke less than 3 months platelet count <70000/mm^3, hematocrit <30% were excluded from the study. Written informed consent was obtained from all the study participants. Patients recruited for the study were put on clopidogrel 300 mg after diagnosed ACS followed by 75 mg OD, and Patients undergoing PCI received 600 mg followed by 150 mg OD for atleast for 7 days and 75 mg later for a period of one year.

DNA extraction and polymerase chain reaction (PCR)

2 -5 ml of blood sample was taken from each patient and stored at -20°C for DNA analysis. Genomic DNA was isolated using standard proteinase-K digestion, phenol/chloroform extraction, and ethanol precipitation method from whole-blood samples of both cases and controls. The primers used were *Cyp19*2* F-5′*AAATTGTTTCCAATCATTTAGCT-3′* and R-5′*ACTTCAGGGCTTGGTCAATA-3′* whereas for *Cyp19*3 F-5′-CAGAGCTT GGCA TATTGTAT C-3′ and R-5′GTAAACACACA ACTAGTCAA TG-3′* producing 321 bp and 271bp PCR products respectively. PCR was carried out in a final volume of 25 uL containing 50 ng genomic DNA template, 1x PCR buffer (Biotools, B and M Labs, Madrid, Spain) with 2 mmol/L $MgCl_2$, 0.4 mmol/L of each primer (Genscript, Piscataway, NJ), 50 mmol/L dNTPs (Biotools, B and M Labs), and 0.5 U Taq Polymerase (Biotools, B and MLabs). For PCR amplification, the standard protocol was used as follows: one initial denaturation step at 94°C for 7 minutes, followed by 35 denaturation cycles of 30 seconds at 94°C, 30 seconds of annealing at 53°C, and 30 seconds of extension at 72°C, followed by a final elongation cycle at 72°C for 5 minutes.

Restriction Fragment Length Polymorphism (RFLP)

Two principle alleles of *CYP2C19*2* (rs4244285) and *CYP2C19*3* (rs4986893) were analyzed by PCR-RFLP. Exon 4 and Exon 5 of *CYP2C19* were amplified by PCR that contain the *CYP2C19* *2 and *3 polymorphisms respectively. 681G>A in Exon 5 of *CYP2C19* (*2) creates an aberrant splice site and destroys *SmaI* restriction site and 636G>A in *CYP2C19*3* gene, creates a premature stop codon and abolishes *BamHI* restriction site. 10ul of PCR amplified DNA were digested with 5 Units of each *SmaI* and *BamHI* (Fermentas Life Sciences, USA) restriction enzymes for both polymorphisms *CYP2C19* *2 and *3 respectively while keeping the other conditions as per instructions of manufacturer. The digested products were electrophoresed using 2% agarose gel and visualized by Ethidium Bromide staining. The PCR products (271bp) of *3 yielded two bands of 175bp and 96bp in wild type and three products of 96, 175 and 271bp in heterozygous variants (Figure 1A). The PCR product (321bp) encompassing allele *2 digested into two smaller bands of 109bp and 212bp in wild type individuals whereas heterozygous variants for this allele yielded three bands of 321, 212 and 109bp and in homozygous variants the PCR product would not be digested (Figure 1B).

Genotyping classification

Patients were classified into different metabolizer phenotype groups. Patients with two *CYP2C19*2* or *CYP2C19*3* alleles (*2/*2 or *2/*3) were classified as having the poor- metabolizer phenotype, those with one *CYP2C19*2* or *CYP2C19*3* allele (*1/*2 or *1/*3) were classified as having the Intermediate – metabolizer phenotype and those without *CYP2C19*2* or *CYP2C19*3* allele (*1/*1) were classified as having extensive-metabolizer phenotype.

Statistical Analysis

All continuous variables of study have been shown in terms of Mean ± SD and the categorical variables in terms of frequency and percentage. The standard statistical tests like T-test. Chi square test and Fischer -exact test have been used to analyze the data, moreover odds ratio with 95% confidence interval have been calculated at appropriate places. All the results obtained have been discussed on 5% level of significance (p value<0.05) considered as significant, also suitable statistical charts have been used to represent the data, SPSS version 20 was used for analysis.

Results

The mean age of our patients was 61.1 ± 8 (range 43 to 75) and majority of patients belonged to age groups of 50-60(38%) and 60-70 years (39%) where 80% of patients were males and 20% females. Hypertension and diabetes mellitus was present in 84% and 39% of patients respectively. Our study included 71% patients with ST Elevation

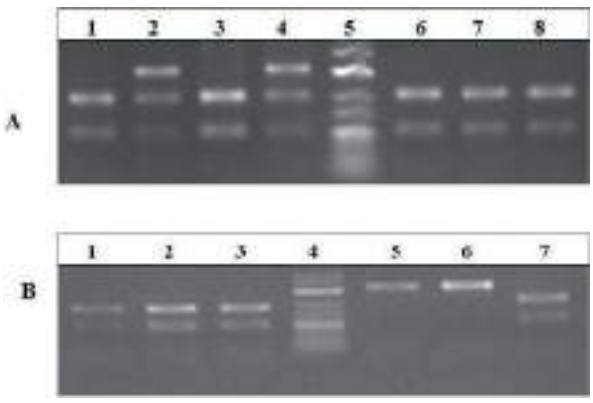

Figure 1: RFLP of CYP2C19*3 (A) on 2% Agarose gel: Lane 1, 3, 6, 7, 8 = Homozygous wild, Lane 2, 4 = Heterozygous mutant, Lane 5 = DNA ladder 25 bp (B) : CYP2C19*2: Lane 1,2,3 and 7= Homozygous wild: Lane 5, 6= Homozygous mutant, Lane 4= DNA ladder 25 bp.

Myocardial Infarction (STEMI), 20% Non-ST elevation Myocardial Infarction (NSTEMI) and 9% of patients were having unstable Angina (USA) Table 1.

Table 1 shows all the clinical details of Acute Coronary Syndrome (ACS) patients classified with cardiovascular events in different conditions. Ninety two patients went for Coronary Angiography (CAG) after Acute Coronary syndrome (ACS) while as in eight patients CAG could not be done due to financial constraints. Of the 92 patients 69(75%) had single vessel disease (SVD), 13 (14%) of patients had double vessel disease (DVD) and 10 patients had triple vessel disease (TVD).

In our study 15 patients had Cardiovascular events (CV) on follow up in which there were 04 deaths (possible stent thrombosis), 06 patients had definite stent thrombosis, 02 patients had probable stent thrombosis (who presented with MI in the same territory), 02 patients had MI in the same territory who had not undergone percutaneous coronary intervention (PCI) after coronary angiography (CAG) and 01 patient had instant restenosis (ISR) and presented with ACS. The cardiovascular events were noticed over a study period mostly during the first year of follow up.

The distribution of *CYP2C19**2 allele wild *1/*1 (no loss of function), heterozygous *1/*2 (Loss of function allele) and homozygous mutant *2/*2 (Total loss of function) genotypes were 56%, 34% and 10% respectively and *CYP2C19*3* wild*1/*1, and heterozygous *1/*3 genotypes were 84% and 16% respectively. No patient had homozygous mutant *3/*3 genotype. The frequency of compound heterozygotes (*2/*3) was found in 9% (9 of 100 patients) Table 2.

Frequency distribution of *CYP2C19**2 and *CYP2C19*3* having different metabolizer phenotypes in ACS patients on clopidogrel and their relation to Cardiovascular Events are shown in detail in Table 3. *CYP2C19* *1/*2 allele (loss of function allele) was found in 03 of 34 (8.8%) patients who had CV events, only 2 of 10 (20%) patients with mutant genotype *CYP2C19**2 (*2/*2) had CV events on follow up and patients with wild genotype *1/*1, 10 of 56 (17.9%) had CV events. In the *CYP2C19**3, l5 out of 16 patients (31.2%) having heterozygous genotype (*1/*3) had CV events as compared to 10 out of 84 (11.9%) with *1/*1 type (31% v/s 11.9% p value 0.061). The distribution of these genotypes among the CV events did not match statistical significance ($p<0.05$) (Table 3).

In the poor-metabolizer group of patients 20.1% of patients had CV events on follow up compared to 15.6% in the extensive metabolizer group of patients, whereas in the intermediate group only 10% of patients had CV events (Figure 1). All these figures were not statistically significant ($p <0.05$) (Table 3).

Discussion

Clopidogrel is an antiplatelet drug used in atherothrombotic diseases, such as myocardial infarction and stroke, which is an inactive prodrug that needs to be bioactivated by a liver enzyme, *CYP2C19*. Several loss-of-function alleles have been previously identified [11]. *CY2C19*2* and *CYP2C19*3* are the two most frequent variants in Occidentals and Asians, respectively. *CYP2C19* alleles are associated with reduced conversion of clopidogrel to its active metabolite. Many studies support the observations regarding reduced-function of *CYP2C19* polymorphisms and platelet aggregation among clopidogrel-treated subjects [20,23,24]. Ethnic difference also exists and they play an important role in optimization of therapy for the individual patient and drug administration. To our knowledge this study is the first from the subcontinent, to investigate the *CYP2C19* polymorphism in our patients on clopidogrel treatment, and its effect on the cardiovascular outcomes in patients with acute coronary syndrome.

The present report observed frequency of wild *CYP2C19**2, heterozygous *1/*2 and mutant homozygous *2/*2 genotypes as 56%, 34% and 10% respectively while as for *CYP2C19*3* wild*1/*1 and heterozygous*1/*3 genotypes were 84% and 16% respectively. Homozygous mutant *3/*3 contributing for loss of function was not found in any case of our study. Nine percent (9%) were compound heterozygotes (*2/*3).

In a study conducted by Dirk et al. [14] on a large sample size, 1805 patients, (73%) were *CYP2C19* wild-type homozygotes (*1/*1), 633 (25%) were *CYP2C19**2 heterozygotes (*1/*2), and 47 (2%) were homozygous (*2/*2) for the mutant *CYP2C19* *2 allele. Despite comparatively small sample size, we observed high frequency of mutant *CYP2C19* *2 allele as against Dirk et al. (2% v/s 10% our study). Simon et al. [12] also observed the same pattern of *CYP2C19* *2 allele frequency as Dirk et al. [14] and thus both differing more on the frequency of mutant allele *2/*2 (2% Dirk et al. [14] v/s 2.5% Tabossam et al. [12] v/s 10% our study). For *CYP2C19*3* allele, although genotype distribution frequency differs from our report (*1/*1; 84%, *1/*3; 16% our study v/s 99% and 1% Tabossam et al. [12]) but both studies report a similar finding where no subject were having mutant (*3/*3) genotype. Prasanthi et al. [25] in northeastern patients of India found 36%, 24% for *1/*1 and *1/*2 respectively with *CYP2C19**2 allele and 60% in *CYP2C19*3* had *1/*1 genotype which show a complete disagreement with our cohort giving a more evidence for the ethnic difference of this polymorphic gene.

Our patients carrying *CYP2C19**2 heterozygous allele *1/*2 had 8.8% CV events and homozygous mutant genotype *2/*2 carriers had 20% against 17.9% CV events in patients with wild genotype *1/*1. Likewise genotype *1/*3 in *CYP2C19*3* had 31.2% CV events as compared 11.9% with *1/*1 (31% v/s 11.9%), however, all these figures did not match statistical significance thus disagreeing with many studies [20, 24].

In the poor-metabolizer group of patients 20.1% of patients had CV events on follow up compared to 15.6% in the extensive metabolizer group of patients, whereas in patients in the intermediate group only 10% of patients had CV events. All these figures were not statistically

Characteristic		Patients		Cardiovascular event		p value
		N=100	%	NO	YES	
Age (yr)	40-50	13	13.0	13(100%)	0(0%)	*0.038*
	50-60	38	38.0	34(89.5%)	4(10.5%)	
	60-70	39	39.0	30(76.9%)	9(23.1%)	
	70-80	10	10.0	8(80%)	2(20%)	
	Mean ± SD	61.44 ± 8.0				
Gender	Male	80	80.0	68(85%)	12(15%)	1.0
	Female	20	20.0	17(85%)	3(15%)	
Hypertension	Yes	84	84.0	71(84.5%)	13(15.5%)	*0.555*
	No	16	16.0	14(87.5%)	2(12.5%)	
Diabetes Status	Non Diabetics	61	61.0	52(85.2%)	9(14.8%)	
	Diabetics	39	39.0	33(84.6%)	6(15.4%)	
Smoking Status	Smokers	82	82.0	71(86.6%)	11(13.4%)	*0.574*
	Non smokers	18	18.0	14(77.8%)	4(22.2%)	
Type of ACS	STEMI	71	71.0	59(83.1%)	12(16.9%)	*0.586*
	NSTEMI	20	20.0	17(85%)	03(15%)	
	USA	09	9.0	09(100%)	(0)0%	
PCI	PCI	82	82.0	69(84.1%)	13(15.9%)	*0.465*
	Non-PCI	18	18.0	16(88.9%)	2(11.1%)	
Coronary Arteries with significant disease	SVD	69	69.0	62(89.9%	07(10.1%)	*0.18*
	DVD	13	13.0	08(61.5%)	05(38.5%)	
	TVD	10	10.0	07(70%)	03(30%)	
Previous MI	No Previous MI	85	85.0	76(89.4%)	9(10.6%)	0.009
	Previous MI	15	15.0	9(60%)	6(40%)	
Previous PCI	No previous PCI	86	86.0	76(88.4%)	10(11.6%)	0.034
	Previous PCI	14	14.0	9(64.3%)	5(37.7%)	
LV function	Normal LV function	60	60.0	55(91.7%)	5(8.3%)	0.024
	LV dysfunction ***(< 45%)***	40	40.0	30(75%)	10(25%)	

Table 1: Clinical details of Acute Coronary Syndrome (ACS) patients classified with cardiovascular events in different conditions.
STEMI: ST Elevation Myocardial Infarction, NSTEMI: Non- ST Elevation Myocardial Infarction, USA: Unstable Angina, SVD: Single Vessel Disease, DVD: Double Vessel Disease, TVD: Triple Vessel Disease, LV: Left Ventricular, MI: Myocardial infarction, PCI: percutaneous coronary intervention

CYP2C19 allele	Genotype	Frequency of genotype n
CYP2C19*2 Allele (rs4244285)	*1/*1 (GG)	56 (0.56)
	*1/*2(AG)	34 (0.34)
	*2/*2 (AA)	10(0.10)
CYP2C19* 3 Allele (rs4986893)	*1/*1 (GG)	84(0.84)
	*1/*3 (AG)	16(0.16)
Compound heterozygous	*2/*3	09 (0.09)

Table 2: Frequency of genotypes with CYP2C19*2 and CYP2C19*3.

significant ($p > 0.05$) and these results were consistent with study of Guillame [19].

The previous studies other than conducted by Guillame et al. [19] had shown attenuated benefits of clopidogrel among carriers of loss of function alleles. Our study matched the results of the study conducted by Guillame et al. [19], although poor metabolizer group of patients had increased frequency of CV events than extensive metabolizer group but could not achieve significance. All these results can be explained to some extent by potential pleotropic effects of loss of function alleles (independent of their effects on active metabolite of clopidogrel). Moreover since variation of non-CYP genes may also have effect on responsiveness to clopidogrel and the likelihood of ischemic events.

Despite increased prevalence of heterozygous and mutant allele in our population, we could not see significant CV events in our patients on the basis of *CYP2C19* *2 and *CYP2C19*3* alleles (loss of function alleles), which justifies us to some extent to continue clopidogrel in our patients, the majority of which belong to low economic class who may not be able to afford the antiplatelet agents like prasugrel and ticagrelor. The FDA has added boxed warning to clopidogrel which is for patients who do not effectively metabolize the drug (poor metabolizers). However this study with comparatively less sample size prompts us to enroll more patients to elucidate whether such approach can be applied to the patients of this geographical area.

