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Research Article

Evaluation of Safety and Efficacy of Amniotic Mesenchymal Stem Cells for POI in Animals

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ABSTRACT

The efficacy of human Amniotic Mesenchymal Stem Cells (hAMSCs) ovarian injection for improving ovarian function of POI (Premature ovarian failure) patients has been showed in some reports. However, the safety and efficacy of the hAMSCs vein injection remains unclear. In this study, we evaluate the safety and efficacy of hAMSCs intravenous injection in *Cynomolgus macaques* and SD rats, and to provide the evidence for clinical trials. The hAMSCs were transplanted three times in SD rats at low, medium and high doses, respectively. The animal behavior, biochemical and biophysical parameters were routinely monitored on a 2-month period posttransplantation, and the histopathologic examinations were also performed. Experiments on the acute toxicity, allergy test and hemolysis test showed that hAMSCs possesses good biocompatibility. Our results showed that maximum tolerated dose of hAMSCs in SD rats was 4.0×10^7 cells/kg. The maximum safe dose with three injections of hAMSCs in SD rats was 5.0×10^6 cells/kg. In addition, the results demonstrated that hAMSCs could restore the POI rat's ovarian function after twice injected with 2.5×10^6 cells/kg or 5.0×10^6 cells/kg dose, which through improving the disturbed estrous cycle, hormone levels and ovarian lesion induced by pZP3. In conclusion, the preclinical results suggested that the transplantation of hAMSCs is safe and efficacious for SD rats at 5.0×10^6 cells/kg and lower doses.

Keywords: hAMSCs; POI; Ovary; Acute toxicity; Allergy test; Hemolysis test

Abbreviations: SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; hAMSCs: human Amniotic Mesenchymal Stem Cells

INTRODUCTION

POI refers to the symptoms of amenorrhea in women below the age of 40 caused by ovarian failure, which characterized by elevated follicle-stimulating hormone levels (FSH>25 mIU/ml on two occasions >4 weeks apart) and low Estradiol (E2) levels [1,2]. POI without treatment can induce cardiovascular disease, depression, anxiety, a decline in cognition and infertility. Approximately 25% of POI is caused by chemotherapy [3], and 4%-30% of POI is caused by autoimmunity [4]. At present there is no effective therapy for POI. Some studies have shown that Mesenchymal Stem Cells (MSC) transplantation may restore ovarian function and repair ovarian injury in animals and patients [2,3,5,6]. Ding, et al. have reported that umbilical cord MSCs on a collagen scaffold can activate primordial follicles in vitro and rescue POI patients overall ovarian function, evidenced by elevated estradiol levels, improved follicular development, and increased number of antral follicles [5]. Li, et al. study has demonstrated that hAMSCs may be a promising seed cell for regenerative medicine and found that hAMSCs improved POI rats ovarian function through a paracrine mechanism [6]. In addition, Ding, et al. reported that hAMSCs exerted better therapeutic activity on POI mouse ovarian function and promoted the proliferation rate of ovarian granular cell from POI patients compared to human Amniotic Epithelial Cells (hAECs) [7]. According to the previous studies, ovarian function can be improved by intravenous or intra-ovarian injection of hAMSCs. However, there is no systematic evaluation on safety and efficacy of

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hAMSCs transplanted into animals or patients.

A systematic process study has been conducted to optimize the process parameters for clinical-grade hAMSCs preparation, including the isolation, characterization, and identification.

In this study, we conducted acute toxicity test, long term toxicity test, allergy test, immunogenicity study, toxicokinetic study, and effective experiment of clinical-grade hAMSCs therapy for POI. We found that intravenous administration of hAMSCs at the dose of 5.0×10^6 cells/kg or lower is safe and effective for the treatment of POI.

MATERIALS

Preparation of clinical-grade hAMSCs

The human placentas were obtained from the Second Hospital of University of South China. Written informed consent was obtained from all women with undetectable HIV 1/2, TP, HAV, HBV, HCV, CMV, EBV and HTLV. The application of human amniotic membranes for this project was approved by the institutional ethics committee. According to the previous reports [8], the human amniotic membranes mechanically isolated from the chorion were cut into 1 × 1 cm2 pieces, and digested with TrypLE (Gibco, America) and NB6 (Nordmark, Germany) at 37°C for 1 hour to obtain hAMSCs. The isolated hAMSCs were cultured with complete medium containing MSC-T4 (STi, Japan) supplemented with 5% UltraGRO™-Advanced (GMP Grade, Helios Bioscience, America) and incubated at 37°C, 5% CO2. The hAMSCs were seeded at 0.89×10^4 cells/cm^2 and reach 80% –90% confluence after 3-5 days, then were digested with TrypLE and passaged. The P5 hAMSCs mixed with DMSO (Jiudian, China, Multiple Electrolytes Injection (Baite, China) and Human Serum Albumin (HSA, CSL Behring, Switzerland) were frozen in liquid nitrogen in 20 ml/package, which were called hAMSCs Preparations. The test results of bacterium, fungi, virus, mycoplasma, abnormal toxicity, endotoxin and oncogenicity for clinical-grade hAMSCs were negative. Karyotype analysis, Short Tandem Repeats (STR) and MSC identify analysis were performed for clinical-grade hAMSCs. Clinical-grade hAMSCs should differentiate into adipocytes, osteocytes, chondrocytes by using Adipogenesis, Osteogenesis, Chondrogenesis Differentiation Kit (Gbico, American) respectively. And in our study, the clinical-grade hAMSCs should have the good immunosuppressive ability through human Lymphocyte inhibition assay.

