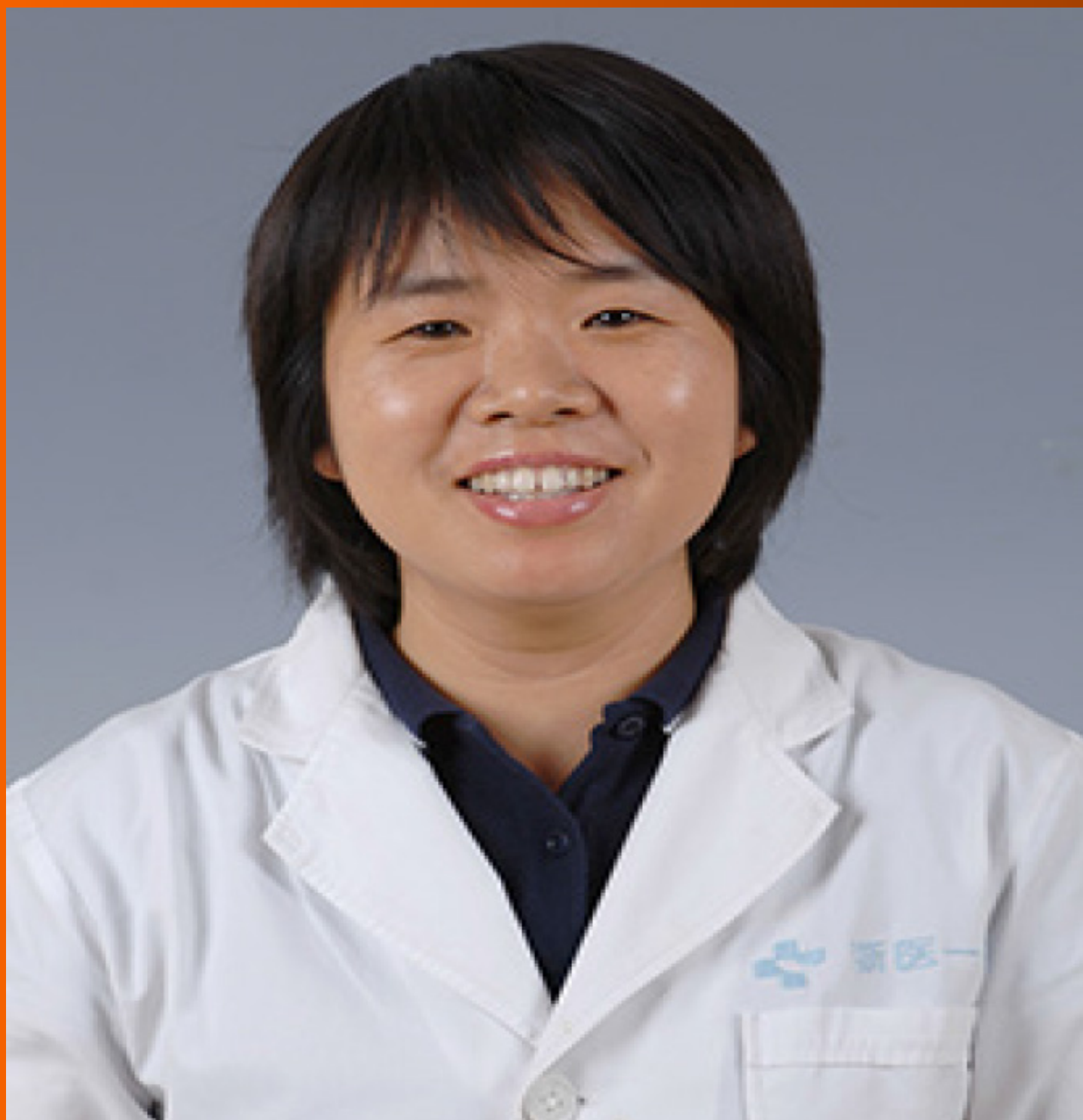


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## Basic Study

## Safety of menstrual blood-derived stromal cell transplantation in treatment of intrauterine adhesion

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**Author contributions:** Chang QY was responsible for the experiment design and conception and data collection; Zhang SW was responsible for experiment design and data analysis; Li PP was responsible for the experiment design and manuscript revision; Yuan ZW provided the technical guidance; Tan JC designed the work, provided the technical guidance, and approved the final version of the manuscript; Chang QY, Zhang SW, and Li PP wrote the manuscript; all authors read and approved the final manuscript.

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**committee statement:** All procedures involving animals were reviewed and approved by the

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## Abstract

## BACKGROUND

Intrauterine adhesion (IUA) can cause serious damage to women's reproductive health, yet current treatment methods are difficult to achieve satisfactory results. In our previous studies, we demonstrated that menstrual-derived stromal stem cells (MenSCs), with high proliferative capacity and self-renewal ability, have a powerful therapeutic effect in patients with severe IUA. However, safety assessment of MenSCs transplantation is essential for its further application.