Conclusion

We conclude that patients carrying *CYP2C19* loss-of-function alleles had a higher rate of subsequent cardiovascular events as against those

Allele	Genotype	Cardiovascular Event		Total	P VALUE
		NO	YES		
CYP2C19*2 (rs4244285)	*1/*1 (GG)	46(82.1%)	10(17.9%)	56	0.460
	*1/*2 (AG)	31(91.1%)	03(8.8%)	34	
	2/2 (AA)	8(80%)	2(20%)	10	
	T0TAL	85	15	100	
CYP2C19*3 (rs4986893)	*1/*1 (GG)	74(88.1%)	10(11.9%)	84	0.61
	*1/*3 (AG)	11(68.8%)	5(31.2%)	16	
	Total	85(85%)	15(15%)	100	
Compound heterozygote	*2*/3	7	2	9	0.404
Metabolizer Phenotype	Extensive	43(84.4%)	8(15.6%)	51	0.570
	Intermediate	27(90%)	03(10%)	30	
	Poor	15(78.9%)	04(20.1%)	19	
	Total	85	15	100	

Table 3: Frequency distribution of CYP2C19*2 and CYP2C19*3 having different metabolizer phenotypes in ACS patients on clopidogrel and their relation to cardiovascular events.

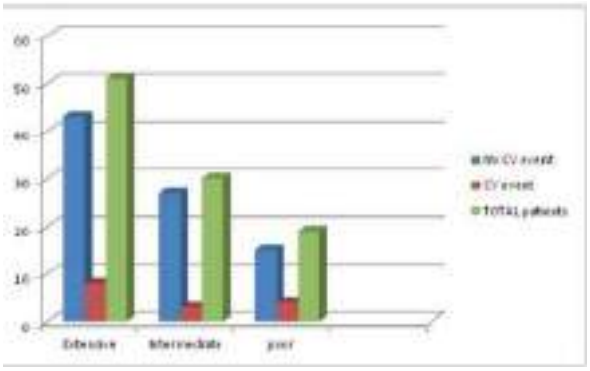

Figure 2: Cardiovascular events in patients with Metabolizer phenotype Extensive metabolizer (with genotype *1/*1), Intermediate metabolizer (with genotype *1/*2,*1/*3) and Poor metabolizers (with genotype *2/*2 and *2/*3).

with normal allele. These events in *CYP2C19* loss-of-function alleles were particularly marked among the patients undergoing percutaneous coronary intervention. Lack of significant events even in presence of variant alleles justifies us to some extent to continue clopidogrel in our patients.

Acknowledgment

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Review Article Open Access

Heart Follows Thyroid

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Abstract

In humans both hyper- and hypothyroidism can have different clinical manifestations. Among these symptoms, there is a numerous group dealing with the cardiovascular system. Thyroid hormones affect the hemodynamic state of organism and regulate expression of some cardiomyocyte structural genes. Thyroid disorders may also contribute to accelerate the underlying heart problems. Unfortunately, since thyroid dysfunction remains undiagnosed, cardiological treatment is not effective enough. In this paper, the authors show and explain the main cardiac consequences of an overactive and underactive thyroid gland. Additionally, some new data about thyroxine application in cardiac remodeling and fetal phenotype creation as a result, are included in this review.

Keywords: Thyroid hormones; Hyperthyroidism; Hypothyroidism; Cardiovascular system

Introduction

Thyroid gland disorders are most frequently endocrinological disturbances. Their prevalence varies according to the studied population. In Poland hyperthyroidism is present at 1-2% and hypothyroidism at 1-6 % until the age of 60 years [1]. According to the PolSenior Study, thyroid disturbances are present in over 10% of people from the age of 55 (hyperthyroidism – 2,95% and hypothyroidism – 7,95%) [2]. Among the US population it was shown, that 4.6% of them suffer from hypothyroidism (0.3% clinical and 4.3% subclinical) and 1.3% had hyperthyroidism (0.5% clinical and 0.7% subclinical) [3]. The main cause of hyperthyroidism is Graves' disease (60-80% of all hyperthyroidism) with a peak onset at 20-50 years [1]. Hypothyroidism, in areas of iodine sufficiency is in most cases caused by Hashimoto's thyroiditis [4]. Thyroid disturbances are more common among women. From 6 (in hypothyroidism) to 10 (in hyperthyroidism) more times it is more likely to occur in woman [1]. Thyroid hormones are one of many most important hormones strongly affecting cardio-vascular system [5,6]. The hemodynamic effects of hyperthyroidism are opposite to those connected with hypothyroidism, but the later may be less obvious in clinical symptoms, at least at the beginning of disease. Moreover, for those with existing cardio-vascular disease (CVD), disorders of the thyroid gland can worsen old cardiac symptoms or cause new ones [7]. This review integrates some mechanisms of thyroid hormone action in cardiomyocyte and show how hormone insufficiency and excess can impact on cardio-vascular system. Some new data have also been reporter regarding the possibility of heart remodeling process with the help of thyroid hormones delivery.

Physiology of Thyroid Gland

Thyroid gland (lat. glandula thyroidea) is an unpaired endocrine organ regulating all the metabolic processes in the human body. It's located in the lower front part of the neck and consists of two lobes. Their upper border reaches the half of thyroid cartilage of larynx. Thyroid hormone synthesis is a multistep process localized in thyrocytes, which requires iodine environmental sufficiency. Figure 1 shortly illustrates this process.

Hypothalamic–pituitary–thyroid axis (HPT axis) regulates thyroid hormone blood concentration according to the negative feedback loop. Triiodothyronine (T_3) regulates the metabolism of the whole body, adjusts to its currently needs and stimulates human's growth during their whole life.

Mechanism of T3 Action in Cardiovascular System

It has been extensively demonstrated that disturbances in functioning of the thyroid gland can modify cardiac performance

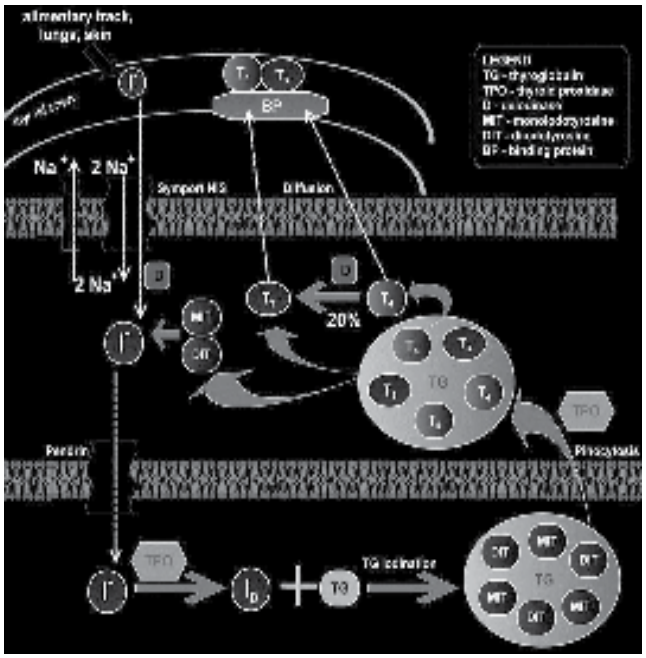

Figure 1: Synthesis of thyroid hormones.

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affecting the heart and the vascular system [8-11]. In some cases, hyperthyroidism can have the form of a tyreocardiac state concealing hyperthyroidism and can be the only symptom of elevated level of T_3 [12].

There are two groups of thyroid hormone action in the cardiovascular system. These are genomic processes, when triiodothyronine interacts with cardiomyocyte nucleus receptor and non-genomic ones, affecting sympathetic nervous system, peripheral circulation and heart rate [8,13]. T_3, being an active form of thyroid hormones plays a greater role in binding to the nucleus receptor in cardiomyocytes [14]. That is why, authors will use this term talking about its thyroid effect. T_3 commonly binds to specific nuclear thyroid hormone receptors (TRs), which belong to the steroid receptor group (mainly retinoid X receptor, RXR). It regulates the transcriptional and posttranscriptional processes. Two TRs genes, α and β, encode four T_3-binding receptor isoforms (α1, β1, β2, and β3) [15]. After T_3 and TRs connection, homo- or heterodimers have been made and they attach to the thyroid hormone response elements (TRE) in DNA promoter region. T_3 regulates positive or negative expression of some genes encoding structural sarcomere's elements and regulating proteins for cardiomyocytes [16].

One of the most important among them is myosin heavy chain (MHC), being the main structural part of sarcomere. There are 2 cardiac MHC isoforms, α- and β-MHC with genes encoding them (MYH6 and MYH7 respectively) located on 14 chromosome. Human heart has 20-30% of α-MHC mRNA of the total MHC mRNA and the rest constitutes as isoform β but this isoform is related to having lower activity than α-MHC [16,17]. Quantitative changes between its two isoforms impact on contractile velocity. Human hemodynamic status, heart diseases and thyroid status can alter expression of the MHC genes. Namely, T_3 promotes α-MHC expression and causes increase of speed contraction and cardiac growth. In hypothyroidism was observed lower α-MHC concentration with compensatory bigger cell size and stimulation of anabolic processes in cardiomyocyte. Contractile velocity was poorer [18]. Similar α-MHC changes have been observed in failing heart and fetal phenotype [19]. After the T_3 delivery, there was a increase of α-MHC and decrease of β-MHC [18]. Moreover, some DNA and proteins caring DNA are of importance in this regulation. Haddad et al. have investigated, that altered thyroid state induces histone modifications in the chromatin associated with the cardiac MHC genes [20].

A novel regulating mechanism of cardiac myosin heavy chain gene by naturally occurring anti - sense transcription was elucidated via pre-mRNA analysis. Haddad et al. reported the expression of an antisense myosin heavy chain RNA in the normal rodent myocardium [21]. It was also found, that hypothyroidism and diabetes were correlated with an increased expression of the sense β-MHC pre-mRNA and a dramatically decreased expression of both the antisense β-MHC RNA and sense of α-MHC pre-mRNAs [22]. The study of Danzi et al. [23] confirmed the above mentioned results. In hypothyroid rat ventricle, it's been proved that β-MHC antisense RNA expression is minimal, while in the euthyroid rat ventricle, there is a maximal β-MHC antisense RNA. In hypothyroid humans, there was a study suggesting, that after T_3 therapy there was also an α-MHC increase but with no change in β-MHC activity. It was positively correlated with better clinical stage of the patient [24].

Proportion in MHC isoforms depends on the heart's condition. In the course of heart failure there was effectively no detectable α MHC protein in the left ventricles [25]. Physiological consequence of this state can be perturbed contractibility and increased cardiac work and as a result cardiac hypertrophy [23].

Next important gene, which expression depends on T_3 is an ATP- Ca^{2+} activated pomp localized in sarcoplasmic reticulum (SR), called SERCA2. It plays a key role in relaxation time during diastolic phase by Ca^{2+} active transport from the cytosol to the SR. A period of time, when Ca^{2+} leaves cytosol and doesn't bound to troponin C determines the length of cardiomyocyte contractibility and relaxation. T_3 stimulates SERCA2 gene expression and accelerates relaxation time in diastolic phase. It shows a positive lusitropic activity [26]. It was also reported, that both in hyperthyroidism and short-term cold exposure was observed an increase in oxygen consumption and heat production during Ca^{2+} transport via SERCA2 in cardiomyocyte [27]. SERCA2 action is conjugated with phospholambam (PLB) activity. It is an integrative protein in the SR, which blocks SERCA2 activity. It is activated, when being in dephosphorylated form. Its activity is necessary to keep a properly length of systolic phase. Its phosphorylation by protein kinase A (PKA) stops SERCA blockage and Ca^{2+} is transported into the SR [16]. There is a T_3 negative relationship to PLB gene expression. T_3 leads to a decreased PLB quantity and makes SERCA2/PLB ratio higher [28]. Mouses PLB depleted revealed better contractility parameters [29]. Beside this, T_3 increases cAMP production in cardiomyocytes and as the consequence increases the activity of PKA and phosphorylates PLB. That is why, it can be said that PLB regulates the inotropic heart effects [16].

Thyroid hormones regulate also the expression of some ion channels located in cell membrane. Among them are Na^+/K^+ ATP-ase pomp [13,15], voltage-gated K^+ channels, (Kv1.5, Kv2,1, Kv4,2, Kv1,2, Kv1,4) [30] and Na^+/Ca^{2+} exchanger (NCX) [29,31]. Working together, they are responsible for electrochemical responses of the myocardium. First Na^+/K^+ ATP-ase is under positive T_3 regulation [13]. An increase in Na^+/K^+ ATPase expression occurs independently of increased cardiac work [31]. NCX exchanger expression is opposite to T_3 regulation [32,33]. Depending on the form of voltage-gated K^+ channels, their expression differs. There was investigated that Kv1,5; Kv2,1 and Kv4,2 are in direct proportion to T_3 presence. Whereas expression of Kv4,3 is not altered due to thyroid state [30]. These observations are similar with other study conducted by Shimoni et al. beside the Kv4,3 expression, which was hound to increase due to hyperthyroidism [34]. Moreover, is was investigated that expression of Kv1.5 channels is selectively present on the ventricular cardiomyocytes [35].

It isn't without importance, that T_3 contributes to down regulating the expression of angiotensin receptors in vascular smooth muscle cells. Angiotensin II type 1 receptor (AT1R) mRNA and its protein were down regulated in the aorta of T3-treated rats. It becomes an essential point for T3-induced vascular relaxation [36]. The opposite effect is for sympathetic nervous system by increasing β1 adrenergic receptor gene expression in ventricles. It works by double effect, that is, increase of β1 receptor function and density [37]. These genes and others being under T_3 regulation one are presented in Figure 2. To sum up, these genomic effects lead to an increased cell proliferation and as a result of higher anabolic cardiomyocyte rate can be one of the causes of pathological heart hypertrophy.

Thyroid hormone non-genomic action acts independently to nuclear thyroid receptor. Thus, these effects start quickly and include changes in various membrane ion channels, effects on cytoskeleton like actin polymerization and a variety of intracellular signaling pathways in heart and vascular smooth muscle cells. Thyroid hormones cross-couple to the phosphatidylinositol 3-kinase (PI3-kinase) localized under the cell membrane and the signaling process incorporates to

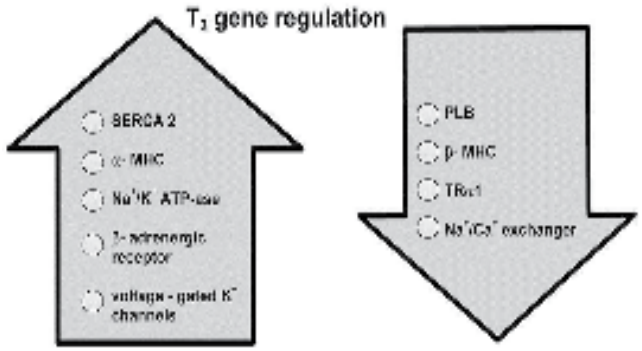

Figure 2: T3 regulates expression of some genes.

protein kinase B (AKT) pathway involved in cell proliferation and survival processes.

In vascular endothelial cells, through α1 isoform of thyroid receptor (TRα1), T_3 leads to activation of endothelial nitric oxide (eNOS) [38]. Acting together, in association with decreasing density of angiotensin receptors, vasodilatation effect becomes larger (mp).

This vascular dilatation effect contributes to maintain the homeostasis of systemic blood pressure. Smooth muscle cells isolated from a rat's aorta relax rapidly during T_3 exposure. It leads to decreased arterial resistance and as a result decreased blood pressure and increased cardiac output [39]. Studies on hypothyroid human model confirm this suggestion. Organism depleted of thyroid hormones revealed increased blood pressure, particularly diastolic one, connected to the peripheral vascular resistance. There was observed higher blood level of catecholamine's and decreased arterial compliance [40,19]. Thus, the cardiac preload increases and accordingly to Frank-Starling's mechanism, cardiac output elevates until a certain value (mp). Figure 3 is an illustration of cooperating processes leading to increase a cardiac output.

The role of T_3 and thyroxine (T_4) on microcirculation has also been evaluated. The application of T_3 and T_4 induced dose-dependent dilation of arterioles within 2.0 ± 0.5 and 16 ± 2 min from administration, respectively. It also seems that local conversion of T_4 to T_3 represents a crucial step for the dilation of the microcirculatory system, which can be now considered a target for a TH action [41].