To track hAMSCs, hAMSCs were labeled with DiR'; DIIC18(7) dye (Invitrogen, D12731). 2.5 mg/mL working solution was prepared by combining 10 mg DiR with 4 mL DMSO. 6×10^7 hAMSCs were resuspended with 12 mL DMEM and 18 ul DiR working solution, and incubated at 37°C for 30 minutes. Labeled hAMSCs were mixed with DMSO, Multiple Electrolytes Injection and HSA.

Animal protocols

All animals were purchased from Beijing Weitong Lihua Laboratory Animal Technology. All projects were approved by the Animal Ethic Committee of Tianjin Tiancheng New Drug Evaluation, and conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

Acute toxicity test and *Cynomolgus macaques* assay

30 healthy SD rats, 15 male and female each, were randomly divided into control group (Solvent), low dose (3.0×10^7 cells/kg hAMSCs) group and high dose (4.0×10^7 cells/kg) group. Solvent represents that cell-free hAMSCs preparation containing HSA, DMSO and Multiple Electrolytes Injection. Each SD rat was injected with 2 ml solution. After hAMSCs injected intravenously, all rats were observed for 30 days and dissected the next day. Urine tests were performed on the same day of administration, 1 day after administration, and 3 days after administration.

Two *Cynomolgus macaques* were purchased from Xusheng Biotech, one male and one female, aged 3-5 years, weighted 2-3 kg. Two monkeys were injected intravenously with 6×10^7 cells/kg hAMSCs. The administration volume was 10 mL/kg and the administration rate was 2 ml/min. The clinical symptoms of two monkeys were constantly observed for 7 days, including weight test, electrocardiogram, urinalysis, etc.

Long term toxicity test and tissue distribution assay

Long term toxicity tests are defined as tests that characterize adverse effects following repeated administration of a test substance over a significant portion of the life span of the test species [9]. Healthy SD rats 120, half male and female were divided into control group (30 rats, normal saline), low dose (30 rats, 2.5×10^6 cells/kg hAMSCs) group, medium dose (30 rats, 5.0×10^6 cells/kg hAMSCs) group and high dose (30 rats, 1.0×10^7 cells/kg hAMSCs) group. All rats were given hAMSCs intravenously three times, each two weeks apart for 29 days. After the last dose, each group of 20 rats were dissected on the next day, and the remaining 10 rats in each group were dissected after recovering for 57 days. The rats were subjected to clinical observation once a day, body weight examination twice a week during the administration period and once a week during the recovery period, and food intake examination once a week. The urine examination and eye examination were conducted on the 29th of administration period and 57th day of recovery period, respectively. And hematology, coagulation function, blood biochemistry and electrolytes, anatomical examination, bone marrow smear, organ weight and histopathology were performed.

To detect the cell tissue distribution, 42 rats were divided into one control group and six satellite groups, half female and male. The 36 rats of satellite groups were treated hAMSCs three times, each two weeks apart. After the last dose, 3 male rats and 3 female rats were dissected at 0.5 hours, 4 hours, 24 hours, 7 days, 14 days, 57 days, respectively. The lung, heart, liver, spleen, kidney, brain, bone marrow, ovary/epididymis, uterus/testis were collected to extract the DNA, which used to track the distribution of hAMSCs. Human anti-Mitochondrial antibody (Abcam, American) was used for the detection of hAMSCs location through immunohistochemical method.

Hemolysis and allergy test

The hemolysis assay is used to determine the hemolytic effect of hAMSCs [10]. 2% sheep erythrocytes (YUDUOBIO, China) suspension was blended with clinical-grade hAMSCs (3.0×10^6 cells/ml) to detect the hemolysis of hAMSCs, then were constantly observed at 15 minutes, 30 minutes, 45 minutes, hour, 2 hours and 3 hours. Red blood cells were deposited at the bottom of the tube and supernatant of the mixture solution was colorless, indicating hAMSCs did not cause hemolysis, otherwise hAMSCs caused hemolysis. Deionized water was used as the hemolysis positive control and normal saline was used as the hemolysis negative control, which were mixed with sheep erythrocytes respectively.

Guinea pigs were used for allergy test of hAMSCs [11]. The 0.5×10^6 cells/ml (low dose group), 1.0×10^6 cells/ml (high dose group) and 1.0×10^6 cells/ml (high dose group without HSA) hAMSCs preparation

were used to detect the hAMSCs active systemic anaphylaxis. Ovalbumin was used as a positive allergen, and normal saline was used as the negative control. After intraperitoneal injected with cells, guinea pigs were stimulated with cells intravenous injection on 14th or 21th day. The clinical symptoms of guinea pigs were observed daily during experiments period.

The hAMSCs treated POI rats

To establish an immune POI rats model, healthy female SD rats were treated with 0.5 mg/ml pellucida protein 3 (pZP3) by subcutaneous abdomen and plantar injection, three times, two weeks apart. The rats estrous cycle was longer than 6 days and rats showed disorganized hair, lethargy, and reduced activity, indicating the immune POI rats were successfully established [12]. 50 POI rats were randomly classified into five groups: POI group (POI+ Solvent), low dose group (POI+1.25 × 10^6 cells/kg hAMSCs), medium dose group (POI+2.5×10^6 cells/kg hAMSCs), high-dose group (POI+5.0 × 10^6 cells/kg hAMSCs), Kuntai (Xintian, China) group (POI+Kuntai). At 0 and 2 weeks, the rats in hAMSCs group were injected with 2 ml of the clinical-grade hAMSCs via the tail vein, while the control group were injected with 2 ml of Solvent. The rats in Kuntai group were given 0.6 g/kg Kuntai everyday by gavage for 4 weeks, which is traditional Chinese medicine used to treat POI. The levels of E2, FSH, AMH, PRL, LH and P in rat plasma were evaluated by enzyme-linked immunosorbent assay (ELISA) kits purchased from Shanghai Enzyme-Linked Biotechnology according to the manufacturer's instructions.