## AIM

To evaluate the short-, medium-, and long-term biosafety of MenSCs *via* intrauterine transplantation in a rat model of IUA, with a focus on toxicity and tumorigenicity.

## METHODS

MenSCs were injected into the sub-serosal layer of the uterus in an IUA rat model, for 3 d, 3 mo, and 6 mo separately, to monitor the corresponding acute, sub-chronic, and chronic effects. Healthy rats of the same age served as negative controls. Toxicity effects were evaluated by body weight, organ weight, histopathology, hematology, and biochemistry tests. Tumorigenicity of MenSCs was investigated in Balb/c-nu mice *in vivo* and by colony formation assays *in vitro*.

## RESULTS

Compared with the same week-old control group, all of the IUA rats receiving MenSC transplantation demonstrated no obvious changes in body weight, main



Institutional Animal Care of Shengjing Hospital Affiliated to China Medical University and were conducted in accordance with the AAALAC and IACUC guidelines.

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organ weight, or blood cell composition during the acute, sub-chronic, and chronic observation periods. At the same time, serum biochemical tests showed no adverse effects on metabolism or liver and kidney function. After 4 wk of subcutaneous injection of MenSCs in Balb/c-nu nude mice, no tumor formation or cell metastasis was observed. Moreover, there was no tumor colony formation of MenSCs during soft agar culture *in vitro*.

## CONCLUSION

There is no acute, sub-chronic, or chronic poisoning, infection, tumorigenesis, or endometriosis in rats with IUA after MenSC transplantation. The above results suggest that intrauterine transplantation of MenSCs is safe for endometrial treatment.

**Key words:** Menstrual blood-derived stromal cells; Endometrial treatment; Intrauterine adhesion; Stem cell transplantation; Biosafety; Toxicity

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**Core tip:** Menstrual-derived stromal stem cells (MenSCs) with high proliferative capacity and self-renewal ability have a powerful therapeutic effect in patients with severe intrauterine adhesion. However, safety assessment of MenSC transplantation is essential for its further application. Here, we evaluated the short-, medium-, and long-term biosafety of MenSCs *via* intrauterine transplantation in an intrauterine adhesion rat model, with a special focus on toxicity and tumorigenicity. There was no acute, sub-chronic, or chronic poisoning, infection, tumor, or endometriosis in rats with intrauterine adhesions after MenSC transplantation, highlighting that intrauterine transplantation of MenSCs is safe for endometrial treatment.

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## INTRODUCTION

Intrauterine adhesion (IUA) is a traumatic disease mostly associated with intrauterine surgery<sup>[1]</sup>, mainly characterized by endometrial functional disorders, including abnormal menstruation (menstrual reduction or amenorrhea), thin endometrium, pelvic pain, implantation abnormality, infertility, and abortion<sup>[2]</sup>. With the increasing frequency of intrauterine operation, the incidence of IUA is gradually increasing. Approximately 2.8%-45.5% of secondary infertility cases are related to IUA<sup>[3]</sup>. However, for patients with severe IUA, conventional treatment methods, such as surgical isolation and hormone supplementation, cannot achieve the desired therapeutic effect. Especially, it is difficult to improve the fertility in patients with IUA.

Recent studies have shown that tissue and organ damage can be repaired effectively by stem cell transplantation. Mesenchymal stem cells (MSCs) are adult stem cells that can be easily collected and cultured from tissues and organs<sup>[4]</sup>. Due to the advantages of strong proliferative ability, high genetic stability, chemotactic properties, and low immunogenetic effects, MSCs play an important role in the field of regenerative medicine<sup>[5-7]</sup>. Recently, MSCs have been used in stem cell-based infertility treatment. Bone marrow-derived MSCs (BMSCs), umbilical cord-derived MSCs (UCMSCs), and endometrial-derived MSCs were all proved to be effective in recovering damaged endometrium<sup>[8-11]</sup>.

Menstrual blood-derived stromal cells (MenSCs) are shedding endometrial stem cells that are obtained from menstrual blood, and were first reported by Meng *et al*<sup>[12]</sup>. These cells exhibit classic MSC characteristics, such as automatic cloning, high proliferation, and pluripotency<sup>[13]</sup>. Recent studies have shown that MenSCs improved a variety of diseases, including type 1 diabetes<sup>[14]</sup>, liver disease<sup>[15,16]</sup>, premature ovarian failure<sup>[17]</sup>, osteochondral defects<sup>[18]</sup>, heart disease<sup>[19]</sup>, and cartilage damage<sup>[20]</sup>. It is worth

noting that MenSCs are easily obtained from abandoned menstrual blood in a non-invasive manner, which can be obtained periodically and autologously transplanted without trauma or ethical risk. Therefore, compared with BMSCs and adipose tissue-derived mesenchymal stem cells (ADSCs), MenSCs have greater clinical application potential on the premise of similar efficacy<sup>[21]</sup>. Our previous research confirmed that autologous MenSC transplantation can significantly promote endometrial morphology regeneration and functional recovery in seven patients with severe IUA, and achieved four positive pregnancies<sup>[22]</sup>. After MenSC transplantation, the endometrial pathology and uterine fertility of an IUA rat model were also improved<sup>[23]</sup>. Therefore, MenSC transplantation is a promising treatment for endometrial injury.