Looking at the blood volume value and its regulation due to T_3, it is necessary to remember about the interplay between thyroid, kidneys and higher erythropoietin release [42,43]. Furthermore, it was found, that thyroid hormones increases accumulation of hypoxia induced-factor 1 protein (HIF-1) by increasing HIF-1 protein synthesis rather than leading to its proteasomal degradation. Increased synthesis of HIF-1 may also contribute to the adaptive response of increased oxygen demand under hyperthyroid conditions [44].

Thyroid hormones have also proangiogenic effect and can stimulate vascular growth both in normal heart and after myocardial infarction [45]. In this process an adhesive molecule integrin avb3 is included, acting as the key element. There is a place in structure of this integrin, where thyroid hormones join and lead to activation of mitogen-activated protein kinase and induction of angiogenesis [46]. Beside this, it's known that vascular endothelial growth factor (VEGF) is also involved in thyroid hormone-induced angiogenesis process [47-49]. This is mainly in the course of Grave's disease, when VEGF serum concentrations are higher than in euthyroid people [49]. That is why, greater vascularity is observed within the thyroid. VEGF and additionally fibroblast growth factor (FGF) increased expression can also affect the hypertrophy of cardiomyocytes [50,51].

Hyperthyroidism

Cardiological symptoms of hyperthyroidism may display as a result of both hyperthyroidism per se and its impact on already existed heart disease [52]. Thyroid affects cardio-vascular system on different ways. It has direct effect by chronotropic and inotropic positive effect. Additionally by the intervention in peripheral resistance and blood volume cardio-vascular effect is amplified or more evident. An increase of β-adrenergic receptors within heart is also significant thyroid hormone action as well. All cardiac and vascular events correlated to hyperthyroidism are called a hyperdynamic circulation state. This typically occurs as spontaneous increase of cardiac output and increase in systolic pressure due to reduction of peripheral vascular resistance and decrease in diastolic blood pressure [53]. Short-lived concentration of the overt T_3 blood is associated with positive and time-limited cardiac events [54]. When this state prolongs, it can cause established cardiac disturbances (mp). T_3 positive inotropic effect occurs as increased left ventricular ejection fraction (LVEF) at rest but paradoxically its significant fall during exercise is to be observed. Characteristic for hyperthyroid state is a decrease of cardiac output with clinical left ventricular failure symptoms during exercise [54].

In hyperthyroidism, at rest, myocardial contractility has been already increased due to molecular changes as altered synthesis of MHC, increase of SERCA2 expression. Physical exercises can't raise it more and lead to opposite effect (mp). Due to peripheral decrease of vascular resistance and increased blood flow, afterload reduces. Cardiac output rises as well. Heart metabolism accelerates and glucose uptake is also higher. Thus, an increase of heat production is observed. When there is additional burden of exercise it induces increase in afterload and heart cannot increase its capacity, because heart, even at rest, still works near its value being [55]. This paradoxical response disappeared within a few weeks of the patients becoming euthyroid [56]. A patient with undestroyed heart can feel, like mentioned above, a fall of physical tolerance, but a person with an organic heart disease can feel much

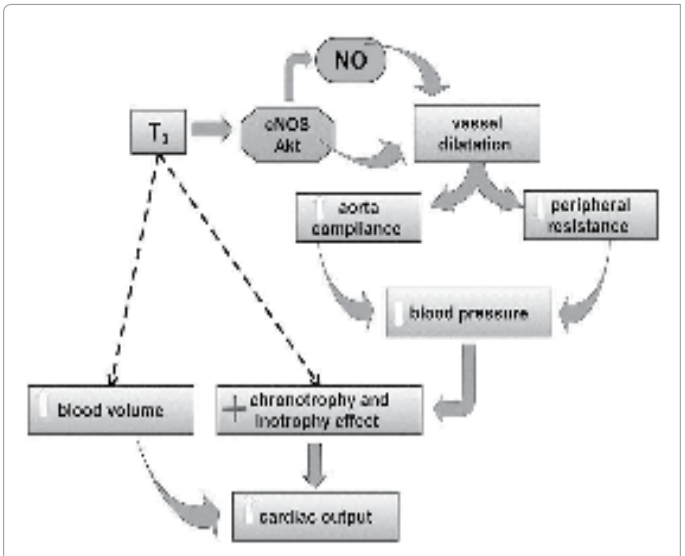

Figure 3: T3 coordinates blood pressure and cardiac output.

worse all the time. Dyspnea can be a main clinical symptom of it. Additionally, in the course of hyperthyroidism respiratory muscles work poorer and heart contraction is less efficient, which can intensify shortness of breath [57]. Myocardial ischemia is presumably caused by the increased demands of the thyrotoxic myocardium. However, a coronary spasm may be an additional factor and myocardial infarction can occur in the absence of significant atheroma [58].

Due to the fall of vascular resistance, kidneys' vessels relax and as a consequence, serum concentrations of angiotensin converting enzyme and erythropoietin increase. It causes absorption of renal Na^+ (increase of $Na^+\backslash H^+$ATP-ase activity) and polycythemia [59]. It leads to expanded blood volume and increase of end diastolic pressure (mp). As positive chronotropic cardiac event, sinus tachycardia is the most popular. The combination of rapid diastolic depolarization and shortage of the action potential of the sinoatrial cells causes this effect [54].

It can be clinically silent in young people, because human body can adaptate to persistent tachycardia. As a consequence this state can be revealed during a period appointment. Sometimes, mainly among children, tachycardia can be masked in special behavior. They could be nervous, unable to concentrate for a longer time or irritated. This state can be wrongly correlated to the psychological base of this behavior, like hyperactivity syndrome (mp).

Among a variety of atrial and ventricular tachycardia described in hyperthyroidism, atrial fibrillation (AF) is the most common one [55]. Pathophysiological view on AF is the shortage of refractory time in atrial cardiomyocytes' and increased sinoatrial activity [60], which causes difficulties to keep sinus rhythm, particularly in older people with some accompanying cardiac disorders like e.g. angina, arteriosclerosis or valvulopathies (mp). AF occurrence in population rises with age, from 0.1% among adults younger than 55 years to 9.0% in person in the age of 80 years or older. It makes AF the main tachyarrhythmia in society [61].

A Danish study, conducted by Frost et al. [62] included more than 40,000 patients with hyperthyroidism and it was shown that nearly 8.3% of them were diagnosed with AF or atrial flutter in the time of 30 days of the hyperthyroidism lasted. Because of its high percentage, it's necessary always to check thyroid function, when AF has been pronounced [63]. It's also important to exclude also subclinical hyperthyroidism (SH). AF can be the first symptom of that disorder [64].

It has been investigated, that prolonged atrial electromechanical coupling time and impaired mechanical atrial functions can be in the relationship with the increased prevalence of arrhythmias. Positive correlation has been found between intra- and interatrial delay and THS blood level in subthyroid patients, whereas negative correlation between TSH and interatrial delay in subclinical hyperthyroid patients have been observed [65].

Clinically patient may feel 'palpitations', which he/she explains as rapid, irregular heart rate. During patient's auscultation, doctor can recognize arrhythmia absoluta and a peripheral pulse deficit (mp) 60% of patients with hyperthyroid AF revert spontaneously to sinus rhythm within a few weeks after restoring normal tests of thyroid function. But it is often necessary to add a β-adrenoceptor antagonist to achieve adequate rate control [63,64].

Additionally, in hyperthyroidism there is a well-proved action of β-blockers in inhibiting T_4 ⊠ T_3 conversion (non-selective ones). Newest data shows, that during paroxysmal AF in euthyroid patient who had suffered from hyperthyroidism in the past and need amiodarone treatment to prevent sudden cardiac death, preventive radioiodine therapy (RAI) can be considered. Retrospective analysis showed, that this procedure, applied to 17 patients aged from 65 to 87 years old, was safe. Amiodarone-induced hyperthyroidism has performed in 5 patients and additional RAI was investigated. After 6 and 12 months, 14 patients revealed sinus rhythm during control examination [66].

When thinking about AF, there is a point of anticoagulation to prevent stroke due to embolization. However, some statistical data about the risk or benefits associated with this therapy in hyperthyroidism remain difficult to quantify [67,68].

That is why, the decision about anticoagulant therapy delivery should be analyzed in the context of patient age and coexistence of cardiovascular disorders, which lead to greater risk of stroke [69].

Despite the fact, that among younger patients with structurally normal hearts benefit from anticoagulation is not known at all, there are some case reports of arterial thromboembolism associated with AF in the course of hyperthyroidism [70].

It would be recommended to check, if during transoesophageal echocardiography (TEE) there is no atrial thrombus assertion. Beside this, patients with hyperthyroidism are associated to present an increased sensitivity to warfarin and its enhanced effects [69]. Doses of warfin should be reduced to avoid severe coagulopathy [71].

Many of clinical symptoms of hyperthyroidism seem to be close to these performed in the course of pheochromocytoma. However, serum and urinary catecholamine concentrations remain in correct range or they are even low in hyperthyroidism. It may be an argument for independently cellular action of hormones and catecholamines but with other signaling pathways within cardiomyocyte [55].

An isolated right ventricular failure is a possible, yet not very common disorder, due to hyperthyroidism. In literature, there are some clinical cases correlated with this state [72,73].

Lozano et al. conducted a case report with a young woman suffering from rapid progressive right-sided heart failure together with pulmonary hypertension and with no secondary causes of these disorders. Her only concurrent illness identified was Grave's disease. This state totally reversed after thyreoistatics therapy [74].

Giovambattista has also described similar kind of right failure case. It was a 51 year old woman with overt hyperthyroidism. She has stopped tiamazole therapy at the same month, when she presented obligatory semi-orthopnoic decubitus, severe edema, ascites and bilateral pleural effusion and echocardiographic findings of right ventricle volume overload was also observed [75]. Potential cardiac abberations performed in hyperthyroidism are seen in Figure 4. Long-term exposure to thyroid hormone excess, affect pathologically effects on cardiac morphology and function because of cardiomyocyte hypertrophy [76]. Morphological chances lead to an increase of left ventricular mass and its index (increase of left ventricular posterior wall together with the increase of intraventricular septum) [77-79].

LV hypertrophy is correlated with cardiac remodelling, that is overgrown cardiomyocytes, fibrosis of parenchyma and increase of apoptosis is observed. Missing cells are replaced with fibroblasts, which leads to less effective cardiac work [80].

Adrenergic activity is also increased. As a result, diastolic dysfunction exists and symptoms of heart failure can occur [54, 81]. β-blockers were proved to inhibit cardiomyocyte hypertrophy and

fibrosis effect caused by many disorders [80, 82].

Additionally, suppressive thyroxine treatment as obligatory treatment procedure after thyroid cancer also leads to cardiac hypertrophy even in the first year of therapy. Left ventricular mass and left ventricular index are increased. When β-blocker (e.g. bisoprolol) is added to suppressive therapy, after 6 months combination treatment hypertrophic changes inhibits and come back to range before suppressive therapy [83].

Whereas, big Framingham Heart Study evaluated that TSH was not related to LV mass, LV wall thickness or left atrial size and LV systolic function in either sex. Only an inversed correlation to LV contractility was observed [84].

NT-pro brain natriuretic peptide (NT-pro-BNP) as a cardiac dysfunction marker was measured to change its blood concentration level due to thyroid state. In hyperthyroidism, both overt and subclinical one, there was an increased NT-pro-BNP level than in control group (overt hyperthyroidism: 1,129.7 ± 1,119.8 pg/ml vs. 138.9 ± 173.3 pg/ml; subclinical hyperthyroidism: 598.1 ± 639.2 pg/ml vs. 138.9 ± 173.3 pg/ml), whereas in hypothyroid patients this effect wasn't observed. Moreover, L-thyroxine treatment increased plasma levels of this parameter [85].

In 2012 an interesting study was conducted with the aim to estimate thyroid function and morphology state before and after a cardiac invasive treatment - Percutaneous Coronary intervention (PCI) and percutaneous transluminal coronary angioplasty (PTCA) with single burden iodine contrast. The study showed that thyroid function alterations seen in laboratory blood tests are transmitted but both measurement of fT_3 and TSH blood level before cardiac invasive treatment and monitoring after this procedure should be necessary [86].

Hypothyroidism

In contrast to hyperthyroidism, deficiency of thyroid hormones in bloodstream is associated with opposite cardiac events. Due to a slow-down of the metabolism rate and nearly whole body functions, cardiac work diminishes as well. However cardiac symptoms aren't so evident as during excess of TH and are usually only prominent in patients with profound longstanding thyroid failure. A decreased cardiac output, heart rate, stroke volume, and myocardial contractility are observed, whereas systemic vascular resistance increases [55].

The authors want to present a development of coronary heart disease (CHD) as the first disorder connected to the hypothyroidism. Overt hypothyroidism contributes to its evolution on two ways. First theory is, that it exacerbates lipid profile, endothelium damage and hypertension. The second point of view is because of chrono- and inotropic activity reduces and oxygen deficit improves, which may provoke underlying coronary ischemia [87,88]. There is prevalent increased risk of CHD events and mortality correlated with different hypothyroidism state [89]. The hazard ratio (HR) for CHD was 1.89 for a TSH level of 10 to 19.9 mIU/L [90]. Increased prevalence of ischemic heart disease has also been reported in patients under 65 of age, affected patients affected by subclinical hypothyroidism (SH) [91].

Mayer et al. [92] tried to assess association with hypothyroidism as conventional cardiovascular risk factor in Czech Republic. Among 1240 participants taking part in the study, the overall prevalence of hypothyroidism was 6.8% in males and 13.8% in females. The relative risk of hypothyroidism was increased in males with manifest vascular disease, in females above 55 years old. Hypertension also contributed to that state. Moreover, the risk ratio increases in males and females with a positive TPOAb among euthyroid subjects.

What is characteristic for hypothyroidism is a change in lipids levels. In many cases its incorrect result, that is elevated LDL concentration and hypertriglyceridemia are the main point of appointment [87]. Moreover, sometimes patient can have revealed distorted lipid profile and hypothyroidism during periodical medical checkup. Organism can be adapted to this state, that is why no clinical symptoms are visible, but distantly consequences of that silent process are obvious (mp). Disturbed lipid profile probably results from reduced catabolism of lipoproteins, and simultaneously there is a decreased expression of lipoprotein receptors in the liver [93].

Tromsø's study has investigated, that TSH blood level remains in direct proportion with LDL and total cholesterol serum increases [94].

Inflammatory markers and homocysteine level are also elevated and contribute to creating an inflammation and oxidative stress in atherosclerosis [89,95].

These lipid disturbances together with slow energy metabolism, increased blood pressure can become the components of metabolic syndrome (MS). Roos et al. evaluated that in low, but still within range, fT_4 blood levels are related to abdominal obesity, triglycerides, high-density lipoprotein cholesterol, and blood pressure. What is more, low normal thyroxine level was found to increase insulin resistance [96]. Similar findings were obtained in a bigger study called Health ABC [97]. Due to the investigation of the thyroxine treatment was total cholesterol level reduced by 0.4 mmol/l and slight effect on HDL fraction of cholesterol were observed [98].

Among elderly patients with a cardiological burden, start of thyroid hormones supplementation therapy can even intensify myocardial ischemia or acute coronary events [55,87]. That is why, doses of thyroxine are increased gradually and more extended in time, moving towards euthyroid state. Hypertension, mainly diastolic one, occurs in about 30% of patients. Pathophysiological base of hypertension is due to impaired vascular smooth muscle cells relaxation. Reduced NO production and endothelial dysfunction lead to increased vascular resistance. However, this disorder can reverse rapidly after thyroxine therapy [99].

Cardiac-echo parameters and changes in left ventricle's (LV) work are not so noticeable and clinically evident as the opposite ones performed in hyperthyroidism. Its severity depends on hypothyroid progression (mp). Due to the decreased heart rate, low cardiac output is observed. Moreover, the afterload increases because of hypertension [10,100].

Due to overt hypothyroidism systolic and diastolic functions are reduced. It was observed both at rest and during exercise, and may display as dyspnea and fall of physical tolerance [54].