Statistical analysis

Data were analyzed using SPSS 25 software. One-way Analysis of Variance (ANOVA) and Dunnett T3 test were calculated to evaluate the effects of clinical-grade hAMSCs. The average, standard errors and standard deviation were calculated and included in the tables.

RESULTS

Acute toxicity and hemolysis analysis of clinical-grade hAMSCs

Acute toxicity test methods measure the adverse effects that occur within a short time after administration of a test substance [13]. In this project, the clinical dose of hAMSCs was intended to be 1×10^6 cells/kg. The results showed that 3 × 10^7 cells/kg and 4 × 10^7 cells/kg hAMSCs both induced part of the rats activity decreased and prostrated, but those symptoms went away within a day. The rats treated with hAMSCs showed hematuria and pilo-erection, but returned to normal within 3 and 10 days, respectively (Table 1 and S1). No abnormal manifestations were observed for 30 days after transplantation of hAMSCs in rats, including the body weight change (Supplementary Tables 2 and 3) and no obvious lesions were found in the autopsy of the rats at the end of the experiment (Table 2). These results suggested that the maximum tolerated dose of hAMSCs in SD rats was 4.0 × 10^7 cells/kg. The figures displayed that hAMSCs did not cause hemolysis at 3.0 × 10^6 cells/ml (Figure 1 and Table 3).

Table 1: Clinical observation for female rats in acute toxicity experiments.

Group	Number	d1	rd1	rd2	rd3	rd4	rd5	rd6	rd7	rd8	rd9	rd10	rd11-rd30
Control	24	—	G1	G1	G1	G1	G1	G1	G1	—	—	—	—
group	26	—	G1	G1	G1	G1	G1	G1	G1	—	—	—	—
	28	—	G1	G1	G1	G1	G1	G1	G1	—	—	—	—
	30	—	G1	G1	G1	G1	G1	G1	G1	—	—	—	—
	32	—	G1	G1	G1	G1	G1	G1	G1	—	—	—	—
Low dose group	20	I2	G1	G1	G1	G1	G1	G1	G1	—	—	—	—
	22	I2	G1	G1	G1	G1	G1	G1	G1	—	—	—	—
	25	I2	G1	G1	G1	G1	G1	G1	G1	G1	—	—	—
	29	I2	G1	G1	G1	G1	G1	G1	G1	G1	G1	—	—
	34	I2	G1	G1	G1	G1	G1	G1	G1	—	—	—	—
High dose group	19	I2	G1	G1	G1	G1	G1	G1	G1	G1	—	—	—
	21	I2	G1	G1	G1	G1	G1	G1	G1	G1	—	—	—
	23	I2	G1	G1	G1	G1	G1	G1	G1	G1	G1	—	—
	27	I2	G1	G1	G1	G1	G1	G1	G1	G1	G1	G1	—
	33	I2	G1	G1	G1	G1	G1	G1	G1	G1	G1	—	—

Note: r: represents restore period, — represents no abnormalities, I2 represents red urine, G1 represents pilo-erection.

Table 2: The data of rats dissection in acute toxicity experiments.

Group	Gender	Number	Rats tissues
Control group	♂	2	-
		8	-
		9	-
		16	-
		17	-
	♀	24	-
		26	-
		28	-
		30	-
		32	-

Low dose group	♂	1	-
		3	-
		10	-
		11	-
		13	-
	♀	20	-
		22	-
		25	-
		29	-
		34	-
High dose group	♂	4	-
		5	-
		7	-
		12	-
		15	-
	♀	19	-
		21	-
		23	-
		27	-
		33	-

Note: - represents no obvious abnormality.