It is undeniable that MenSCs represent a new type of therapeutic stem cells. Many related treatment studies are still in the preclinical or phase 1 clinical trial phase. At present, the effectiveness of MenSCs in treating traumatic diseases has been confirmed. However, long-term observational data of clinical application of MenSCs is scarce, and systematic biosafety evaluation is still lacking<sup>[24]</sup>.

In this study, we aimed to investigate the biosafety of intrauterine transplantation of MenSCs to treat endometrial injury over acute, sub-chronic, and chronic periods. Based on an IUA rat model, the safety of MenSC treatment was systematically evaluated for toxicity, tumorigenicity, and abnormal differentiation. Our results provide a theoretical basis for the clinical application of MenSCs in endometrial injury treatment.

## MATERIALS AND METHODS

### **Culture and identification of MenSCs**

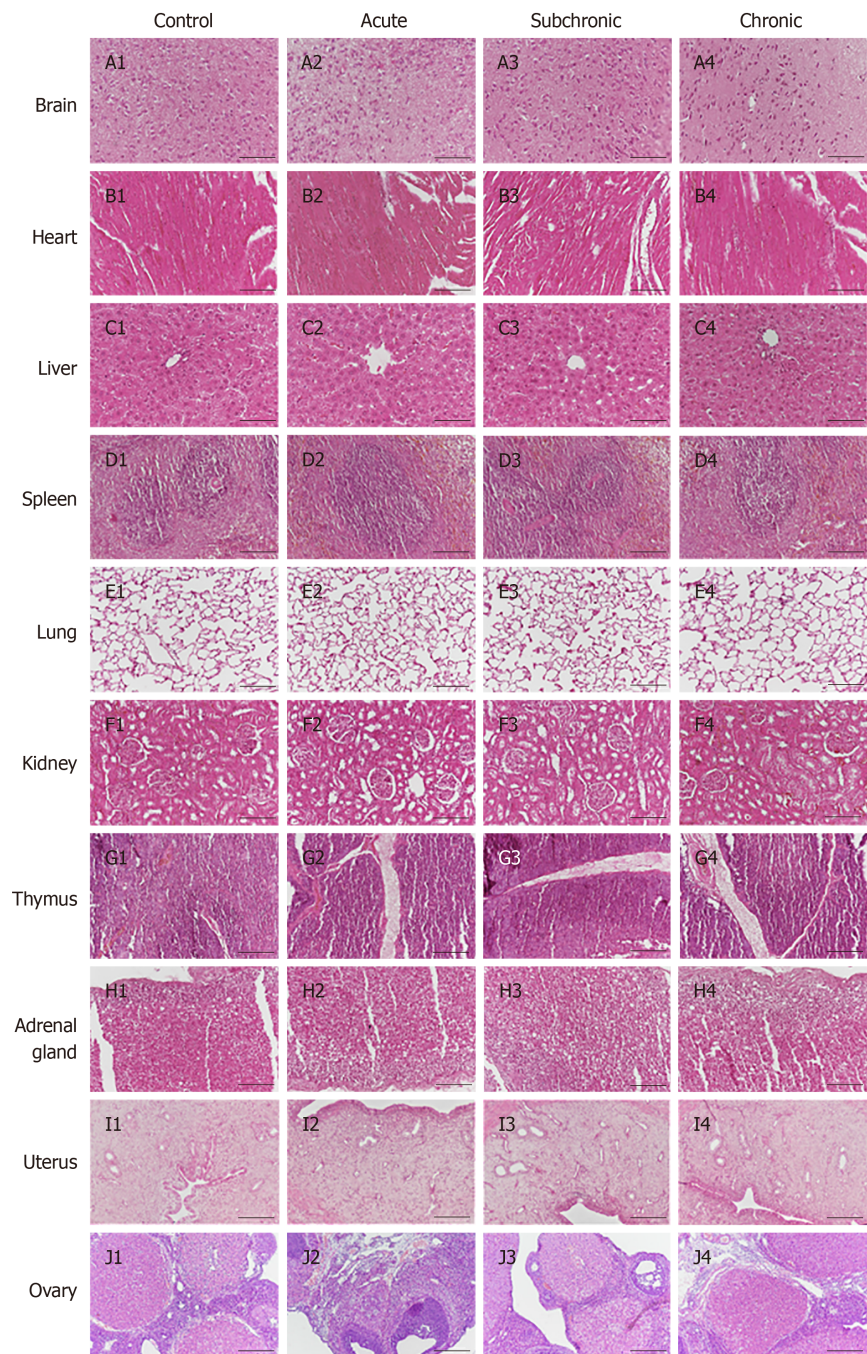
MenSCs were cultured and identified as described previously<sup>[23]</sup>. In brief, sterile techniques were used to collect menstrual blood from three healthy volunteers, aged 25 to 30 years. After mixing with PBS, the samples of menstrual blood were lightly placed on the upper layer of an equal amount of Ficoll. The intermediate cell layer was separated and cultured in DMEM/F12 medium (1:1; HyClone, Logan, UT, United States) containing 10% fetal bovine serum (Gibco, Waltham, MA, United States) at 37 °C in a 5% atmosphere. MenSCs at passage 3 (P3) were collected and MSC surface markers were evaluated by flow cytometry (CD34, CD38, CD44, CD45, CD73, CD90, and CD105) (**Supplemental Figures 1**). Only well-grown and verified P3 MenSCs were used in this study.