In a study, which evaluated subclinical hypothyroidism correlated with LV impairment (30 cases of SH and 15 of healthy control), a significant diastolic dysfunction in SH compared to healthy control (mean Ei/Ai = 1.35 ± 0.53 vs. mean Ei/Ai = 2.11 ± 0.26) has been shown with no significant impairment of systolic function [101].

Similar study has described the effect of T_4 therapy. It was demonstrated, that after 1 year of thyroxine therapy, only balance in hormones blood level was obtained and there were no signs of better cardiac-echo parameters. However, after a yearly follow-up, diastolic

Figure 4: Deviations in physical examination in hyperthyroid patients and additional tests.

dysfunction and echocardiographic features improved in these patients [102].

Cardiac preload decreases as well, due to the impaired diastolic function and the decreased blood volume [54, 103]. Moreover, hypothyroidism leads to chamber dilatation and impaired myocardial blood flow [104-106]. In connection with all the above mentioned hemodynamic changes, a loss of coronary arterioles, reduced cardiac oxygen consumption, higher progression of arteriosclerosis and impaired blood flow, a heart failure (HF) can develop in the course of hypothyroidism [10, 106- 109]. There was an interrelation between reduced both LVEF and total T_3, showing higher mortality than in patients having similar LVEF but normal total T_3 [110]. Disturbances connected with HF are more expressed with already exiting cardiac disorder than unloaded heart (mp).

Changes in thyroid heart metabolism (reduction in biologically active T_3) have been reported in Wassen et al. study, which has evaluated local hypothyreosis in cardiomyocytes within overloaded and failured heart. It is because of increase in activity of deiodinase D3, inactivating T_3 [111]. The same effect was seen after myocardial infarction [112]. This mechanism can be interpreted as a cardiac compensatory process of overloaded heart [113].

Molecularly, in the absence of T_3, concentration of β-MHC isoforms increases, containing low ATP-ase activity. Thus, cardiac contractibility decreases [114].

Cardiac remodeling in hypothyroidism is presented in chronic HF. Cardiomyocytes change its shape and disorganize. The pathophysiological changes in HF and hypothyroid have similar components, that is why, authors want to underline this theme [115-117].

More recently, cases of hypothyroidism and reversible dilated cardiomyopathy have been reported [24,118]. Selvaraj's et al. [119] study tried to estimate the correlation between Brain Natriuretic Peptide (BNP) value and diastolic LV dysfunction and determine, whether BNP and diastolic dysfunction were independently associated with T_3 level. The study consisted of 89 consecutive patients (mean age of 67±14 years and female predominance) with HFpEF (HF with Preserved Ejection Fraction). Patients were divided into two groups based upon median T_3 level (108 ng/dl). T_3 reference range was 87-178 ng/dl with the clinical cut-point for reduced T_3 was 87 ng/dl. Results indicated, that 22% of HFpEF patients had T_3 under range rate. Moreover, they had higher NYHA functional class and BNP levels. The study showed that T_3 is inversely associated with markers of HFpEF severity (BNP and diastolic heart dysfunction) . Heart failure progression performed in hypothyroidism is illustrated in the Figure 5.

Going to physical examination, delayed relaxation after ankle jerk reflex examination is special and its escalation correlates positively with TH deficit [4]. Although cardiological physical symptoms are not so obvious and characteristic in hypothyroidism (mp). That is why differential cardiac diagnosis should be always undertaken. As the first sign, doctor usually auscultates bradycardia and discovers bradycardia. Heart sounds seem to be quitter due to pericardial effusion but it's not an evident symptom, because an overweight or obese patient can demonstrate the same. X-ray examination can show an accumulation of fluid in pulmonary space. When there is concurrent HF, cardiac X-ray shadow can be enlarged as well. ECG reveals low QRS voltage as the one of more popular signs of hypothyroidism. Because of impaired ventricular blood-supply in the course of CHD, ventricular conduction can be disturbed and QRS complex can last more than 150 ms. Among arrhythmias performed in hypothyroidism the most common sinus bradycardia, is mentioned above (mp). Sometimes, when bradycardia becomes more intense, A-V block can occur [120]. The prolongation of the QT interval can also perform and it has similar morphology to the one, seen in euthyroid patients receiving class 3 antiarrhythmic agents [121]. More cardiac and laboratory symptoms can be seen in Figure 6. To better illustrate the interrelation between hemodynamic changes performing in hypo- and hyperthyroidism authors have created Figure 7.

Kidneys function is very important from a cardiological point of view. Their good work helps to maintain circulatory system competent and doesn't overload the heart (mp). In hypothyroidism kidneys reveal decreased perfusion with a consequent reduction in glomerular filtration, impaired free water clearance and hyponatremia [10].

On the other side, the prevalence of hypothyroidism associates with chronic kidney disease. An increasing hypothyroidism prevalence (overt and SH) directly proportional to lower levels of GFR was shown,

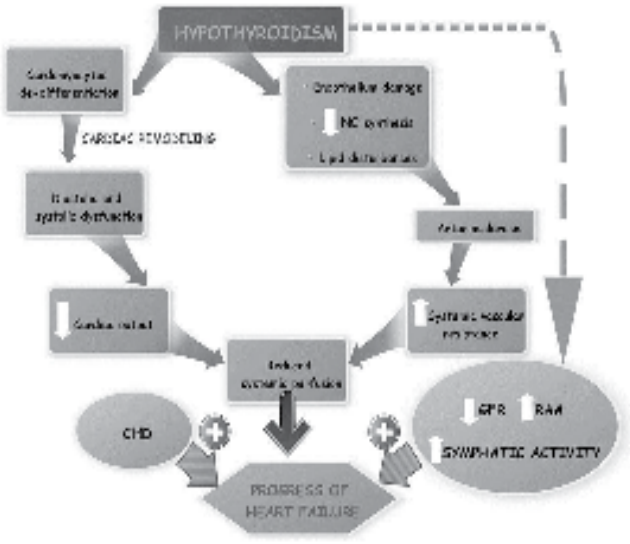

Figure 5: Heart failure progression due to hypothyroidism.

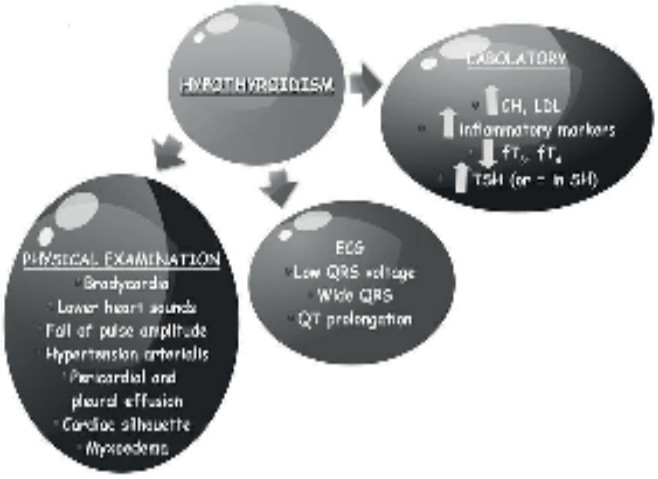

Figure 6: Deviations in physical examination of hypothyroid patient and additionally tests.

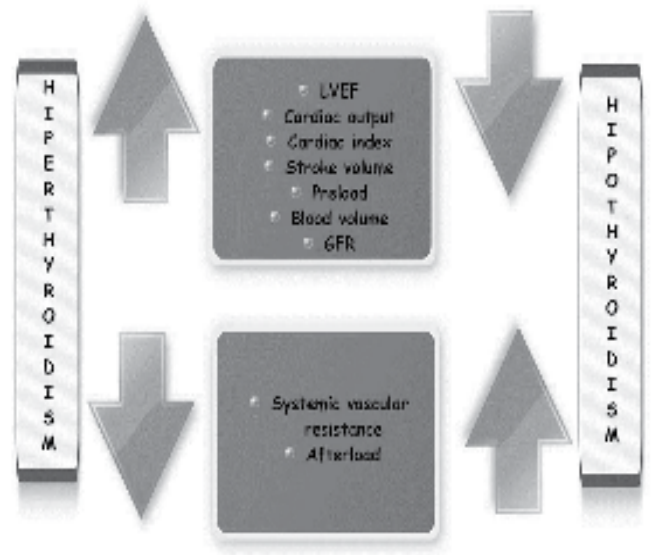

Figure 7: Interrelation in hemodynamic effects in hyper- and hypothyreosis.

occurring from 5.4% of subjects with GFR >/=90 mL/min/1.73 m^2, to 23.1% with GFR <30 mL/min/1.73 m^2 [122].

Cardiac Remodelling

Chronic hypothyroidism leads to induction of maladaptive changes in the shape of myocytes and their disorganization [106]. It leads to cardiac phonotypical remodelling as a stress response and contributes to pathological events such as ischemia, mechanical loading, and metabolic changes. In the short term, this response seems to be time-limited [123]. When it prolongs, it was investigated, that cardiac phenotype reminds the fetal one. Cells are described as de-differentiated [123]. Thus, features of fetal heart metabolism occur and glucose metabolism over fatty acids is presented as energy substrates. Transcription of some genes alters and among them are sarcomeric proteins such as these, already performed in hypothyroidism, increase in β-MHC expression [124]. Fetal replacement acts like "low-energy state", adapting and protecting already damaged myocardium [125]. The hypothesis about the opportunity to regenerate overloaded heart, was gained by the fact, that the return to fetal phenotype and cell dedifferentiation become an introductory allowable state for regeneration after stress. De-differentiated cells seem to have the ability to proliferate and/or grow and then to re-differentiate to specialized cells, affecting the regenerated structure or organ [126]. This regenerative potential is regained in adult life after return to fetal phenotype. However, cells' ability to re-differentiate may be diminished upon intense and sustained stressful stimuli [127]. Our point in this chapter is to show, that thyroid hormones delivery can enhance re-differentiation and restore damage. It was investigated that thyroid hormones' pathways signalling via TRα1 receptor may promote endogenous regeneration of damaged myocardium [128]. Several data of experimental evidence support this notion [129-132]. Moreover, thyroid hormones are shown to involve redox regulated signaling pathways [133], leading to altering cardiac cell shape, their differentiation process and up-regulating some molecules like heat shock proteins, which can increase tolerance of the cell against ischemia [134- 136].

Furthermore, TRα1 receptor appears also to mediate TH-induced cardioprotection [123]. Here, a paradox of TH action has been shown, being rather protective than detrimental for the ischemic heart, although it increases oxygen consumption (by accelerating heart rhythm and increasing cardiac contractility) and depletes the heart from glycogen [136].

In 2009 the phase II has been started, randomized, double blind, placebo-controlled Thyroid Replacement Therapy and Heart Failure Study (THiRST) by the use of substitutive doses of synthetic L-triiodothyronine in patients with STEMI (ST-Elevation Myocardial Infarction) infarction, having borderline or reduced circulating T_3 level. The study is conducted by Dr Iervasi [137].

Conclusions

Thyroid hormones have significant cellular actions, which determine homeostasis in cardio-vascular system and contribute to its god work. Both hyper- and hypothyroidism have its own characteristic way of cardiac symptoms connected to excess or insufficiency of T_3. An excess of thyroid hormones can cause an increase of cardiac output, heart action, ejection fraction, systolic and diastolic function, blood volume and systolic cardiac pressure. Simultaneously a decrease of peripheral resistance and diastolic blood pressure is observed [5]. Insufficiency of T_3 isn't so evident in clinical practice and its symptoms are opposite to hyperthyroidism. Authors hope that this paper give the reader broad point of view on thyroid cardiac clinical manifestations and explain cellular base of them.

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Editorial Open Access

Meningitis Outbreak in Nigeria: Public Health Alert

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Meningococcal meningitis is a life threatening bacterial infection caused by Neisseria meningitidis (13 serogroups), which can result in severe damage to the brain, with a case fatality rate of 50%, if left untreated [1]. Although, outbreaks of meningococcal meningitis have been reported across the world, it has been rated as one of the leading public health concerns in sub-Saharan Africa [1]. In-fact, the region has been named as the meningitis belt (extending from Senegal to Ethiopia - comprising of 26 nations), because of the large number of cases being reported in the region [2]. In addition, in the year 2014 alone, close to 12000 cases and 1146 deaths have been reported among the 19 nations of the meningitis belt [1,2].

The epidemiological analysis of the trends of the disease has shown that the disease has a seasonal variation, with maximum number of cases / outbreaks being reported in the dry season (December to June) [1]. This is probably because of the interplay of various factors like dust winds, cold nights, overcrowding, increased risk of upper respiratory tract infections, and significant population displacement because of the large number of pilgrims coming in the region during the season [2,3]. As anticipated according to the prevalent trends, since the beginning of 2015, a new outbreak of the meningococcal meningitis (caused predominantly by the serogroup-C) has been reported in Nigeria, in which 5855 cases, including 406 deaths (case fatality rate - 7%) have been notified till the first half of May month [2,4]. The number of suspects has increased at an alarming rate, with number of cases being tripled in the last couple of weeks, which is a serious concern [4]. Another area of concern is that for the first time a large-scale meningitis outbreak has been reported because of serogroup-C, and hence there is a significant shortage of the appropriate vaccine [2,4,5].

It is really a major public health concern that so many people are losing their lives because of a disease which can be completely prevented through the vaccines, some of which are available since the last 30 years [1,6]. In-fact, documented evidence is available to suggest that since the introduction of a new meningococcal-A conjugate vaccine (MACV) in the targeted age-group of 1-29 years, the number of cases have declined remarkably in the region [5,6]. Realizing the utility and scope of vaccine in reducing the burden of the disease, it has been advocated to facilitate prompt detection of cases and outbreaks through enhanced surveillance; appropriate management of cases with a complete course of antibiotic; to administer serogroup-specific vaccines in the affected region; prophylactic vaccination of the general population with MACV; and to introduce MACV into national routine immunization schedule [1,2,4]. In addition, there is a crucial need to constitute a national epidemic committee to respond to such outbreaks, and every attempt should be taken to mobilize and actively involve the national and international partners [2,4,5].

To conclude, as a part of preparedness and effectively contain and manage the outbreaks of meningococcal meningitis in the sub-Saharan African region, the need of the hour is to strengthen the existing resources, work in a concerted manner with the stakeholders, and effectively address the issue of vaccine shortage, so that any such future outbreaks can be averted.

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Research Article Open Access

Nanoheating without Artificial Nanoparticles

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Abstract

Our aim is to describe the physical and physiological effects of local modulated electro-hyperthermia (mEHT). The method concentrates on the energy absorption of cluster rafts of the plasma membrane. The targeted cluster contains transmembrane proteins, which can form coherent working voltage-gated ion channels and/or TRP receptors in one domain. This targeting is analogous to nanoparticle heating, except this uses readily available nanoparticles on the cell membrane instead of the artificially placed nano-object to induce selective energy absorption from the outside field. In the discussed non-artificial nanoparticle heating, selectivity is the consequence of the synergy of various interactions embedded into each other in their effective volume in macro-, micro- and nano-ranges.

Keywords: Membrane rafts; TRP receptors; Selection; RF current; Cancer; Hyperthermia

Introduction

Modulated electro-hyperthermia (mEHT, trade-name: oncothermia) is a type of hyperthermia applied in oncology for the curative and palliative treatment of malignant tumours [1]. It is highly selective for malignant cells, and solves the targeted therapy mostly on cancer cells. The selection is solved by the modulated radiofrequency carrier signal (13.56 MHz). The electronic excitation allows special laddering-type selection, step-by-step focusing from macro- to micro- and finally to the nano-range. The method has been widely applied in numerous research activities [2], and also in clinical practice [3-7]. Our objective in this paper is to discuss the mechanism of nanoscopic heating in detail, allowing a better understanding of the cancer-killing effect of mEHT.

The macroscopic selection of mEHT is based on certain differences in the metabolic rate of malignant and healthy cells (Warburg effect [8-10]). The high glucose influx of malignant cells changes their extracellular surrounding, where the ionic concentration increases dramatically, creating a more conductive path directing the electric current and focusing automatically on the malignant lesion.