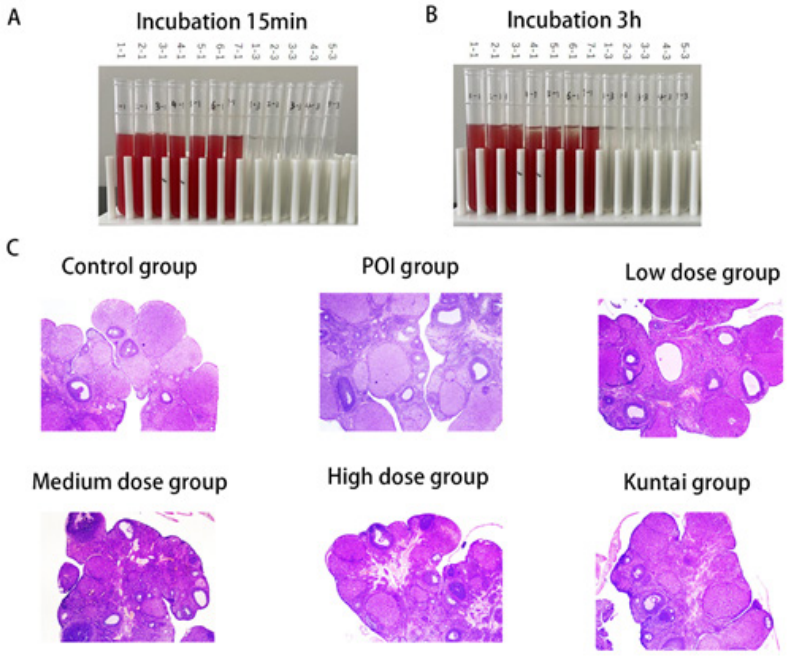

Figure 1: hAMSCs did not cause hemolysis and retore the ovarian function of POI rats. **Note:** A,B showed that hAMSCs mixed with sheep erythrocytes were incubated for 15 min and 3 h, respectively. C. The HE picture of rat ovaries. POI group represents POI+cell free hAMSCs preparations), Low dose group represents POI+1.25×10^6 cells/kg hAMSCs, Medium dose group represents (POI+2.5 × 10^6 cells/kg hAMSCs), High dose group represents POI+5.0 × 10^6 cells/kg hAMSCs, Kuntai group represents POI+Kuntai.

Table 3: The scheme of hemolysis experiment.

Solution (ml/Number)	01-Jan	02-Jan	03-Jan	04-Jan	05-Jan	06-Jan	07-Jan	01-Mar	02-Mar	03-Mar	04-Mar	05-Mar
Normal saline	2	2.1	2.2	2.3	2.4	2.5	–	4.5	4.6	4.7	4.8	4.9
ddH_2O	–	–	–	–	–	–	2.5	–	–	–	–	–
hAMSCs	0.5	0.4	0.3	0.2	0.1	–	–	0.5	0.4	0.3	0.2	0.1
2% erythrocyte	2.5	2.5	2.5	2.5	2.5	2.5	2.5	–	–	–	–	–

Active systemic anaphylaxis test

Our results showed that the hAMSCs preparation containing DMSO and Human Serum Albumin (HSA) leaded to the extremely positive allergic reactions in guinea pigs. But the hAMSCs preparation without HSA did not cause the guinea pigs allergic reactions (Supplementary Tables S4-S8).

hAMSCs was safe in SD rats at 5.0 × 10^6 cells/kg

In this study, we found that one female cynomolgus macaque was died after injected with 6 × 10^7 cells/kg of hAMSCs, which may due to the accumulation of hAMSCs in the lung of the monkey. However, no abnormal symptoms were observed in another male monkey injected with the same dose of hAMSCs. Above data suggested that hAMSCs may be toxicity for monkeys at 6 × 10^7 cells/kg.

In long toxicity assay, hAMSCs were administered intravenously every other week in 1 month (3 times in total) at 2.5 × 10^6 cells/kg, 5 × 10^6 cells/kg, 1 × 10^7 cells/kg respectively. There is no weight change in rats among all groups (Supplementary Table 4). The results demonstrated that six rats died in the high dose group (66 rats) after the second cell administration, which was considered as pulmonary embolism death induced by the excessive cell dosage. All rats showed decreased and disorder of movement, shortness of breath, ptosis and red urine on hAMSCs administration days. In addition, after the second hAMSCs injection, female rats in the high-dose group showed symptoms including increased urinary specific gravity, decreased pH in urine, increased occulted blood, protein, and bilirubin in urine (Table 4). After the third hAMSCs injection, the data demonstrated that hAMSCs induced rats decreased %Lymph (Lymphocyte percentage) and PLT (Platelet Count), while the other test indexes were increased including %Neut (Neutrophils percentage), %Mono (Monocyte percentage), spleen weight, spleen weight/body weight and spleen weight/brain weight, the infiltration of perivascular inflammatory cells in lung, the area of white pulp and marginal of spleen (Tables 5-7). The above abnormal symptoms were dose-dependent. And all the animals in each dose group recovered completely by the end of the recovery period.

Table 4: The urinary Specific Gravity (SG) and pH of fresh rat urine.

Gender	Time	Project	Control group	Low dose group	Medium dose group	High dose group
♂	Administration 29d	SG	1.014 ± 0.002	1.015 ± 0.003	1.016 ± 0.002	1.019 ± 0.003**
		pH	8.1 ± 0.3	8.2 ± 0.5	7.9 ± 0.5	6.9 ± 1.1*
	Recovery 57d	SG	1.015 ± 0.006	1.014 ± 0.002	1.014 ± 0.002	1.012 ± 0.004
		pH	7.9 ± 0.5	7.7 ± 0.3	8.0 ± 0.0	7.8 ± 0.6
♀	Administration 29d	SG	1.013 ± 0.003	1.013 ± 0.003	1.016 ± 0.003	1.022 ± 0.004**
		pH	8.1 ± 0.4	8.2 ± 0.2	7.7 ± 0.5	6.8 ± 1.1*
	Recovery 57d	SG	1.014 ± 0.004	1.016 ± 0.005	1.015 ± 0.005	1.014 ± 0.004
		pH	7.9 ± 0.4	7.8 ± 0.6	7.3 ± 0.3	7.8 ± 0.4

Note: *p ≤ 0.05, **p ≤ 0.01.

Table 5: The data of hematological and coagulation tests in female rats (Mean ± SD).