### **Toxicology study**

**Establishment and treatment of a rat model of IUA:** Forty-five eight-week-old female Sprague-Dawley rats were purchased from HFK Bioscience Co. (Beijing, China) and housed in a Specific-Pathogen-Free (SPF) laboratory (SYXK 2017-0004, China) at a temperature of 22 °C ± 1 °C, a relative humidity of 50% ± 1%, and a light/dark cycle of 12/12 h. Sterilized food and water were available *ad libitum*. All animal studies (including euthanasia procedures) were conducted in accordance with the regulations and guidelines of China Medical University institutional animal care and with the AAALAC and IACUC guidelines. A rat model of IUA was established according to the procedures outlined in our previous study. In brief, 30 female rats in estrus cycle were selected for surgery ( $n = 10$  for each group). After anesthesia with 3% pentobarbital, the uterine horn was surgically exposed. The endometrium was then damaged mechanically using a 16 G syringe. After two estrus cycles, the abdominal wall was reopened and  $5 \times 10^5$  MenSCs were injected into each uterine serosa. Five rats in each group received a placebo (PBS) and acted as controls.

**Sample acquisition:** The study involved three experimental groups according to observation time: Acute group (3 d), sub-chronic group (3 mo), and chronic group (6 mo). All rats were weighed prior to sacrifice and 5 mL of blood was collected from the abdominal aorta. Sodium citrate was added to 2 mL of peripheral blood to analyze the blood cell composition. The remaining peripheral blood was quickly centrifuged and the serum was separated for biochemical detection. After removing the surface adipose tissue, the brain, heart, liver, spleen, lungs, kidneys, thymus, adrenal glands, uterus, and ovaries of each rat were weighed and recorded. Subsequently, these organs were fixed in 4% paraformaldehyde, dehydrated, and then embedded in paraffin. Then, we prepared 5 µm serial sections of each tissue for staining.

**Blood cell composition test and serum biochemical test:** Freshly collected rat peripheral venous blood was immediately tested for blood cell composition (Procyte DX, IDEXX Laboratories, United States). Red blood cells, hematocrit, hemoglobin,



**Figure 1** Histopathological analysis of intrauterine adhesion rats after menstrual-derived stromal stem cell treatment. Representative H&E staining of various organs (brain, heart, liver, spleen, lungs, kidneys, thymus, adrenal glands, uterus, and ovaries). No structural changes or injuries were detected in these organs. Scale bar = 100  $\mu$ m.

average red blood cell volume, average hemoglobin concentration, red blood cell distribution width, reticulocytes, white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, basophils, neutrophils, and platelet related concentrations or percentages were evaluated. Subsequently, blood glucose, urea, creatinine, total protein, albumin, globulin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and sodium, potassium, and chloride ion concentrations in the serum of each group of rats were detected (Catalyst One, IDEXX Laboratories, United States).

### **Histopathology**

Paraffin sections of various organs were subjected to hematoxylin-eosin (HE) staining after dewaxing and dehydration. The tissue morphology of each tissue was then evaluated by light microscopy (NikonECLIPSE N80-i).

### **Tumorigenicity tests**



**In vivo:** P3 MenSCs were fluorescently labeled by transfection with green fluorescence protein (GFP)-labeled lentivirus (multiplicity of infection = 20). Subcutaneous injection in Balb/c-nu mice was applied to detect tumor-forming properties of MenSCs *in vivo*. Eight-week-old female Balb/c-nu mice weighing 20 g, were purchased from HFK Bioscience Co. (Beijing, China), and housed in a specific-pathogen-free (SPF) laboratory (SYXK 2017-0004, China). We then injected GFP-labeled  $10^7$  MenSCs into the epidermis on the groin of Balb/c-nu mice. The intensity and range of fluorescence were then evaluated every 7 d by 470-535 nm excitation. Each mouse was placed onto the scanning stage of the *in vivo* MS FX Pro system (Carestream, United States). Bioluminescence imaging was carried to identify the location of GFP-MenSCs. Images were acquired and analyzed with Carestream MI SE software.