The microscopic selection is determined by the differences of the dielectric constant of the extracellular electrolyte in the immediate vicinity of the malignant and healthy cells (Szentgyorgyi effect [11]). The dielectric permittivity of the extracellular electrolyte depends on its molecular order and fixed bonds, which are mostly absent around malignant cells [12-14]. Healthy cells have homeostatic electrolyte concentrations in different regions, while the cancer cells definitely modify it, losing the connections (adherent and junctional) between cells, which disorients part of the order and increases the electric permeability [15]. The dielectric forces decrease along with the increased dielectric permittivity, adding to the separation of the cells (autonomy) [16,17]. The energy which hits a cell in the healthy network can be shared immediately with the connected neighbours, while this mechanism is mostly missing in individual autonomic malignant cells; they disseminate the energy slower, using only thermal effects.

Malignant tissue has a special and distinguishable pattern, which is studied by pathology. In addition to the above localisations, mEHT also uses these structural differences for selection (pathological pattern recognition) of the malignant and healthy tissues (fractal physiology [18]).

The cellular membranes have further dielectric specialties in their parts in the nanoscopic range (Schwan effect [19]). The membrane-bound water has the upper part of the β-dispersion, denoted by δ [20,21], meaning that this part is highly selective for the various cell membrane states. In particular, all of the electrolyte and membrane properties differ between malignant and healthy tissue [22,23]. The proper selection uses the dipole relaxation of beta-dispersion connected to membrane-bound water [24]. This allows the zero-order electric-field action, which has no noise-induced thermal limit of the field [25]. The huge local dielectric permittivity of the transmembrane proteins and their clusters (rafts), are active in the electro-orientation of cells [26].

Artificial (standard) nanoparticle heating has very good selectivity, due to the targeted absorption of the locally-heated nanoparticles on cell membranes.

The electric field strength gain (ratio of the induced field in the material compared to the outside inducer field) is the largest in the cell membrane, as we know from *in silico* models [27]. The gain of up to a few tens of MHz is constantly $\approx 5 \cdot 10^3$, and it decreases by power law 1/f approaching higher frequencies [21]. In the case of more realistic tissue models, the gain of the membrane depends on the position of the cell in the tissue, but it is not lower than 10^2 in tissue arrangements [28]. In the case of cancer cells, the intracellular gain is the same as in non-cancerous cells, but the membrane gain in malignant cells is double that of their healthy counterparts [22].

The described concept of cell membrane rafts [29] is well studied nowadays in the relevant literature [30]. Important observations indicate a coherent cluster structure of a large number ($\sim 10^5$) of voltage-gated ionic channels [31,32], which could have transient receptor potential receptors (TRP, especially the TRPV subtypes) in one temperature-sensing domain [33]. These clusters are the targets of our

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method targeting the 'built-in' nanoparts of the membrane for heating selectively. Selection is based on the high specific absorption rate (SAR) of the nanoparts. The targets of heating could be these clusters of cell membrane in malignant cells.

Amongst the receptors belonging to the TRPV family, a strong correlation between clinicopathological findings and expression of different TRPV channels (e.g. TRPV1, TRPV2 and TRPV6) in cancer growth has recently been reported [34,35]. Changes in TRPV1 expression occur during the development of human urothelial cancer [36]. Recently, the role of TRPV1 mRNA down-regulation as a negative prognostic factor in patients with bladder cancer has been assessed [37]. However, increased TRPV1 expression is suggested as an inhibitory factor of cancer development [38]. Therefore, we hypothesised that any factors which can exhaust TRPV1 receptor expression can reduce tumorigenesis.

The TRPV1 ion channels are capable of exercising a regulatory effect on intracellular calcium concentration, which, in turn, plays a key role in cell function. The short-term regulation of cell function depends primarily on several cell components which are affected by the free calcium ions in the cytosol. However, long-term activation of the TRPV1 receptor and the consequent increase in intracellular calcium concentration results in cytotoxicity. Moderate heat (≥ 43°C) can activate TRPV1, demonstrating that thermal heat and the pungent ingredient in 'hot' foods elicit their effects through the same channel [39].

Non-excitable cells also have TRP channels [40], and thermosensors are one of these groups. Thermosensors are charged transmembrane proteins which are displaced by heat'. TRP is an 'electrochemical diode' which rectifies ionic transport through the membrane and is loaded in the closing direction under standard (resting, negative membrane potential, cell is positive outside) physiological conditions. These channels are primarily involved in Ca^{2+} transport. Due to their closed resting (default) value, the Ca^{2+} influx into the cell is rather limited. The diode characteristics (rectification parameters) are different in hot- [28] and cold-sensing [28] TRPs. Hot-sensing shifts the diode to the opening direction [41], while cold-sensing shifts the diode in the opposite direction [42], to the closing side. The regulation is dynamic, and the transmembrane protein is displaced by the temperature action [43]. The TRP receptors could be clustered in membrane rafts which are effective thermosensors of the cell [27]. These domains could considerably increase Ca^{2+} influx to the cytoplasm by heating.

The concentration of intracellular Ca^{2+} is low under normal conditions. However, Ca^{2+} has an important controlling function in the homeostatic balance of proliferation. Intracellular Ca^{2+} concentration affects the cellular decision between division and apoptosis [44]. The balance of proliferation and apoptosis is well regulated by Ca^{2+} entry. The Ca balance also acts on cell membranes; the membrane potential decreases in cells with enhanced concentrations of Ca^{2+} in the cytoplasm [45,46]. The lowered membrane potential, however, is pro-proliferative, both in healthy and malignant cases [47]. Malignant differentiation increases Ca^{2+} entry [48], which is pro-proliferative, reaching an oscillatory equilibrium of Ca^{2+}. Extracellular ATP induces oscillations of intracellular Ca^{2+} and membrane potential [49]. The ATP released from inflamed, damaged or metabolically impaired cells represents a 'danger signal' that plays a major role in activating the innate immune system, and intracellular Ca-overload also promotes apoptosis [50].

The aim of this study is to demonstrate a mathematical model which describes the TRP receptor function between heat and carcinogenesis.

Materials and Methods

The cellular membrane could be considered as fluid mosaic [51], where the transmembrane proteins move by lateral diffusion [52]. The inhomogeneous electric field in the case of mEHT acts on transmembrane proteins by dielectrophoretic force, drifting them forwards. This interaction is rather selective, because the dielectric permittivity of the transmembrane proteins is at least two orders of magnitude higher than the membrane permittivity in which they move [53]. As a consequence, the direction of the drift movement points to higher energy density, where the specific energy absorption (SAR) is also higher. SAR increases on these points of the membrane (micro-contacts), and consequently the increase in local temperature also affects the local TRPs ('Micro-contact' means the place on the cellular membrane where the electric field strength induced by the outside field is the highest. This point is roughly identical to the in/out point of the RF current or its imaginary part in direct mechanical contact with the neighbouring cells). As a consequence, the heat-sensing TRPs displace to the opening while the cold-sensing TRPs displace to the closing direction. The motility of TRPs is greater in the membrane of malignant cells [54], facilitating more TRPs in the high SAR places, allowing the certainly high Ca^{2+} influx to the cytoplasm. This changes the homeostatic equilibrium of the actual cell, promoting apoptotic cell death, as observed in mEHT treatments [55,56]. The increased BAX level in the affected cells [55] also indicates this apoptotic effect.

In this calculation, we assume the equal dielectric and diffusion properties of the various ion channels. In this case, the following equations describe the above drifting process:

$$\frac{\partial \rho}{\partial t} + div\mathbf{J} = 0, \qquad (1)$$

$$\mathbf{J} = \mathbf{J}_{drift} + \mathbf{J}_{diff} = -\mu \mathbf{F}\,\rho - D\,grad\,\rho$$

where $\rho(\mathbf{r},t)$ is the concentration of the ion channels in the $\mathbf{r}$ point of the membrane in the t time, $\mathbf{J}(\mathbf{r},t)$ is the resultant current density of the ion channels, $\mathbf{J}_{drift}(\mathbf{r},t)$ and $\mathbf{J}_{diff}(\mathbf{r},t)$ are the drift and diffusion current density, respectively, μ is the motility of t ion channel with D diffusion constant in the membrane and $\mathbf{F}$ is the dielectrophoretic force, which is [57,58]:

$$\mathbf{F}(\mathbf{r},t) = -a^3\varepsilon\frac{\varepsilon_{TRP}-\varepsilon}{\varepsilon_{TRP}-2\varepsilon}\,grad\frac{\left[\mathbf{E}(\mathbf{r},t)\right]^2}{2} \qquad (2)$$

where a is the radius of the ionic channel, ε and ε_{TRP} are the dielectric permittivity of the membrane and the TRP channels, respectively, and $\mathbf{E}(\mathbf{r},t)$ is the electric field strength in the $\mathbf{r}$ point of the membrane at t time. Since the relation of $\varepsilon < \varepsilon_{TRP}$ holds, from Eq. (2), a simple form of dielectrophoretic force follows:

$$\mathbf{F}(\mathbf{r},t) = -grad\left\{a^3\varepsilon\frac{\left[\mathbf{E}(\mathbf{r},t)\right]^2}{2}\right\} \qquad (3)$$

where we assume that the dielectric permittivity, ε, of the membrane does not depend on the position of the membrane. Due to the radiofrequency supply, the electric field is time-dependent. Let us

consider the average of the field in a period of time in Eq. (1), since the expected time constants in the processes are larger. In this case, Eq. (1) and its connected values are:

$$\begin{aligned}&\frac{\partial\langle\rho\rangle}{\partial t}+div\langle\mathbf{J}\rangle=0,\\&\langle\mathbf{J}\rangle=\langle\mathbf{J}_{drift}\rangle+\langle\mathbf{J}_{diff}\rangle=\mu\langle\mathbf{F}\rangle-Dgrad\langle\rho\rangle,\\&\langle\mathbf{F}\rangle=-grad\left\{a^3\varepsilon\frac{\mathbf{E}_{eff}^2(\mathbf{r})}{2}\right\}\\&\mathbf{E}_{eff}(\mathbf{r})\coloneqq\sqrt{\langle[\mathbf{E}(\mathbf{r},t)]^2\rangle}\end{aligned}\tag{4}$$

where we introduced the effective value of the electric field and the ‹› signs denote the averages. We are looking for the stationary solution of equations (4) when the resultant current density is zero; the drift and diffusion current densities are equal. In this case, we have:

$$\begin{aligned}&\frac{\partial\langle\rho\rangle}{\partial t}+div\langle\mathbf{J}\rangle=0,\\&\langle\mathbf{J}\rangle=\langle\mathbf{J}_{drift}\rangle+\langle\mathbf{J}_{diff}\rangle=-\mu\langle\mathbf{F}\rangle\langle\rho\rangle-Dgrad\langle\rho\rangle,\\&-Dgrad\left\{\frac{\mu}{D}a^3\varepsilon\frac{\mathbf{E}_{eff}^2(\mathbf{r})}{2}-\ln\langle\rho\rangle\right\}=0\Rightarrow\langle\rho\rangle=\langle\rho_0\rangle e^{a^3\varepsilon\frac{\mathbf{E}_{eff}^2(\mathbf{r})}{2kT}},\\&\frac{1}{kT}=\frac{\mu}{D}\end{aligned}\tag{5}$$

where k is the Boltzmann constant, T is the temperature of the membrane (introduced by Einstein's relation), and $\langle\rho_0\rangle$ is the ion channel density when the applied outside field strength is zero.

The increase in the density of ion channels is expected at the high field-strength micro-contact places, when the following condition is valid:

$$0<<a^3\varepsilon\frac{\mathbf{E}_{eff}^2(\mathbf{r})}{2kT}\tag{6}$$

This criterion is a simple consequence of Eq. (5). When this value in Eq. (6) is 1, the density of ion channels is nearly three times higher than the field-free equilibrium concentration, and the field strength would in this case be $E\approx10^7V/m$ [31], which is unrealistic. Consequently, this model failed.

Results

The polarisability of the biopolymers is well known [59]. The field-strength of the cell membrane is huge: $E\approx10^7V/m$ consequently, its effect on the ionic channels is expected and it will likely polarise the transmembrane proteins until their saturated polarisation value is reached because the rotational diffusion is stronger in the membrane than the translational [60] diffusion. Consequently, the transmembrane proteins have to have strong enough dipoles to block their rotation, which would likely be due to the thermal excitation. It would cause definite disturbances of their functions, so that saturation by the extreme huge membrane field maximises the electric dipole moment of the transmembrane proteins, ensuring their stability. The mathematical condition of the stability is:

$$1<<\frac{E_m p_{ich}}{kT}\tag{7}$$

where p_{TRP} is the dipole moment of the TRP. To estimate the value of this dipole moment, we consider the low frequency permittivity of the muscle tissue, which has a value in the range of 10^7 [61]. Assuming a linear polarisation effect until saturation polarisation is reached, we obtain for the value of saturation polarisation vector:

$$P=\varepsilon_0\left(\varepsilon_{TRP}-1\right)E_m\cong10^{-11}\times10^7\times10^7=10^3As/m^2$$

Choosing the volume of the ionic channel on the realistic $V_{TRP}\cong10^{-24}m^3$, the dipole momentum of the channel is $p_{TRP}=P_{TRP}=10^3\times10^{-24}=10^{-21}Ams$. With this assumption, we obtained a definite high value for the critical value in Eq. (7):

$$\frac{E_m p_{TRP}}{kT}\cong10^7\tag{8}$$

When the electric field in the membrane is E_m, and assuming the isotropy of polarisability of the channel, then in the saturated case, the polarisation vector turns to the direction of the resultant electric field keeping its absolute value (Figure 1). When no saturation occurs, the forced rotational orientation will not happen.

When $E<E_m$, then the E field induces $P(E)$ value of the polarisation vector, and:

$$P(E)\cong P\frac{E}{E_m}\tag{9}$$

Due to the small angles, the polarisation $\mathbf{P(E)}$ could be well approached as parallel with field $\mathbf{E}$. This allows the two cases to be described identically. In this way, the induced dipole momentum of the TRP channel is:

$$p_{TRP}(E)=V_{TRP}P(E)\cong PV_{TRP}\frac{E}{E_m}=p_{TRP}\frac{E}{E_m}\tag{10}$$

With this, the dielectrophoretic force acting on the TRP channel will be:

$$\mathbf{F}(\mathbf{r},t)=\left[\mathbf{p}_{TRP}(\mathbf{E}(\mathbf{r},t))grad\right]\mathbf{E}(\mathbf{r},t)=\frac{p_{TRP}}{E_m}grad\frac{\mathbf{E}^2(\mathbf{r},t)}{2}\tag{11}$$

Also, the drift diffusion equations according to Eq. (4) will be:

$$\begin{aligned}&\frac{\partial\langle\rho\rangle}{\partial t}+div\langle\mathbf{J}\rangle=0,\\&\langle\mathbf{J}\rangle=\langle\mathbf{J}_{drift}\rangle+\langle\mathbf{J}_{diff}\rangle=\mu\langle\mathbf{F}\rangle-Dgrad\langle\rho\rangle,\\&\langle\mathbf{F}(\mathbf{r},t)\rangle=\frac{p_{TRP}}{E_m}grad\frac{\langle\mathbf{E}^2(\mathbf{r},t)\rangle}{2}=\frac{p_{TRP}}{E_m}grad\frac{\mathbf{E}_{eff}^2(\mathbf{r})}{2}\\&\mathbf{E}_{eff}(\mathbf{r})\coloneqq\sqrt{\langle[\mathbf{E}(\mathbf{r},t)]^2\rangle}\end{aligned}\tag{12}$$

We solve these equations in the stationary case when the resultant current density is zero. Therefore, when the drift and diffusion current densities eliminate each other, then:

$$\begin{aligned}&\frac{\partial\langle\rho\rangle}{\partial t}+div\langle\mathbf{J}\rangle=0,\\&\langle\mathbf{J}\rangle=\langle\mathbf{J}_{drift}\rangle+\langle\mathbf{J}_{diff}\rangle=-\mu\langle\mathbf{F}\rangle\langle\rho\rangle-Dgrad\langle\rho\rangle,\\&-Dgrad\left\{\frac{\mu}{D}\frac{p_{TRP}}{E_m}grad\frac{\mathbf{E}_{eff}^2(\mathbf{r})}{2}-\ln\langle\rho\rangle\right\}=0\Rightarrow\langle\rho\rangle=\langle\rho_0\rangle e^{\frac{p_{TRP}}{E_m}\frac{\mathbf{E}_{eff}^2(\mathbf{r})}{2kT}},\\&\frac{1}{kT}=\frac{\mu}{D}\end{aligned}\tag{13}$$

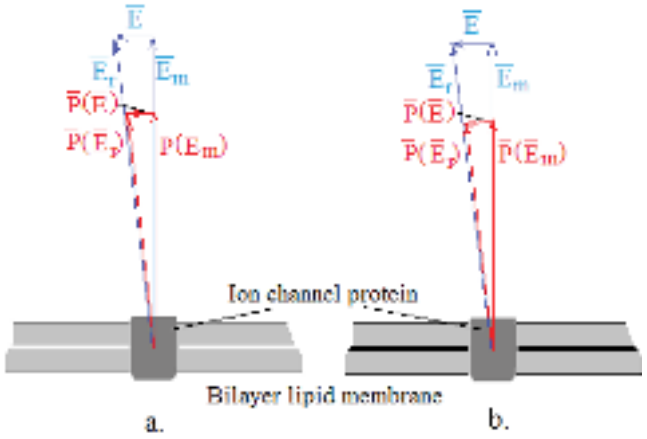

Figure 1: For calculation of the electrophoretic force (the angle of the resulting P(E) vector is small and negligible in comparison to the relative large components).