Time	Project	Control group	Low dose group	Medium dose group	High dose group
The day after last dose	WBC (×10 ^ 9/L)	4.11 ± 1.31	4.43 ± 1.08	4.73 ± 1.52	3.65 ± 1.77
	%Neut (%)	18.4 ± 4.8	17.5 ± 5.7	20.4 ± 8.5	29.3 ± 8.5
	%Lymph (%)	76.4 ± 4.8	76.3 ± 5.8	72.0 ± 10.2	62.7 ± 9.1**
	%Mono (%)	1.6 ± 0.3	2.3 ± 0.9	2.4 ± 0.6	2.6 ± 0.6**
	%Eos (%)	1.5 ± 0.5	1.4 ± 0.4	1.0 ± 0.3	1.7 ± 0.8
	RBC (×10^12/L)	8.11 ± 0.39	7.94 ± 0.33	8.03 ± 0.37	7.58 ± 0.57*
	HGB (g/L)	160 ± 8	157 ± 4	156 ± 7	149 ± 11
	HCT (%)	47.0 ± 2.2	46.3 ± 1.5	46.6 ± 2.2	44.2 ± 3.4
	MCV (fL)	58.0 ± 1.1	58.4 ± 1.1	58.0 ± 0.9	58.4 ± 1.6
	MCH (pg)	19.7 ± 0.6	19.8 ± 0.5	19.4 ± 0.5	19.6 ± 0.6
	MCHC (g/L)	339 ± 6	340 ± 4	334 ± 6	336 ± 3
	PLT (×10^9/L)	1176 ± 134	1072 ± 135	879 ± 125**	552 ± 131**
	Retic (×10^12/L)	0.23 ± 0.05	0.24 ± 0.06	0.25 ± 0.07	0.30 ± 0.03
	PT(s)	16.4 ± 0.5	15.8 ± 0.4	16.2 ± 0.6	15.9 ± 0.4
	APTT(s)	18.1 ± 1.0	18.4 ± 0.6	19.6 ± 1.2*	18.5 ± 1.5
The day after the recovery period	WBC (×10^9/L)	3.24 ± 0.55	2.85 ± 0.77	2.96 ± 0.92	3.99 ± 0.72
	%Neut (%)	13.2 ± 3.7	12.9 ± 2.7	13.7 ± 3.6	13.2 ± 3.9
	%Lymph (%)	81.0 ± 4.3	82.0 ± 4.2	81.5 ± 3.1	82.6 ± 3.7
	%Mono (%)	1.5 ± 0.3	1.3 ± 0.7	1.3 ± 0.3	1.0 ± 0.5
	%Eos (%)	1.7 ± 0.6	1.7 ± 0.9	1.4 ± 0.9	1.0 ± 0.2
	RBC (×10^12/L)	8.71 ± 0.41	8.41 ± 0.06	8.21 ± 0.71	8.50 ± 0.38
	HGB (g/L)	159 ± 7	153 ± 4	145 ± 9	155 ± 8
	HCT (%)	50.5 ± 2.2	48.5 ± 1.2	46.6 ± 3.0	49.2 ± 2.1

MCV (fL)	57.9 ± 0.7	57.6 ± 1.1	56.9 ± 2.0	57.9 ± 1.2
MCH (pg)	18.2 ± 0.1	18.2 ± 0.3	17.7 ± 0.9	18.2 ± 0.4
MCHC (g/L)	315 ± 2	316 ± 5	312 ± 5	315 ± 5
PLT (×10 ^ 9/L)	1107 ± 145	1131 ± 103	1163 ± 136	1168 ± 101
Retic (×10 ^ 12/L)	0.18 ± 0.04	0.21 ± 0.06	0.20 ± 0.07	0.21 ± 0.04
PT (s)	16.2 ± 1.4	16.5 ± 1.8	16.7 ± 1.0	16.7 ± 1.5
APTT (s)	20.2 ± 1.7	19.6 ± 1.2	18.1 ± 1.4	18.1 ± 1.1

Note: Compared to control group, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$

Table 6: The abbreviations used in the project.

Projects	Abbreviations
White Blood Cell count	WBC
Neutrophils percentage	%Neut
Lymphocytes percentage	%Lymph
Monocytes percentage	%Mono
Eosinophils percentage	%Eos
Red Blood Cell count	RBC
Haemoglobin	HGB
Red blood cell volume	HCT
Mean red blood Cell Volume	MCV
Mean red blood Cell Hemoglobin	MCH
Mean Crythrocyte Hemoglobin Concentration	MCHC
Reticulocyte Count	Retic
Platelet Count	PLT
Prothrombin Time	PT
Activated Partial Thrombin Time	APTT

Table 7: The organ weight and coefficient/ body weight in female rats (Mean ± SD).