**In vitro:** The tumorigenicity of MenSCs was evaluated by soft-agar colony formation assays *in vitro*. HELA cells were used as a positive control. These cells were first suspended in complete culture medium with 0.35% low melting agarose, then the mixture was transferred onto solidified 0.6% agarose in a 6-well plate. Approximately  $2.5 \times 10^3$  and  $5 \times 10^3$  cells were uniformly inoculated into the upper layer of each well and cultured in DMEM/F12 medium containing 10% fetal bovine serum at 37 °C in an atmosphere containing 5% CO<sub>2</sub>. The monoclonal formation of MenSCs and HELA cells was observed under a microscope (Nikon ECLIPSE Hi) to investigate for malignant proliferation for a total period of 2 wk.

### Statistical analysis

All data in this study are presented as the mean  $\pm$  standard deviation (SD), and comparisons between groups were analyzed using one-way analysis of variance (ANOVA). Bonferroni post hoc tests were used to further investigate significant differences. Statistical analyses were carried out with Prism 8 software (GraphPad, San Diego, United States) and  $P < 0.05$  was considered to represent a statistically significant difference.

## RESULTS

### Toxicity

**Body weights:** Over the entire experimental period, no deaths or adverse response were evident in either the control group or the MenSC transplantation groups. All rats represented with normal behavior without surgical complications. As shown in Table 1, there was no difference between each experimental group and the healthy controls in terms of body weight throughout the entire experimental period ( $P_{\text{acute}} = 0.207$ ,  $P_{\text{sub-chronic}} = 0.255$ , and  $P_{\text{chronic}} = 0.696$ ).

**Organ weights:** Next, we weighed the major organs of all rats. As shown in Table 2, there was no difference between the control groups and the treatment groups in terms of the relative weight of the brain, heart, liver, spleen, lung, kidney, thymus, adrenal glands, uterus, and ovaries, indicating that organ weights were within the normal range. No morphological change or color change was observed in any of the examined organs.

**Hematology and biochemistry:** Next, blood cell composition and serum biochemical and metabolic parameters were examined in two groups of rats (Tables 3 and 4). The blood cell compositions in the MenSC transplantation groups fluctuated slightly, but were all within the range of normal values. In addition, compared with controls of the same age, there were no changes in terms of serum biochemical or metabolic parameters in the MenSC transplantation groups.

### Histopathology

Compared with controls of the same week-old age, there were no changes in the main organ morphology or size in the MenSC transplantation groups. In addition, we carefully examined the abdominal cavity of every rat, and no endometriosis or tumor formation was observed at 3 d, 3 mo or 6 mo after MenSC transplantation.

Next, histopathological examinations of the brain, heart, liver, spleen, lung, kidney, thymus, thyroid, adrenal glands, uterus, and ovaries were carried out by HE staining. Representative histological images are shown in Figure 1. Compared with the control group of the same age, there were no obvious structural changes or tumor formation in the MenSC transplantation group.

### Tumorigenicity studies

Table 1 Body weight changes of mice in toxicity study

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Body weight (g)	228.0 ± 18.18	228.8 ± 9.175	267.4 ± 26.16	265.8 ± 9.378	326.0 ± 22.75	303.7 ± 35.06

Measurements are given as the mean ± SD. Group 1: Control for acute group; Group 2: Acute group; Group 3: Control for subchronic group; Group 4: Subchronic group; Group 5: Control for chronic group; Group 6: Chronic group ( $n = 5$  for each control group,  $n = 10$  for each experimental group).

**In vivo assay:** The tumorigenesis of MenSCs was examined *in vivo* (Figure 2) via hypodermic injection into Balb/c-nu mice. Four weeks post-injection, there was no tumor formation in the nude mice injected with MenSCs and the fluorescent-labeled MenSCs gradually disappeared over time. No fluorescent signals were detected in other parts of the body. In contrast, Balb/c-nu mice with HELA cell injection showed subcutaneous tumors and had surface gangrene (Supplementary Figure 2).

**In vitro assay:** The tumorigenesis of MenSCs was also investigated *in vitro* using soft-agar assays. The tumorigenicity was negative for MenSCs *in vitro*, and no cell colonies were formed during 2 wk of culture (Figure 3A). In contrast, as for Hela cells, there were numerous colonies observed in the soft agar (Figure 3B). These tumor colonies grew in stacks and spread outward with strong tumorigenicity.