Similarly to Eq. (5), the expected concentration of the ionic channels in the high SAR micro-contacts could be from Eq. (13), when:

$$0 << \frac{p_{ich}}{E_m}\frac{\mathbf{E}_{eff}^2(\mathbf{r})}{2kT} \tag{14}$$

Using the value in Eq. (8), we obtain at $E_{eff} = 4\times10^3 V/m$ a relatively small field strength:

$$\frac{p_{TRP}}{E_m}\frac{\mathbf{E}_{eff}^2(\mathbf{r})}{2kT} \cong 1 \tag{15}$$

when the concentration is at least three times higher than the field-free equilibrium. This ***E*** value of the field strength is small in the membrane, due to the fact that the membrane gain at 13.56 MHz (carrier frequency of mEHT) is more than 100 [21,62]. In this consideration, the $E_{eff} = 4\times10^3 V/m$ field could be obtained by 40 V/m outside the electric field and causes a 0.04 mV field on the membrane. When considering the physiological safety limit for the outside field, which is 200 V/m, we get:

$$\frac{p_{TRP}}{E_m}\frac{\mathbf{E}_{eff}^2(\mathbf{r})}{2kT} \cong 25 \tag{16}$$

(This could be reached by 7×10^{10} time concentration gain.)

According to these considerations, there is a realistic physical basis in having large ion channel concentrations in the areas of the membrane where the SAR is large.

Due to safety reasons, /standards allow 200 V/m outside field strength. This could induce only 2× 10^4 V/m field strength in the membrane, which is negligible compared to the huge 107 V/m filed strength there. field strength there. Calculating conductivity of the membrane with $\sigma_m \cong 3\times10^{-7} S/m$, the connected SAR value is $SAR \cong 10^7 W/m^3$ dissipation, which is considerably larger (a thousandth of this value could heat a tumour up to 45°C). Nanoparticle heating often uses one to two orders of magnitude higher SAR than this certainly huge value. The phospholipid, the membrane's base material, is a very effective heat and electric isolator; therefore, the observed (at least not too great) conductivity properties must be produced by the transmembrane proteins and their channel constructions. When these are homogeneously distributed in the membrane, they heat it equally everywhere. The metabolic heat liberated from the cell machinery interacts with the environment in this way. The membrane conductivity could be increased locally by the concentration of the ion channels and the transmembrane proteins as a part of the membrane. Due to the stable membrane potential, in those places where conductivity is increased, the SAR also increases, while at the expense of this, other parts have a lower load. When the direction of ion channels in relation to the membrane does not change during their drift, the membrane conductivity (σ_{mlocal}) will be proportional to the local concentration of these channels. Hence:

$$\sigma_{mlocal} = \sigma_m \frac{\langle\rho\rangle}{\langle\rho_0\rangle} = \sigma_m e^{\frac{p_{TRP}}{E_m}\frac{\mathbf{E}_{eff}^2(\mathbf{r})}{2kT}} \tag{17}$$

where σ_m characterises the membrane conductivity when the channel distribution is homogenous. It can be assumed that the SAR gain at least follows the gain of the conductivity, so:

$$SAR_{local} = \sigma_{mlocal}E_m^2 = \sigma_m E_m^2 e^{\frac{p_{TRP}}{E_m}\frac{\mathbf{E}_{eff}^2(\mathbf{r})}{2kT}} = SAR\, e^{\frac{p_{TRP}}{E_m}\frac{\mathbf{E}_{eff}^2(\mathbf{r})}{2kT}} \tag{18}$$

where SAR is the value when the ion channels are distributed homogeneously. Using the conditions of Eq. (16), this means 7×10^{10} -times larger SAR at the concentration of the channels. This obviously increases the membrane temperature by a few centigrade without the addition of external SAR. The local temperature increase opens the heat-sensitive TRP channels, which further increase the local SAR and gains of the Ca^{2+} ion influx to the given cell.

The heat conduction coefficient of the extra- and intracellular electrolyte is considered equal in the first approximation [63]. We also assume that the large SAR induced by the concentrated channel distribution in a micro-contact is small, cylinder-shaped, and has no heat exchange on its jacket, and also that the heat flux conducted and flowing on the cylinder is connected to free space at its two ends (Figure 2). Let us consider a ΔT heat increase due to the I_q heat flux at λ heat conduction value (Figure 2).

In this case:

$$\Delta T = I_q R_q \tag{19}$$

where R_q is the thermal resistance of the cylinder, which could be determined from the stationary electric and thermal analogy, as follows:

$$R_q = \frac{R}{\rho\lambda} \tag{20}$$

where *R* is the electric resistivity of a circle plate having *a* radius and ρ specific resistivity [64]

$$R = \frac{\rho}{2a} \tag{21}$$

In the stationary state, the connection between the SAR_{local} and the I_q heat flux on the cylinder is:

$$I_q = \frac{SAR_{local}\, a^2 d}{2} \tag{22}$$

where the SAR_{local} is approximated as constant due to the small volume of the cylinder. From Eq. (19) using Eqs. (18), (20) and (21) we get:

T $$\Delta T = I_q R_q = \frac{\pi}{4}\frac{SAR_{local}\, ad}{\lambda} = \frac{\pi}{4}\frac{ad}{\lambda}\sigma_m E_m^2 e^{\frac{p_{TRP}}{E_m}\frac{\mathbf{E}_{eff}^2(\mathbf{r})}{2kT}}} \tag{23}$$

Calculating an example: when ΔT =7K and α = 1 μm, d= 10 nm, λ=0.6 W/ mK, σm=3×10^7 S/m, Em=10^7 V/m

$$e^{\frac{p_{TRP}}{E_m}\frac{\mathbf{E}_{eff}^2(\mathbf{r})}{2kT}} = 1.75\times10^7 \rightarrow \frac{p_{TRP}}{E_m}\frac{\mathbf{E}_{eff}^2(\mathbf{r})}{2kT} = 16.6 \tag{24}$$

Producing this value, we need $E_{eff} = 16\times10^3 V/m$ locally on the membrane and $E = 160V/m$ outside the applied electric field. These are actually possible values for practical application. In the case of special pink noise modulation, very high effective field strengths could occur (especially, when very long noise representation is given for the modulation. This is the consequence of Shannon's sampling theorem II).

The local temperature increase would be 44°C when the example above is applied. Thermolysis occurs at this temperature [65]. This is shown in Figure 3 [52].

The increased temperature melts the membrane lipids and starts to dissolve in the surrounding electrolytes, thus changing the configuration of the membrane lipids, resulting in the growth of the molecular permeability of the targeted membrane. This effect induces a further local SAR increase which damages the membrane. It is possible that the tumour treating fields effect [66] (NovoTTF, [67]) is based on this phenomenon.

The time function of a Ω_0 circular frequency harmonic carrier signal, which is amplitude modulated by a ___ carrier frequency sinus signal, is:

$$u(t) = U_0\left[1 + m\sin\left(\omega t + \phi\right)\right]\sin\Omega_0 t \tag{25}$$

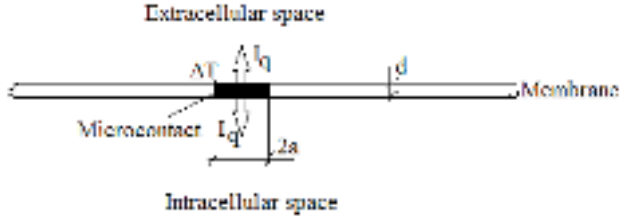

Figure 2: For calculating the increase in local membrane temperature.

where m is the modulation depth. The spectrum of this signal is shown in Figure 4.

Therefore, the effective potential is:

$$u_{eff}^2 = \frac{U_0^2}{2} + m^2\frac{U_0^2}{4} \tag{26}$$

Consequently, the modulation increases the effective value of the potential, and thus increases the effective electric field strength. As a consequence, it increases the local conductivity by Eq. (17) and the local SAR by Eq. (18). In the case of noise modulation, which has a continuous frequency spectrum (also it has no measurable discrete line in its spectrum), the local conduction and SAR could be increased even further.

Let us show an example of a modulated signal as below, where $x(t)$ is pink noise.

$$u(t) = x(t)U_0\sin\Omega_0 t \tag{27}$$

The noise power spectrum of the pink noise in $X(\omega)X^*(\omega) = \frac{A}{\omega}$ frequency interval is:

$$X(\omega)X^*(\omega) = \frac{A}{\omega} \tag{28}$$

In this case, the effective value of the modulated signal is:

$$u_{eff}^2 = \frac{U_0^2}{2} + 2A\ln\frac{\omega_f}{\omega_a} \tag{29}$$

Which in the case of $\omega_a \rightarrow 0$ goes to infinity: $u_{eff}^2 \rightarrow \infty$.

Consequently, this type of pink noise modulation is excellent for local heating (other important effects of pink noise modulation are discussed elsewhere [11]).

A further consequence of modulation with harmonic signals is the higher probability of the opening of voltage-gated ion channels by stochastic resonance [68]. With this, there is a considerable increase in the membrane conductivity, the ion influxes and the local SAR. Since the multiple voltage-gated ionic channels are concentrated in the

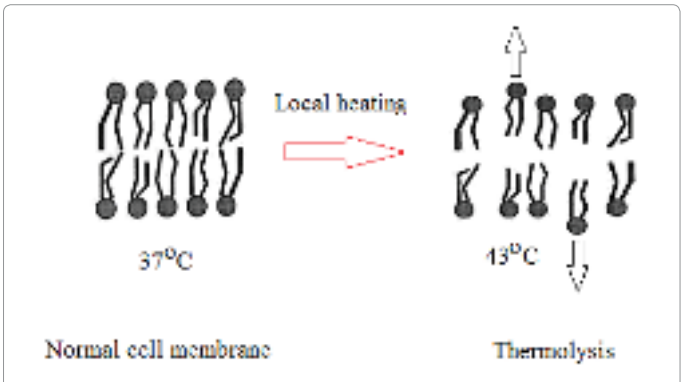

Figure 3: Conceptual view of thermally-driven membrane permeabilisation.

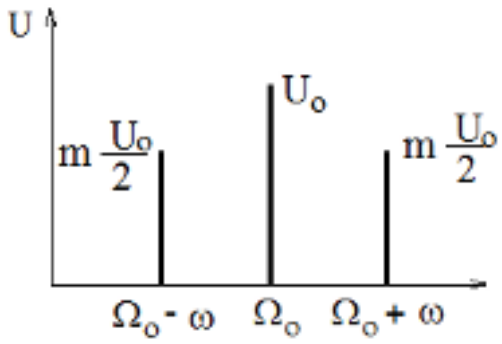

Figure 4: The frequency spectrum of an amplitude modulated harmonic signal.

dense cluster, multiple stochastic resonance frequencies exist there. It is possible that the 'magic' frequencies in [69-71] could be explained in a similar way.

The dynamic (time fractal) behaviour is the effective information exchange between the cells in space and also in time. Due to the malignant autonomy, the collective communication signals (called social signal) are broken [72]. The social signals and their correlation length is determined by different factors, among which is an energy pack-like information transfer e.g. [73-76]. Consequently, the normal (healthy homeostatic) biological processes could be described by self-similar function classes based on the dynamic observations for long-range correlation lengths [77,78]. The missing information exchange could be re-established by the forced delivery of information, for which the best is modulation of the RF carrier. The famous Adey-window, published in the early 1990s, was the first proof of the special modulation effects [79,80]. The modulation of RF carrier frequency then started to be used [81], and became an important new method for cancer therapies [82]. Numerous clinical results show its efficacy [83-86], and it was applied in oncothermia for dynamic selection from the late 1980s. The support of modulation is clearly shown in experimental results [87]. The carrier frequency delivers the information (modulation frequencies), since the cancer cells are much less 'transparent' at these frequencies than their healthy counterparts. Malignant cells are heated up by the selectively absorbed energy. The applied modulation helps to localise the tumour border, it helps to 'clear' the contours, whilst (most importantly) helping to select (self-focus) the energy intake, exciting numerous signal pathways on the outer cell membrane.

Appendix: Temperature On Membrane Rafts

The temperature from SAR in the stationary process can be derived from the non-linear Pennes equation [88,89]. Blood perfusion, metabolic heat and temperature dependence of the target leads to non-linearity. The simple equation in its general form is [90]:

$$\rho c\frac{\partial T}{\partial t}+c_b\rho_b w_b(T)(T-T_b)=\lambda\Delta T+p_0 SAR(T)+q_0 1,1^{(T-36,6)} \qquad (1)$$

The generalisation appears in the second term on the right side of the equation as we note the SAR from the heating process of the target resulting from the change in conductivity.

If we take the stationary case and neglect the part obtained from the conduction, then we get:

$$c_b\rho_b w_b(T)(T-T_b)=p_0 SAR(T)+q_0 1,1^{(T-36,6)} \qquad (2)$$

From this, the increase in temperature compared to the temperature of the blood is:

$$\Delta T=(T-T_b)=\frac{p_0 SAR(T)+q_0 1,1^{(T-36,6)}}{c_b\rho_b w_b(T)} \qquad (3)$$

With no consideration of the SAR and the metabolic heat, we come to:

$$\Delta T=(T-T_b)=\frac{p_0}{c_b\rho_b w_b(T)} \qquad (4)$$

where p_0 is the SAR of the target. Here, the blood perfusion rate is given in the SI system, which is: $\frac{m^3}{\sec m^3}$. The physiological adequate unit is: $\frac{ml}{perc\ 100g}$. To converse between these two:

$$w_b(T)=\frac{10^{-6}}{6}w_{bfiziol}(T) \qquad (5)$$

Putting this into the previous equation:

$$\Delta T=\frac{p_0}{c_b\rho_b w_b(T)}=\frac{p_0}{1,1\times10^3\times4,5\times10^3\times\frac{10^{-6}}{6}w_{bfiziol}(T)}=$$

$$=1.3\frac{SAR}{w_{bfiziol}(T)} \qquad (6)$$

which could be due to the different database applications.