Time	Project	Control group	Low dose group	Medium dose group	High dose group	APTT
The day after last dose	Organ weight (g)	Body weight	242.6 ± 11.7	246.7 ± 12.9	247.7 ± 15.2	246.7 ± 11.1
		Heart	1.047 ± 0.129	1.072 ± 0.125	1.081 ± 0.132	0.998 ± 0.136
		Liver	6.822 ± 0.671	7.351 ± 0.786	7.392 ± 0.481	7.652 ± 0.619
		Spleen	0.505 ± 0.092	0.636 ± 0.106**	0.760 ± 0.087**	0.723 ± 0.082**
		Kidney	1.607 ± 0.167	1.707 ± 0.131	1.715 ± 0.066	1.765 ± 0.200
		Brain	1.857 ± 0.079	1.845 ± 0.081	1.835 ± 0.099	1.847 ± 0.102
		Adrenal gland	0.069 ± 0.009	0.073 ± 0.011	0.067 ± 0.012	0.073 ± 0.006
		Thymus	0.537 ± 0.139	0.492 ± 0.099	0.517 ± 0.087	0.554 ± 0.115
		Uterus	0.557 ± 0.101	0.502 ± 0.074	0.647 ± 0.116	0.739 ± 0.172**
		Ovary	0.183 ± 0.037	0.192 ± 0.032	0.188 ± 0.038	0.236 ± 0.069
	Organ coefficient (mg/g)	Heart	4.311 ± 0.444	4.347 ± 0.462	4.389 ± 0.680	4.051 ± 0.555
		Liver	28.079 ± 1.765	29.791 ± 2.672	29.863 ± 1.375	30.992 ± 1.774**
		Spleen	2.075 ± 0.323	2.570 ± 0.356**	3.065 ± 0.286**	2.927 ± 0.283**
		Kidney	6.624 ± 0.596	6.927 ± 0.477	6.942 ± 0.449	7.146 ± 0.645
		Brain	7.669 ± 0.469	7.495 ± 0.500	7.426 ± 0.539	7.493 ± 0.416
		Adrenal gland	0.285 ± 0.037	0.297 ± 0.045	0.271 ± 0.054	0.294 ± 0.023
		Thymus	2.204 ± 0.516	1.998 ± 0.412	2.092 ± 0.373	2.241 ± 0.437
		Uterus	2.311 ± 0.485	2.042 ± 0.317	2.615 ± 0.477	3.009 ± 0.743*
		Ovary	0.753 ± 0.148	0.777 ± 0.121	0.762 ± 0.174	0.954 ± 0.265
The day after the recovery period	Organ weight (g)	Body weight	302.8 ± 19.3	292.1 ± 20.8	291.9 ± 19.4	306.2 ± 20.4
		Heart	1.111 ± 0.147	1.113 ± 0.123	1.161 ± 0.142	1.168 ± 0.140
		Liver	8.021 ± 0.525	7.314 ± 0.786	7.949 ± 1.495	7.735 ± 0.971
		Spleen	0.547 ± 0.087	0.503 ± 0.069	0.541 ± 0.030	0.648 ± 0.115
		Kidney	1.793 ± 0.037	1.800 ± 0.164	1.781 ± 0.217	1.887 ± 0.112
		Brain	1.967 ± 0.038	1.966 ± 0.031	1.936 ± 0.081	1.932 ± 0.089
		Adrenal gland	0.076 ± 0.009	0.076 ± 0.011	0.079 ± 0.016	0.087 ± 0.014

	Thymus	0.388 ± 0.073	0.341 ± 0.047	0.396 ± 0.071	0.388 ± 0.039
	Uterus	0.740 ± 0.066	0.747 ± 0.118	0.853 ± 0.080	0.910 ± 0.104*
	Ovary	0.196 ± 0.040	0.180 ± 0.018	0.219 ± 0.033	0.200 ± 0.019
Organ	Heart	3.662 ± 0.307	3.814 ± 0.392	3.968 ± 0.305	3.804 ± 0.235
coefficient (mg/g)	Liver	26.518 ± 1.492	25.028 ± 1.857	27.149 ± 3.852	25.253 ± 2.652
	Spleen	1.803 ± 0.234	1.722 ± 0.225	1.854 ± 0.052	2.121 ± 0.369
	Kidney	5.935 ± 0.316	6.174 ± 0.560	6.095 ± 0.512	6.170 ± 0.244
	Brain	6.513 ± 0.346	6.757 ± 0.480	6.650 ± 0.402	6.334 ± 0.543
	Adrenal gland	0.252 ± 0.042	0.261 ± 0.041	0.271 ± 0.056	0.284 ± 0.043
	Thymus	1.279 ± 0.221	1.165 ± 0.095	1.357 ± 0.220	1.267 ± 0.115
	Uterus	2.461 ± 0.340	2.583 ± 0.502	2.941 ± 0.418	2.981 ± 0.369
	Ovary	0.647 ± 0.116	0.618 ± 0.087	0.755 ± 0.128	

Note: Compared with control group, *p ≤ 0.05, **p ≤ 0.01.

The tissue distribution of hAMSCs

Our results showed that there were quantitatively detectable hAMSCs in the blood and lung of rats, which collected at 0.5 hours after the third cells administration, but no in other tissues. The hAMSCs were detected in the lung collected at 4 hours after last cell injection, but no other tissues. All tissues collected at 24 h and 7 d were negative for quantitative detection of hAMSCs (Table 7). In addition, to further detect the location of hAMSCs in positive tissues, the immunohistochemistry was used. We found that there were positive cells only in 0.5 hours lungs (Supplementary Figure 1).

hAMSCs restored rats ovarian function

To evaluate the effects of different dose hAMSCs on the POI rats, the hAMSCs were injected intravenously at different dose, once two weeks (two times in total). The results showed that different doses hAMSCs and Kuntai had no significant effect on the POI rats body weight. Our data demonstrated that the percentage of estrous cycle normalization in POI rats increased in an hAMSCs-dose dependent manner (light dose group: 40%, medium dose group: 60%, high dose group: 70%) (Table 8). After four weeks, the estrous cycle of all POI rats without treatment remained delayed. In addition, we found that medium dose and high dose of hAMSCs, and Kuntai obviously increased the ovarian index (ovarian index=ovarian weight/body weight *100%) compared to the POI group, but not in uterine index (uterine index=uterine weight/body weight *100%) (Supplementary Table 7). Our data showed that serum levels of E2, PRL and AMH were significantly reduced in the POI group compared with the control group, while serum levels of FSH, LH and T were significantly increased. Meanwhile the results suggested that hAMSCs and Kuntai both restored the decreased levels of E2 and AMH and the increased levels of FSH and LH in POI rats (Tables 9-11). In addition, the HE results showed that medium dose and high dose hAMSCs alleviated the increased atresia follicles and stromal cells induced by pZP3. Besides, pZP3 or hAMSCs did not change the morphology of the rat uterus, vagina, and adrenal gland (Figure 1).

hAMSCs homing to the injured ovaries

To track the location of hAMSCs in POI rats, the hAMSCs were labeled with DiR and tracked with extracorporeal imager. Our results showed that hAMSCs were located in ovary in POI rats but not healthy rats on the third and seventh day after the injections (Figure 2). In addition, the results showed that no hAMSCs was detected in ovaries of all rats on the 17 days (Figure 2).