## DISCUSSION

MSCs are the most commonly used stem cells in basic and clinical research. In addition to differentiation potential, MSCs also participate in the regulation of immune balance. Moreover, MSCs have the effect on microenvironment formation that is conducive to tissue regeneration<sup>[25]</sup>. Recent clinical studies have confirmed that BMSCs, ADMSCs, UCMSCs, and vascular endothelial MSCs provide therapeutic effects with regard to organ function improvement and tissue regeneration, and thus were widely used in clinical treatment research of cardiovascular disease, immune system disease, motor system injury, and digestive system disease. During the follow-up of these clinical studies, there were no tumor or serious complications associated with stem cell transplantation<sup>[26-30]</sup>.

Up to now, only two clinical trials of MenSC transplantation have been reported. In 2009, Zhong's study indicated that transplantation of allogeneic MenSCs can effectively improve multiple sclerosis. During the one-year follow-up, there were no complications related to immune response and cell transplantation observed in all four patients<sup>[31]</sup>. Similarly, in our previous clinical trial, no transplant-related complication was found in all seven severe IUA patients after receiving autologous MenSC transplantation<sup>[22]</sup>.

Currently, MenSC treatment studies were mostly in the preclinical or phase 1 clinical research stage<sup>[24]</sup>. Therefore, it is essential to acquire more clinical and basic research data in order to support the further clinical application of MenSCs. To transform MenSCs from an experimental product into a clinical treatment formulation, it is necessary to consider factors directly related to clinical application, such as indications, routes of administration, and dosage. Due to the high proliferative potential and multi-pluripotency, the toxicity and tumorigenicity of stem cells are the main concerns in clinical research<sup>[32]</sup>. Meanwhile, the biological safety should be assessed in appropriate *in vivo* and *in vitro* models. In this study, we transplanted MenSCs in an IUA rat model using a dose and method consistent with the clinical application, and evaluated the safety of MenSCs for acute, sub-chronic, and chronic observations. To our knowledge, it is the first comprehensive preclinical biosafety study of MenSCs.

In our study, 30 IUA rat models received  $10^6$  MenSCs *via* intrauterine sub-serosa injection. During the observation periods from 3 d to 6 mo, all these rats maintained normal body weight, without death, abnormal behavior, or transplant-related diseases. At the same time, the weight, shape, and appearance of the main organs remained normal. Moreover, compared with the control group, there were no differences in blood cell composition or ratio after MenSC transplantation. Serum biochemical results showed that the liver and kidney function in these rats was normal. The above results demonstrated that MenSCs were well tolerated, without initiation of abnormal immune response or organ dysfunction.

It is worth noting that the leukocytes and lymphocytes in all experimental groups were within the normal range, indicating that the preparation and transplantation process of MenSCs was sterile. Menstrual blood is usually obtained non-invasively

**Table 2** Relative organ weights of rats in toxicity study

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Brain	1.314 ± 0.220	1.439 ± 0.176	1.480 ± 0.211	1.477 ± 0.197	1.441 ± 0.047	1.508 ± 0.176
Heart	0.718 ± 0.048	0.750 ± 0.087	0.744 ± 0.076	0.805 ± 0.064	0.826 ± 0.039	0.868 ± 0.039
Liver	8.383 ± 0.764	8.525 ± 1.012	8.563 ± 0.646	9.205 ± 1.385	9.982 ± 0.282	10.01 ± 0.872
Spleen	0.432 ± 0.101	0.490 ± 0.123	0.515 ± 0.051	0.506 ± 0.067	0.500 ± 0.018	0.501 ± 0.082
Lung	1.225 ± 1.104	1.269 ± 0.100	1.375 ± 0.129	1.374 ± 0.088	1.350 ± 0.084	1.374 ± 0.100
Kidney	0.900 ± 0.161	1.016 ± 0.267	1.028 ± 0.032	1.015 ± 0.181	1.012 ± 0.082	1.009 ± 0.144
Thymus	0.298 ± 0.021	0.335 ± 0.051	0.341 ± 0.072	0.339 ± 0.059	0.352 ± 0.032	0.338 ± 0.052
Adrenal gland	0.027 ± 0.003	0.025 ± 0.005	0.032 ± 0.002	0.029 ± 0.006	0.031 ± 0.002	0.030 ± 0.006
Uterus	0.512 ± 0.025	0.524 ± 0.017	0.511 ± 0.021	0.514 ± 0.020	0.536 ± 0.031	0.546 ± 0.045
Ovary	0.047 ± 0.004	0.049 ± 0.005	0.049 ± 0.004	0.051 ± 0.003	0.054 ± 0.005	0.053 ± 0.004

Measurements are given as the mean ± SD. Group 1: Control for acute group; Group 2: Acute group; Group 3: Control for subchronic group; Group 4: Subchronic group; Group 5: Control for chronic group; Group 6: Chronic group.

through the vagina, which is a unique advantage of MenSCs in regenerative medicine. However, this is also the main source of contamination risks during MenSC preparation. Therefore, the establishment of a quality control system is essential for MenSC clinical application.