In case the temperature rises when the heating power is switched on, the simplified Pennes equation can be used:

$$\rho c\frac{\partial T}{\partial t}+c_b\rho_b w_b(T-T_b)=p_0,$$

$$\rho c\cong c_b\rho_b \qquad (7)$$

consequently:

$$\frac{\partial(T-T_b)}{\partial t}+\frac{1}{\tau}(T-T_b)=\frac{p_0}{\rho c},$$

$$\tau=w_b^{-1} \qquad (8)$$

Therefore, the solution is:

$$\Delta T=\frac{\tau}{\rho c}p_0\left(1-e^{-\frac{t}{\tau}}\right) \qquad (9)$$

From here, the starting speed of the temperature rise is:

$$\left.\frac{\Delta T}{t}\right|_{t=0}=\frac{\tau}{\rho c}p_0\left(1-e^{-\frac{t}{\tau}}\right)\cong\frac{p_0}{\rho c} \qquad (10)$$

Substituting the values:

$$\left.\frac{\Delta T}{t}\right|_{t=0}\left[C^0/\min\right]=60\frac{SAR\left[W/kg\right]}{c}\cong\frac{SAR\left[W/kg\right]}{67} \quad (11)$$

Hence, the measurement of the speed of temperature rise at the beginning of heating determines the SAR according to (11).

This makes it possible to calculate the SAR in the target by measuring the speed of the temperature rise in the neighbouring healthy tissue (assuming equal temperature) and to calculate the SAR in the malignant neighbourhood.

The switching-off of the heating power can be approximately described with the following Pennes equation:

$$\frac{\partial\left(T-T_b\right)}{\partial t}+\frac{1}{\tau}(T-T_b)=0,\qquad \tau=w_b^{-1} \quad (12)$$

The solution is:

$$\Delta T\left(t\right)=(T-T_b)=\frac{\tau}{\rho c}p_0e^{-\frac{t}{\tau}}, \quad (13)$$

From this, the $\Delta T(t)$ function, the τ perfusion time constant and the w_b by (8) are also calculable. The starting slope of the clearance of temperature, for example the speed of decrease of the temperature, is:

$$\left.\frac{d\Delta T}{dt}\right|_{t=0}=-\frac{p_0}{\rho c} \quad (14)$$

which is the same in absolute value as that obtained from (10). Substituting the values, we again obtain (11) with the opposite sign:

$$\left.\frac{d\Delta T}{dt}\right|_{t=0}\left[C^0/\min\right]=-60\frac{SAR\left[W/kg\right]}{c}\cong-\frac{SAR\left[W/kg\right]}{67} \quad (15)$$

The starting speed of the temperature rise and the starting slope of the clearance are equal in their absolute value, independent of the perfusion constant. This means that the starting slope of the switch-on and -off depends only on the SAR and is independent from vascularisation.

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Editorial Open Access

Strategies to Facilitate Integration of AYUSH in the Existing Health Set-Up: Program Manager Perspectives

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India is in possession of a wide range of socio-cultural and linguistic variability and is one of the most populous nations of the world [1]. Being a middle-income country with an ineffective public health system (viz. geographical inequitable distribution of health establishments or vacant posts of health professionals, etc.), the masses have been exposed to a range of health concerns [2,3]. However, the impact of all these public health concerns can be remarkably reduced, if quality assured health care services is accessible and available to all [2,4].

Apart from the Allopathic fraternity, distinctive sort of scientifically proven, legally recognized, and acceptable field of medicine such as Ayurveda, Yoga, Naturopathy, Unani, Siddha and Homeopathy (AYUSH) are in operation in variable parts of the country [2,5]. Acknowledging the manpower resources and to combat the significant shortage in number of health care professionals, the National Rural Health Mission (now known as National Health Mission) has recommended to mainstream the AYUSH system of indigenous medicine [6,7]. Mainstreaming of AYUSH basically refers to the process of integrating AYUSH system of medicine with the existing health system in the country, at all levels of health care (viz. co-location in all public health establishments), so that preventive, promotive and rehabilitative health care services can be offered to all sections of society [8].

To ensure successful implementation of the process of mainstreaming all across the country, multiple measures such as strengthening of infrastructure – building, equipments and dispensaries; involving state government to decide which system of medicine should be set-up in a specific state; setting up of specialty centers and clinics in district headquarters hospitals and medical colleges; creating a managerial post for ensuring effective supervision and implementation of different activities at district/state level; building linkages with multiple sectors; encouraging cross-referral between allopathic and AYUSH streams; involving AYUSH practitioners to create awareness about their systems; mobilizing existing AYUSH establishments; integrating AYUSH with accredited social health activists (ASHA) workers by training them on relevant aspects of AYUSH; implementing initiatives for ensuring availability of AYUSH drugs at all levels; strengthening quality control mechanism in laboratories to avoid manufacture and sale of counterfeit and sub-standard drugs; streamlining the process of drug standardization so as to determine the drug potency; expanding the existing laws to encompass the manufacture and sale of drugs; and facilitating research work and promoting publications by exploring the local health traditions and traditional drugs used by experienced local health practitioners; have been proposed and implemented with varying range of success [6,8-10].

However, the proposed strategies have not been achieved the desired results owing to the presence of multiple challenges / barriers such as distinct approach for management of a clinical condition; unrelated rationale involved in different systems practice; an unclear policy for cross referral; inadequate or absent infrastructure, assistance and supplies; potential rise in cross practice; shortage of staff; inequitable emoluments; ethical concerns (viz. no healthy dialogues between practitioners of either system/not disclosing which type of practitioners the patient is seeing); and lack of accountability mechanisms especially at the grassroots levels [8,10-12].

In order to counter the identified challenges, the program managers have come with a comprehensive strategy which includes specific elements like developing/upgrading AYUSH institutes/colleges; conducting re-orientation training program for AYUSH personnel; facilitating international exchange of experts and officers; providing incentives to drug manufacturers, entrepreneurs, AYUSH institutions for international propagation of AYUSH; formulating uniform policy on reimbursement of AYUSH treatment; extending support for international market development and AYUSH promotion-related activities; establishing AYUSH information cells in foreign countries; organizing international fellowships for foreign nationals for undertaking AYUSH courses in premier institutions in India; conducting integrative research to assess the scope of drug formulations or community-based epidemiological studies to assess the utility of AYUSH in health care delivery system; involving pharmacists; and by implementing strategies that have been successfully employed in other countries, to fast-track the process of integrating AYUSH system [9,10,13,14].

To conclude, the program managers have to supervise the process of integration of AYUSH into the existing health system so as to combat the public health concerns of inadequate health professionals and ineffective public health system.

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Editorial Open Access

Supporting the World Health Organization's 2015 Immunization Week Theme: "Are You Up-To-Date?"

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Editorial

Globally, immunization has been acknowledged as one of the crucial elements of the human right to health and a key responsibility for the health care professionals/public health authorities [1]. The recent estimates suggest that every year the intervention of immunization is averting almost 2-3 million deaths from the vaccine-preventable diseases across the globe [2]. In-fact, in the year 2013, almost 1.5 million children died because of not receiving the vaccines which have been recommended by the World Health Organization (WHO) [3].

Even though, the immunization coverage trends over the recent years reveal that it is steady (viz. 84% infants received 3 doses of Diphtheria-Pertusis-Tetanus vaccine), nevertheless, 21.8 million infants still fail to receive basic vaccines [2,3]. However, the alarming concern is that almost 50% of these unimmunized children are from developing nations - India, Nigeria and Pakistan [3]. Such poor immunization coverage has been attributed to the resource constraints; competing health priorities; defective management of the health care delivery system; and limited monitoring/supervision of the different aspects of the immunization program at all possible levels [1,2]. In addition, parameters like un-registered females during the pregnancy; educational status of parents; inadequate support from husband/paternal grandmothers; large family size; family problems; poor maternal knowledge about the need & importance of immunization; fear about side effects attributed to vaccine administration; health professionals knowledge, attitudes, and perceptions about the vaccines; limited accessibility to health establishments; and seasonal migration, plays a significant role in determining the immunization coverage [4-7].

In order to augment the global vaccine coverage, the WHO in collaboration with various stakeholders like UN agencies, global agencies, national governments, health professionals, manufacturers, researchers, etc., has developed a Global Vaccine Action Plan (GVAP) to prevent millions of deaths by ensuring equitable access to the vaccines [8]. The GVAP aims to achieve a target of ≥90% national and ≥80% immunization coverage in all the districts by the year 2020, by ensuring sustainable access to vaccines at affordable prices; facilitating the local manufacture of vaccines to warrant vaccine security; improving the quality of data through the adoption of electronic registries; establishing the practice of risk communication; and by developing customized strategies based on the local priorities and needs [8,9]. Furthermore, in order to counter the challenges and other potential determinants, the need of the hour is to strengthen the immunization services (in accordance with the clients' needs so that the immunization services are convenient, reliable, friendly, and informative as well), especially in those nations which are home to the highest number of unvaccinated children [1,8,9].

Other measures like ensuring registration of all pregnant females during antenatal period so that they can be sensitized about the immunization; strategies to improve education status of females in specific & society in general; increasing awareness and reducing fear about side effects of immunization; increasing the accessibility to health centres; measures to involve the community actively; and building linkages to foster international collaboration, have also been suggested to improve the vaccine coverage [4-7,10]. Finally, the last week of April each year is observed as World Immunization Week, which provides an opportunity to program managers to increase the public awareness of how immunization saves lives, and motivate people to immunize themselves and their children against the life threatening diseases [2].

In conclusion, timely immunization of children against the vaccine-preventable diseases enables them to thrive and reach their complete potential. It is the responsibility of the policy makers to ensure that not only children, but even adults are immunized, as vaccines is a cost-effective approach to improve a nation's future.

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Research Article Open Access

The Role of the Active Oxygen Produced from Gp91phox NADPH Oxidase on the Newborn Weight of Mouse Pups

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Summary

It is known that active oxygen plays an important role in a reproduction. However, no report has so far investigated the influence of active oxygen produced from gp91phox NADPH oxidase on newborns. In this study, we investigated the influence of active oxygen on the weight of newborns using graviditas gp91phox-knockout (gp91phox$^{-/-}$) mice. Gestational C57BL/6j (control), gp91phox$^{-/-}$, and insulin-like growth factor-1-knockout (IGF-1$^{-/-}$) mice were examined and the weight of the newborn mouse pups were analyzed. Gp91phox$^{-/-}$ and IGF-1$^{-/-}$ mouse pups had low weight compared with control mice. When the control mice were treated with an inhibitor of reactive oxygen species (ROS), the newborn weight decreased. Conversely, when the gp91phox$^{-/-}$ mice were treated with an activator of ROS, the newborn weight increased, however, it remained low in the IGF-1$^{-/-}$ mice. Moreover, there were decreased levels of IL-1 in the plasma of graviditas gp91phox$^{-/-}$ mice compared with control and IGF-1$^{-/-}$ mice. Treatment with an IL-1 receptor antagonist in the control mice resulted in a low newborn weight, similar to the gp91phox$^{-/-}$ and IGF-1$^{-/-}$ mice. Furthermore, the expression of NLRP3 and caspase-1 in the uterus of graviditas gp91phox$^{-/-}$ mice was low compared with the control and IGF-1$^{-/-}$ mice. These results clearly indicate that gp91phox NADPH oxidase produces ROS during graviditas. The ROS activate NLRP3, and NLRP3 leads to the production of caspase-1, which subsequently increases IL-1, thereby finally inducing IGF-1. Because the newborn weight is determined by IGF-1, gp91phox appears to be important for promoting fetal growth during graviditas.

Keywords: Gp91phox; Insulin-like growth factor-1 (IGF-1); Reactive oxygen species (ROS); Interleukin-1 β (IL-1 β); Caspase-1

Introduction

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) is a multicomponent enzyme complex originally decreased in phagocytes [1-4]. Nox consists of seven organization members (Nox1-5 and Duox1 and 2) and all the members produce reactive oxygen species (ROS) [5]. Nox2, also known as gp91phox, is mostly expressed in macrophages or neutrophils [6]. Gp91phox has been largely investigated due to its role in the production of ROS by p67 and the regular arrangement of collecting venules [6]. ROS, which are produced by gp91phox, plays an important role during biophylaxis by acting as a disinfectant [7]. However, ROS also cause oxidative stress. It has been suggested that ROS constitutes the pathogenesis of lifestyle-related disease, such as infection, inflammation, cancer, arteriosclerosis and diabetes mellitus, and various nervous system diseases such as Alzheimer's disease. Furthermore, according to recent research, ROS have been demonstrated to be important as a signal molecule. These studies show that ROS activates a specific signaling pathway, similar to other signal transducers, and may regulate cellular protection, cell differentiation propagation, and cell death [8-10].

In addition, it is reported that ROS are involved in sexual organ development or reproductive behavior. Recently, it was suggested that the ROS released from gp91phox NADPH oxidase, expressed in neutrophils, play a vital role in the regulation of ovulation and the estrous cycle [4]. Furthermore, we previously reported the decrement in the newborn weight using gp91phox-knockout (gp91phox$^{-/-}$) mice [11]. Moreover, gp91phox$^{-/-}$ mouse pups also demonstrated decreased growth hormone levels [11]. Thus, although ROS has been shown to participate in ovulation or the estrous cycle, no study has investigated its effect on fetal growth.

We herein examined the role of gp91phox on the weight of newborn mouse pups and investigated the relationship between gp91phox and growth factors.

Materials and Methods

Animals

Female C57BL/6j mice (SLC, Hamamatsu, Shizuoka, Japan), C57BL/6j gp91phox$^{-/-}$ mice (Jackson Laboratories, Bar Harbor, ME, USA) and C57BL/6j insulin-like growth factor-1-knockout (IGF-1$^{-/-}$) mice (Tokyo Metropolitan Institute of Gerontology, Itabashi, Tokyo, Japan) were used. The mice were kept on a 12-hour light/12-hour dark cycle at 23 ± 1°C in SPF conditions. All animals had free access to water and laboratory chow diet (CE-2, Oriental Yeast Co., Tokyo, Japan) *ad libitum*. The animals were randomly allotted to different groups with six mice in each group. The weight of the newborn pups was recorded for the C57BL/6j, gp91phox$^{-/-}$ and IGF-1$^{-/-}$ mice. This study was conducted in accordance with the Official Guide for the Care and Use of Laboratory Animals of the Suzuka University of Medical Science (Approval number: 34). All surgery was performed under sodium pentobarbital anesthesia, and all attempts were made to minimize suffering.

In addition, the blood concentration of IGF-1 of the mother's body and the fetal weight tends to correlate, thus if the level of IGF-1 is high, then the fetal weight will increase [12-14]. Therefore, this study

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examined the change in the amount of IGF-1 in the tissue and blood of graviditas mice. Furthermore, the value of IGF-1 in the gestational age is high compared with after giving birth in C57BL/6j (control) mice (data not shown). In particular, the value of IGF-1 on the 18th (day 18) was the highest among the gestational age; therefore, we used the blood sample on day 18 for the examination.

N-acetyl-L-cysteine (NAC) treatment

Two hundred mg/kg of n-acetyl-L-cysteine (NAC) (ROS inhibitor; Nakarai Tesque, Kyoto, Japan) in 0.08% dimethyl sulfoxide (DMSO) was injected intraperitoneally for a total of 10 times, once a day, starting from the first day of graviditas; DMSO alone was injected into the graviditas control mice [15].

PAC-1 treatment

Ten mg/kg of the PAC-1 (ROS activator; Selleck Chemicals, Houston, TX, USA) in 0.08% DMSO was administrated orally a total of 10 times, once a day, starting from the first day of graviditas, while DMSO alone was administrated to the graviditas control mice [16].

Interleukin-1 (IL-1) β receptor antagonist (IL-1RA) treatment

Ten mg/kg of an IL-1β receptor antagonist (IL-1RA; ATGen Ltd., Gyeonggi-do, South Korea) in saline was injected intraperitoneally into the mice throughout the graviditas period [17]. Saline alone was injected into the control mice.

Anti-tumor necrosis factor (TNF)-α treatment

Three μg/kg of the anti-TNF-α antibody (R&D Systems, Minneapolis, MN, USA) in saline was injected intraperitoneally into the mice throughout the graviditas period [18]. Saline alone was injected into the control mice.

Caspase-1 inhibitor treatment

The mice were treated with the caspase-1 inhibitor, Ac-YVAD-CMK (10 mg/kg S.C.; Calbiochem, La Jolla, CA, USA), throughout the graviditas period [19]. Control mice were treated with vehicle only (1:1 v/v saline/polyethylene glycol 300).