Table 8: The DNA content of hAMSCs in SD rats tissues (ng/reaction).

Tissues	Control group			High dose group1 (satellite group)			High dose group2 (satellite group)			High dose group3 (satellite group)			High dose group3 (satellite group)		
	A 0.5 h			A 0.5 h			A 4 h			A 24 h			A 7 d		
whole blood	0	±	0	0.37	±	0.21	0	±	0	0	±	0	0	±	0
Brain	0	±	0	0	±	0	0	±	0	0	±	0	0	±	0
Bone marrow	0	±	0	0	±	0	0	±	0	0	±	0	0	±	0
Uterus/Testis	0	±	0	0	±	0	0	±	0	0	±	0	0	±	0
Ovary/Epididymis	0	±	0	0	±	0	0	±	0	0	±	0	0	±	0
Spleen															
	0	±	0	0	±	0	0	±	0	0	±	0	0	±	0
Kidney	0	±	0	0	±	0	0	±	0	0	±	0	0	±	0
Liver	0	±	0	0	±	0	0	±	0	0	±	0	0	±	0
Heart	0	±	0	0	±	0	0	±	0	0	±	0	0	±	0
Lung	0	±			±	9.63	0.42	±	0.1	0	±	0	0	±	0

Note: A represents after the last dose.

Table 9: The estrous cycle of control rats, POI rats treated with hAMSCs, Kuntai and cellular solvent, respectively. (Mean ± SEM) (n=10).

Groups	Dosage	Estrous cycle (day)		Normal percent
	cells/kg	Before treatment	After treatment	(%)
Control group	-	4.2 ± 0.1	4.1 ± 0.1	100
POI group	-	11.0 ± 0.4	10.8. ± 0.5DD	0
Low dose group	1.25×10^6	10.1 ± 0.7	6.4 ± 0.7**	40
Medium dose group	2.50×10^6	9.7 ± 0.6	4.4 ± 0.8**	60
High dose group	5.00×10^6	10.6 ± 0.5	4.6 ± 0.9**	70
Kuntai group	0.6 g/kg	10.9 ± 0.5	4.1 ± 0.5**	70

Note: Low, medium, high dose or POI group compared with control group, DP ≤ 0.05, DDP ≤ 0.01. Low, medium, or high dose group compared with POI group, *p ≤ 0.05, **p ≤ 0.01.

Table 10: The levels of E2, FSH, LH and T in rat serum.

Groups	Dosage (cells/kg)	E2 (pmol/L)	FSH (IU/L)	LH (mIU/mL)	T (pg/mL)
Control group		80.76 ± 1.41	11.20 ± 0.29	39.71 ± 1.07	290.7 ± 11.0
POI group	-	67.53 ± 0.60DD	14.32 ± 0.21DD	42.34 ± 0.48DD	339.8 ± 6.3DD
Low dose group	1.25×10^6	69.92 ± 1.21	13.90 ± 0.30	36.77 ± 0.83**	327.7 ± 3.7
Medium dose group	2.50×10^6	70.67 ± 0.75	11.72 ± 0.47**	38.18 ± 0.50**	311.8 ± 5.4**
High dose group	5.00×10^6	76.21 ± 1.00**	11.58 ± 0.21*	38.90 ± 0.87**	309.0 ± 6.0**
Kuntai group	0.6 g/kg	79.34 ± 0.77**	12.15 ± 0.53*	35.68 ± 0.72**	315.1 ± 7.2*

Note: Low, medium, high dose or POI group compared with control group, DP ≤ 0.05, DDP ≤ 0.01. Low, medium, or high dose group compared with POI group, *p ≤ 0.05, **p ≤ 0.01.

Table 11: The levels of PRL, PROG and AMH in rat serum.

Groups	Dosage (cells/kg)	PRL(ng/mL)	PROG(ng/mL)	AMH(ng/mL)
Control group		46.05 ± 0.55	14.73 ± 0.27	4.16 ± 0.06
POI group	-	43.81 ± 0.50D	14.80 ± 0.22	3.26 ± 0.10DD
Low dose group	1.25×10^6	43.00 ± 0.63	15.20 ± 0.23	3.43 ± 0.10
Medium dose group	2.50×10^6	43.33 ± 1.01	15.12 ± 0.34	3.84 ± 0.12**
High dose group	5.00×10^6	44.86 ± 0.91	14.02 ± 0.42	3.80 ± 0.12**
Kuntai group	0.6 g/kg	46.19 ± 0.80*	15.33 ± 0.40	4.13 ± 0.19**

Note: Low, medium, high dose or POI group compared with control group, DP ≤ 0.05, DDP ≤ 0.01. Low, medium, or high dose group compared with POI group, *p ≤ 0.05, **p ≤ 0.01.