Tumorigenicity is one of the most serious risk factors to be considered for the clinical application of MSCs<sup>[33]</sup>, which is strictly related to genomic instability<sup>[34]</sup>. Only a few studies have investigated malignant lesions at MSC transplant sites, suggesting that the potential risk of tumor formation may still exist<sup>[35-38]</sup>. In contrast, some other studies have indicated that MSCs were not associated with tumorigenicity after intravenous or intramuscular application<sup>[39,40]</sup>. To date, only one article has reported spontaneous tumorigenic transformation due to long-term cultivation associated with genomic alterations in culture<sup>[41]</sup>. It is determined that MenSCs has no karyotype changes in long-term culture *in vitro*<sup>[14]</sup>.

In this study, no tumor formation was observed in IUA rat models after 6 mo of intrauterine sub-serosa injection. After MenSC transplantation, no lump formed on the abdominal wall or organs. The physiological structure of all organs remained normal. Furthermore, we used Balb/c-nu mice to detect the tumorigenicity of MenSCs. The subcutaneous GFP fluorescence range gradually decreased without migration. These GFP-labeled MenSCs completely disappeared at the fourth week, indicating that MenSCs are none-tumorigenic *in vivo*. In addition, soft agar assay demonstrated that MenSCs did not form any tumor-like cell population *in vitro*. These results provide good evidence that MenSCs is non-tumorigenic in clinical applications.

In conclusion, our current research confirms that intrauterine transplantation of MenSCs is safe, without toxicities or tumorigenicity. The results indicate that MenSCs are not only safe but also a promise source of cells for treating IUA and other types of endometrial damage. In addition, it is necessary to conduct longer follow-up studies on patients to fully ensure the safety of MenSC application.