Quantification of the levels of IGF-1, IL-1β and TNF-α in the plasma using an enzyme-linked immunosorbent assay (ELISA)

Blood samples were obtained from the mice on day 18 of gestation, and the plasma samples were fractionated. The plasma levels of IGF-1, IL-1β and TNF-α were determined using commercial ELISA kits (IGF-1; Assaypro LLC., St. Charles, MO, USA; IL-1β and TNF-α; R&D Systems) according to the manufacturer's instructions.

Measurement of plasma ROS concentration

The plasma ROS levels were determined using an OxiSelect™ *In Vitro* ROS/RNS Assay Kit (STA-347; Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's instructions.

Western blotting

Uterus samples were obtained from the mice on day 18 of gestation. Fixed whole uterus samples were homogenized in Lysis buffer (Kurabo, Osaka, Japan), and centrifuged at 8,000 x g for 10 min. The supernatant from each sample was then isolated and stored at -80℃ until analysis. After thawing, the samples (amount of protein: 10 μg/lane) were loaded onto a 4-12% BIS-TRIS Bolt gel (Life Technologies, Carlsbad CA, USA) and electrophoresed at 165 V for 30 min. Following separation, proteins were transferred to a nitrocellulose membrane using an iBlot Western blotting system (Life Technologies, Carlsbad, CA, USA), which was subsequently blocked with 5% skim milk overnight at 4°C. After blocking, the membranes were incubated at 25°C for 1h with primary antibodies against nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) (1:1000; Abnova, Taipei, Taiwan), caspase-1 (1:1000; Epitonics, Burlingame, CA, USA), or β-actin (1:5000; Sigma-Aldrich, St. Louis, MO, USA). Immune complexes on the membranes were then visualized using a horseradish peroxidase-conjugated secondary antibody (Life Technologies, Frederick, MD, USA) and ImmunoStar Zeta (Wako, Osaka, Japan). Images were acquired using the Multi-Gauge software program (Fujifilm, Greenwood, SC, USA).

Statistical Analysis

The results obtained from the animal groups were compared using either ANOVA or Student's *t*-test using an ANOVA software program (XHL STAT, Artwork Conversion Software Inc., Santa Cruz, CA, USA). First, we analyzed all data by an ANOVA, and only items with significant differences were further evaluated using the t-test. All data are expressed as the means ± standard deviation, and significance was set at $P<0.05$.

Results

The plasma levels of ROS and IGF-1 and newborn weight in gp91phox$^{-/-}$ and IGF-1$^{-/-}$ mice

The newborns weight of gp91phox$^{-/-}$ and IGF-1$^{-/-}$ mice decreased compared with the control mice (Figure 1A). Additionally, the plasma ROS level was decreased in graviditas gp91phox$^{-/-}$ mice (Figure 1B). On the other hand, the plasma IGF-1 level was low in both gp91phox$^{-/-}$ and IGF-1$^{-/-}$ graviditas mice compared with control mice (Figure 1C).

Effect of NAC or PAC-1 administration on the plasma IGF-1 and ROS levels, and newborn weight in graviditas mice

The newborn weight and the plasma IGF-1 and ROS levels in graviditas mice decreased in the control mice (C57BL/6j) following treatment with NAC (an inhibitor of ROS; Figure 2A). Conversely, graviditas gp91phox$^{-/-}$ mice treated with PAC-1 (an activator of ROS), showed increased newborn weight and plasma IGF-1 and ROS levels, similar to the control mice (C57BL/6j) (Figure 2B).

The plasma IL-1β and TNF-α levels in graviditas mice

We measured the plasma IL-1β (Figure 3A) and TNF-α (Figure 3B) levels in graviditas C57BL/6j, gp91phox$^{-/-}$ and IGF-1$^{-/-}$ mice. The IL-1β and TNF-α levels were decreased in the graviditas gp91phox$^{-/-}$ mice compared with the control mice, whereas the levels remained unchanged in the graviditas C57BL/6j and IGF-1$^{-/-}$ mice.

Effect of IL-1RA and anti-TNF-α treatment on the newborn weight in graviditas mice

In the C57BL/6j, gp91phox$^{-/-}$ and IGF-1$^{-/-}$ graviditas mice, the influence of an IL-1RA on the newborn weight is shown in Figure 4A, and the influence of anti-TNF-α treatment is shown in Figure 4B. Following the IL-1RA treatment, the newborn weight was decreased in C57BL/6j, gp91phox$^{-/-}$ and IGF-1$^{-/-}$ mice. Following the anti-TNF-α treatment, the newborn weight was decreased in gp91phox$^{-/-}$ and IGF-1$^{-/-}$ mice compared with C57BL/6j mice.

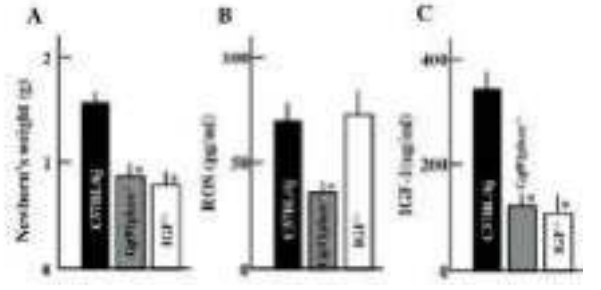

Figure 1: The newborn pups' weight (A) and the plasma levels of ROS (B) and IGF-1 (C) in C57BL/6j (control), gp91phox$^{-/-}$ and IGF-1$^{-/-}$ graviditas mice. The values are presented as the means ± SD derived from six animals. *, P<0.05 in comparison to the control mice.

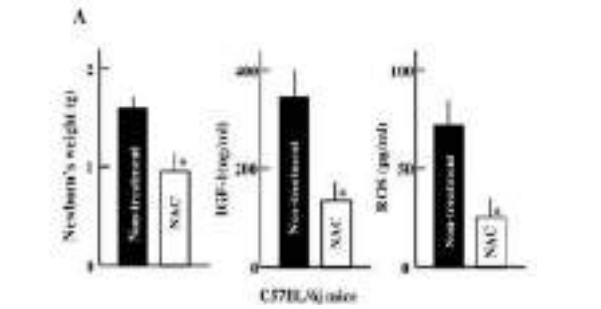

Figure 2A: The effects of NAC (inhibitor of ROS) administration on the newborn pups' weight and the plasma IGF-1 and ROS levels in graviditas C57BL/6j mice.

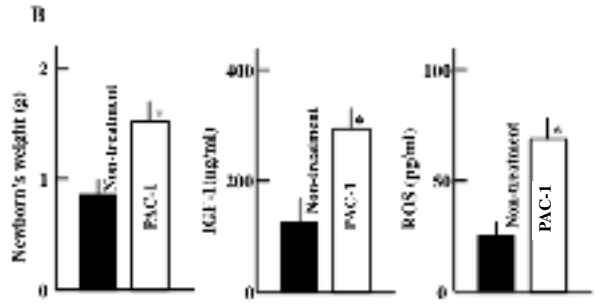

Figure 2B. The effects of PAC-1 (activator of ROS) administration on the newborn pups' weight and the plasma IGF-1 and ROS levels in graviditas gp91phox$^{-/-}$ mice. The values are presented as the means ± SD derived from six animals. *P<0.05 in comparison to the non-treatment mice.

Expression of NLRP3 and caspase-1 in the uterus of graviditas mice, and the influence of caspase-1 inhibitor treatment on the newborn weight

In gp91phox$^{-/-}$ mice, there was little expression of NLRP3 and caspase-1 (Figure 5A) in the uterus compared with the C57BL/6j and IGF-1$^{-/-}$ mice. The newborn weight was low in all groups following caspase-1 treatment (Figure 5B).

Discussion

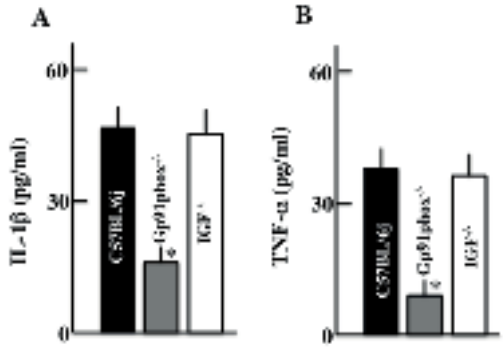

Figure 3. The plasma levels of IL-1β (A) and TNF-α (B) in graviditas C57BL/6j, gp91phox$^{-/-}$ and IGF-1$^{-/-}$ mice. The values are presented as the means ± SD derived from six animals. *P<0.05 in comparison to the C57BL/6j mice.

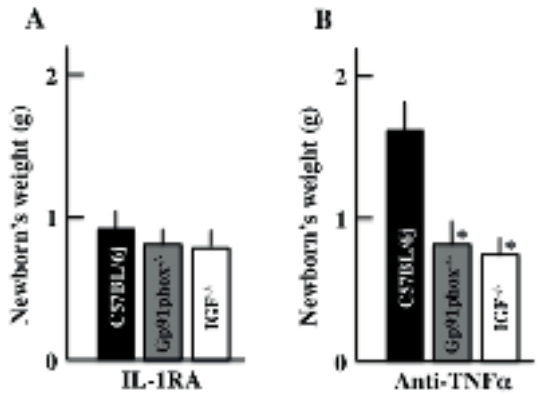

Figure 4: The effects of IL-1β receptor antagonist (A) and anti-TNF-α (B) treatment on the newborn pups' weight in graviditas C57BL/6j, gp91phox$^{-/-}$ and IGF-1$^{-/-}$ mice. The values are presented as the means ± SD derived from six animals. *P<0.05 in comparison to the C57BL/6j mice.

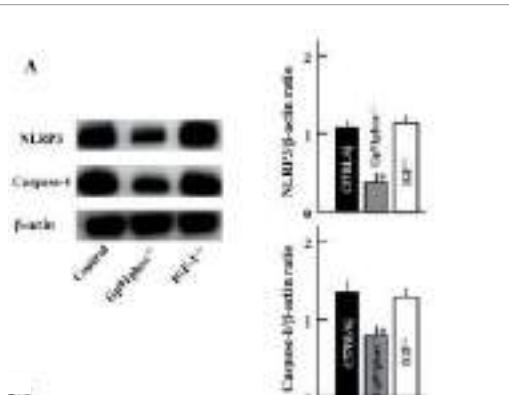

Figure 5A: The expression of NLRP3 and caspase-1 in the uterus of a graviditas C57BL/6j, gp91phox$^{-/-}$ and IGF-1$^{-/-}$ mice.

In this study, a decrement in the newborn weight was seen in gp91phox$^{-/-}$ and IGF$^{-/-}$ mice compared with the C57BL/6j mice. The IGF-1 level in the blood of the graviditas gp91phox$^{-/-}$ and IGF-1$^{-/-}$ mice was also lower compared with the control mice, whereas the ROS level was only low in the gp91phox$^{-/-}$ mice. Moreover, the plasma levels of IL-1β and TNF-α were low in graviditas gp91phox$^{-/-}$ mice, and the

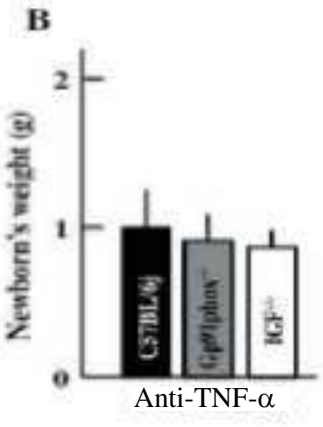

Figure 5B: The effects of caspase-1 inhibitor administration on the newborn pups' weight in graviditas C57BL/6j, gp91phox$^{-/-}$ and IGF-1$^{-/-}$ mice. The data show the results from a representative experiment involving six animals. The values are presented as the means ± SD derived from six animals. *, P<0.05 in comparison to the C57BL/6j mice.

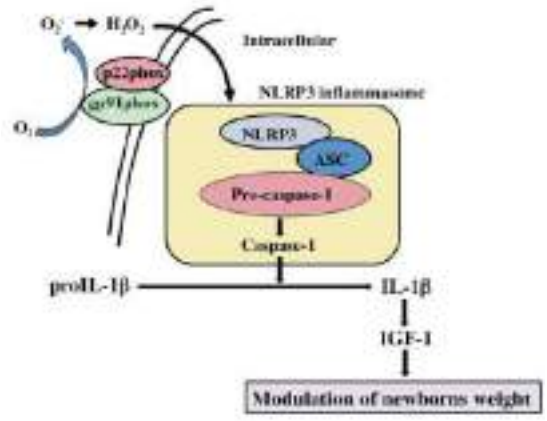

Figure 6: Mechanism of activation of the inflammasome and secretion IGF-1 in graviditas mice.

expression of NLRP3 and caspase-1 in the uterus were also low in the graviditas gp91phox$^{-/-}$ mice. Furthermore, following treatment with an inhibitor of IL-1β receptor or caspase-1, the newborn weight was decreased in all groups.

Gp91phox (Nox2) is a main component of NADPH oxidase, and if gp91phox is stimulated, then O_2^- (active oxygen) will be generated [3]. This active oxygen controls the NLRP3 inflammasome and activates caspase-1 [20]. In this study, the increase in active oxygen led to the activation of NLRP3 and induced an increase in caspare-1 in the graviditas control mice. In the graviditas gp91phox$^{-/-}$ mice, an increase in active oxygen, NLRP3 and caspare-1 was not seen, and a decrement in the newborn weight was observed. In the graviditas IGF$^{-/-}$ mice, although the increase in active oxygen, NLRP3 and caspase-1 was seen, a decrement in the newborn weight occurred, which was likely directly caused by the reduction of IGF-1. Furthermore, active oxygen produced from gp91phox NADPH oxidase was considered to be the first rate-determining step, according to the finding that the newborn weight remained similar to the control mice when gp91phox$^{-/-}$ mice were treated with an activator of ROS.

Although an increase in the plasma IL-1β level was observed in the graviditas control mice, an increase in IL-1β was not seen in the graviditas gp91phox$^{-/-}$ mice. Caspase-1 activates proIL-1β and produces IL-1β [21], which subsequently induces the production of IGF-1 [22]. Through this mechanism, tyrosine kinase JAK-2 and transcription factor STAT-5, downstream of JAK-2, is activated by IL-1β stimulation. Additionally, it has been previously reported that STAT-5 continuously produces IGF-1 [23]. In this study, a decrement in the newborn weight in control mice was observed following IL-1β receptor inhibitor treatment, and the newborn weight of control mice was similar to the newborn weight of gp91phox$^{-/-}$ mice. In addition, the plasma IGF-1 decreased in the control mice treated with IL-1β receptor inhibitor. Taken together, these findings suggest that an increased newborn weight is dependent on increased IL-1β and IGF-1.

Although IL-1β is cytokine which induces inflammation, IL-1β was expressed at a low level in this study and did not induce inflammation. Honsho et al. [23] has reported that low-level efficiently induces IGF-1. Therefore, we speculate that the level of IGF-1 was also efficiently induced by low-level IL-1β in this study.

Taken from the above-mentioned findings, the decrement in the newborn weight in gp91phox$^{-/-}$ mice is likely due to a series of events from the inflammation system stemming from the lack of active oxygen production by gp91phox NADPH oxidase, which, in turn, results in low levels of IGF-1 (Figure 6). Although gp91phox NADPH oxidase has been shown to be involved in the control of ovulation [24] and a retention of graviditas [11] by producing active oxygen, our findings suggest that gp91phox NADPH oxidase also participates in fetal growth.

Conclusion

In this experiment, we showed that the newborn weight is decreased in gp91phox$^{-/-}$ and IGF-1$^{-/-}$ mice compared with control mice. As a mechanism of the weight decrease of these newborns, ROS produced from gp91phox NADPH oxidase activates NLRP3. The increase in IL-1β takes place because the activation of NLRP3 induces caspase-1. It is believed that fetal growth is mediated through IL-1β induction of IGF-1. Our study shows the possibility that active oxygen produced from gp91phox NADPH oxidase may play an important role during fetal growth.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

Authors' Contributions

KH wrote the article and designed the research, KH and YY analyzed and interpreted the data, and TS and EFS contributed the essential reagents and tools.

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