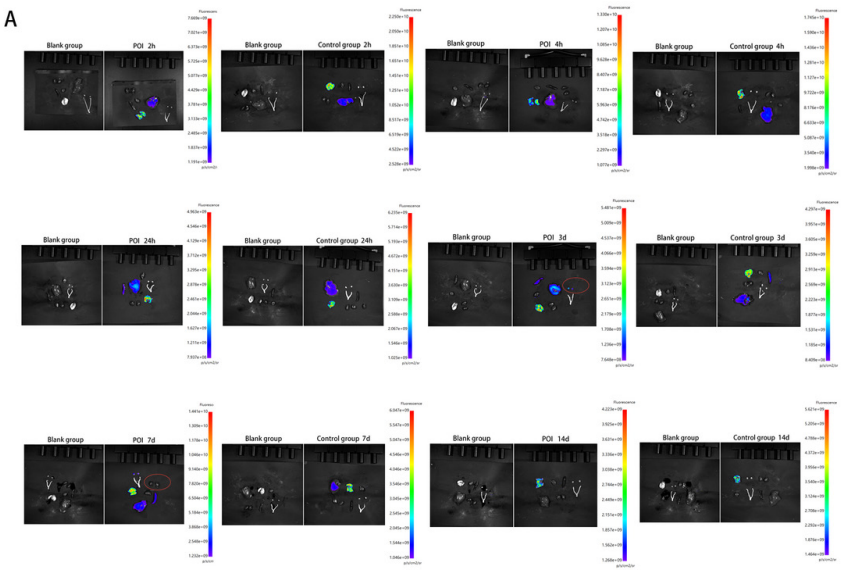

Figure 2: hAMSCs homing to the injured ovaries. **Note:** The pictures of labeled hAMSCs in heart, liver, spleen, lungs, kidneys, ovaries, uterus of rats at different times after injection. The red ovals indicate the ovaries that contained hAMSCs.

DISCUSSION

In our previous studies, to meet the needs of new drugs clinical trials for product large quantity, quality consistency and safety, we investigated the hAMSCs production and preservation technology and established multi-level quality control system, as well as a large-scale production conforming to Good Manufacturing Practice (GMP) regulations. During the production of hAMSCs preparation, no animal extracts were used, which was protected from animal derived viruses. The DMSO and HSA (parenteral drug) were used as pharmaceutical adjuvants in hAMSCs preparations.

To investigate the safety of hAMSCs, we performed the acute toxicity, long term toxicity, hemolysis, allergy test and tissue distribution experiments of hAMSCs. Our data demonstrated that the maximum tolerated dose of hAMSCs in SD rats was 4.0×10^7 cells/kg and the safety dose for repeated administration of hAMSCs was 5.0×10^6 cells/kg, which was much higher than the planned dose (1.0×10^6 cells/kg) of hAMSCs in clinical trial, that referencing to the approved Investigational New Drug (IND) items. The excessive doses of hAMSCs may accumulate in lung, which results in animal breathing difficulties and the lack of oxygen of other tissue, further leads to other symptoms of pulmonary embolism.

In addition, the hemolysis data showed that hAMSCs did not cause hemolysis and the guinea pig allergic reactions. However, HSA caused the allergic reaction in guinea pig, this implied that a higher safe doses of hAMSCs Preparations in human than rodents. The tissue distribution of hAMSCs study suggested that hAMSCs did not stay in any tissue of rat for more than 7 days. Though hAMSCs were found in lungs within 4 h after cell injection, none were found in all tissues collected at all other time points. This suggested that the hAMSCs did not engraft and differentiate in rats. Above data demonstrated that multiple intravenous injections of hAMSCs was safe for animals at doses no higher than 5.0×10^6 cells/kg.

Though some reports showed that hAMSCs could restore the POI animal ovarian function and improve the follicle development. These mechanisms may be mainly due to the fact that hAMSCs migrate to the injured ovaries and decrease the ovarian apoptosis and oxidative stress, regulate the inflammatory reaction and promote angiogenesis by secreting amounts of protein, mRNA, microRNA and exosomes [2,3,14]. The doses of hAMSCs in most reports were higher than the safety dose of hAMSCs in repeated injections [6,7,15]. To investigated the efficacy of safe dose of hAMSCs in repeated treatments of POI rats, the low, medium and high dose of hAMSCs were intravenously injected in POI rats two times. Our results showed that medium and high dose of hAMSCs that not exceeding the safe dose could improve POI ovarian function, decrease atresia follicles, increase the E2 and AMH levels, while reduce the FSH levels [16-18]. Above studies could lay the foundation for cell drug clinical trials, and the results suggested that the hAMSCs preparations might be a safe and effective candidate drug for POI treatment [19].

CONCLUSION

The quality and safety of human Amniotic Mesenchymal Stem Cells (hAMSCs) preparation must be strictly controlled to ensure their final use in patients. We have developed human Amniotic Mesenchymal Stem Cells optimum preparation process and quality control system to meet the needs for new drug according to the China Physicochemistry 2020. In addition, our pre-clinical experiments implied that the human Amniotic Mesenchymal Stem Cells preparations might be safe and efficacious for POI patients.

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AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this article and supplementary information.

HUMAN AND ANIMAL RIGHTS

The Animal Ethic Committee of Tianjin Tiancheng New Drug Evaluation approved the acute toxicity assay (NO: 2021120102), long toxicity and tissue distribution assay (NO: 2022022802), allergy test (NO: 2022051801), cynomolgus macaque assay (NO: 2021120102), POI rats assay (NO: 2022030703). And The Ethic Committee of the Second Hospital of University of South China approved the collection of amniotic membrane sample (NO: 202208-01).

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