**Table 3** Selected hematology analyses of rats in toxicity study

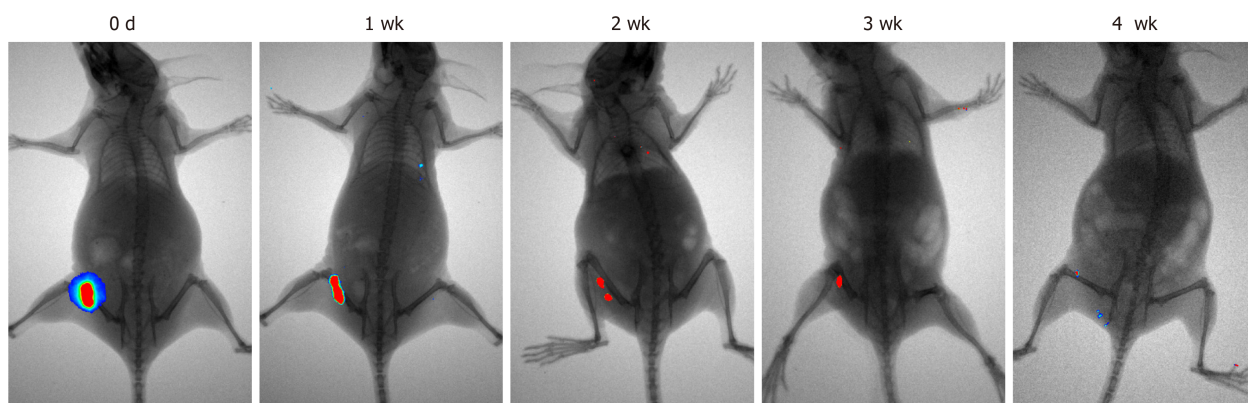
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
RBC ( $\times 10^{12}/L$ )	7.196 $\pm$ 0.090	7.225 $\pm$ 0.340	7.346 $\pm$ 0.418	7.462 $\pm$ 0.430	7.276 $\pm$ 0.421	7.300 $\pm$ 0.347
HCT (%)	39.32 $\pm$ 0.740	38.98 $\pm$ 1.992	39.08 $\pm$ 0.958	38.50 $\pm$ 2.253	38.90 $\pm$ 2.220	37.05 $\pm$ 0.576
MCV (fL)	54.64 $\pm$ 0.541	52.28 $\pm$ 0.784	53.28 $\pm$ 1.767	53.34 $\pm$ 0.845	53.00 $\pm$ 0.714	53.04 $\pm$ 1.681
MCH (pg)	18.66 $\pm$ 0.114	18.01 $\pm$ 0.470	18.10 $\pm$ 0.765	18.39 $\pm$ 0.341	17.82 $\pm$ 1.043	18.51 $\pm$ 0.905
MCHC (g/dL)	34.18 $\pm$ 0.523	34.42 $\pm$ 0.480	33.96 $\pm$ 0.493	34.50 $\pm$ 0.245	34.48 $\pm$ 0.590	35.12 $\pm$ 0.545
RDW (%)	17.42 $\pm$ 0.444	17.87 $\pm$ 0.912	17.78 $\pm$ 0.691	17.29 $\pm$ 1.152	17.30 $\pm$ 0.570	17.92 $\pm$ 0.651
RETIC (K/ $\mu$ L)	2.86 $\pm$ 0.434	3.78 $\pm$ 0.630	2.78 $\pm$ 0.691	2.93 $\pm$ 0.706	2.48 $\pm$ 0.798	2.81 $\pm$ 0.802
RETIC	214.7 $\pm$ 35.25	270.6 $\pm$ 39.98	202.2 $\pm$ 42.64	216.6 $\pm$ 49.50	162.3 $\pm$ 5.118	178.0 $\pm$ 19.04
WBC ( $\times 10^{12}/L$ )	7.644 $\pm$ 1.668	6.932 $\pm$ 2.50	7.592 $\pm$ 2.96	7.504 $\pm$ 2.121	6.440 $\pm$ 1.248	5.883 $\pm$ 2.897
NEU (%)	13.72 $\pm$ 4.217	17.50 $\pm$ 5.977	12.48 $\pm$ 2.104	15.40 $\pm$ 5.918	12.10 $\pm$ 2.351	14.66 $\pm$ 2.831
LYM (%)	75.06 $\pm$ 5.697	73.91 $\pm$ 6.311	80.36 $\pm$ 4.360	79.33 $\pm$ 7.194	78.88 $\pm$ 3.440	76.26 $\pm$ 2.858
MONO (%)	5.180 $\pm$ 0.832	6.800 $\pm$ 0.902	4.520 $\pm$ 1.117	5.540 $\pm$ 1.169	4.540 $\pm$ 0.963	6.160 $\pm$ 1.190
EOS (%)	0.60 $\pm$ 0.123	0.80 $\pm$ 0.340	0.80 $\pm$ 0.200	0.53 $\pm$ 0.200	0.62 $\pm$ 0.356	0.61 $\pm$ 0.166
BASO (%)	0.14 $\pm$ 0.114	0.25 $\pm$ 0.151	0.20 $\pm$ 0.141	0.18 $\pm$ 0.140	0.16 $\pm$ 0.114	0.19 $\pm$ 0.171
NEU ( $\times 10^9/L$ )	1.024 $\pm$ 0.288	1.116 $\pm$ 0.495	0.976 $\pm$ 0.153	1.011 $\pm$ 0.468	1.096 $\pm$ 0.163	1.000 $\pm$ 0.096
LYM ( $\times 10^9/L$ )	6.178 $\pm$ 1.593	4.849 $\pm$ 2.150	6.158 $\pm$ 2.684	6.002 $\pm$ 2.089	5.672 $\pm$ 0.854	5.398 $\pm$ 1.097
MONO ( $\times 10^9/L$ )	0.386 $\pm$ 0.042	0.455 $\pm$ 0.161	0.426 $\pm$ 0.083	0.415 $\pm$ 0.120	0.406 $\pm$ 0.067	0.346 $\pm$ 0.111
EOS ( $\times 10^9/L$ )	0.046 $\pm$ 0.011	0.05 $\pm$ 0.017	0.050 $\pm$ 0.029	0.037 $\pm$ 0.016	0.004 $\pm$ 0.012	0.034 $\pm$ 0.013
BASO ( $\times 10^9/L$ )	0.01 $\pm$ 0.007	0.014 $\pm$ 0.010	0.001 $\pm$ 0.007	0.013 $\pm$ 0.07	0.016 $\pm$ 0.009	0.016 $\pm$ 0.012
PLT (K/ $\mu$ L)	1001 $\pm$ 193.2	1107 $\pm$ 178.6	1032 $\pm$ 142.8	974.4 $\pm$ 121.9	1169 $\pm$ 74.37	1075 $\pm$ 45.99
MPV (fL)	8.50 $\pm$ 0.100	8.43 $\pm$ 0.216	8.42 $\pm$ 0.148	8.63 $\pm$ 0.095	8.44 $\pm$ 0.089	8.47 $\pm$ 0.206
PDW (fL)	8.50 $\pm$ 0.158	8.62 $\pm$ 0.148	8.46 $\pm$ 0.422	8.30 $\pm$ 0.133	8.42 $\pm$ 0.303	8.71 $\pm$ 0.778
PCT (%)	1.037 $\pm$ 0.038	0.962 $\pm$ 0.137	0.870 $\pm$ 0.129	0.80 $\pm$ 0.151	0.844 $\pm$ 0.167	0.88 $\pm$ 0.107

Note: Measurements are given as the mean  $\pm$  SD. RBC: Red blood cells; HCT: Hematocrit; MCV: Average red blood cell volume; MCH: Average hemoglobin concentration; RDW: Red blood cell distribution width; RETIC: Reticulocytes; WBC: White blood cells; NEU: Neutrophils; LYM: Lymphocytes; MONO: Monocytes; EOS: Eosinophils; BASO: Basophils; PLT: Platelet; MPV: Mean platelet volume; PDW: Platelet distribution width.

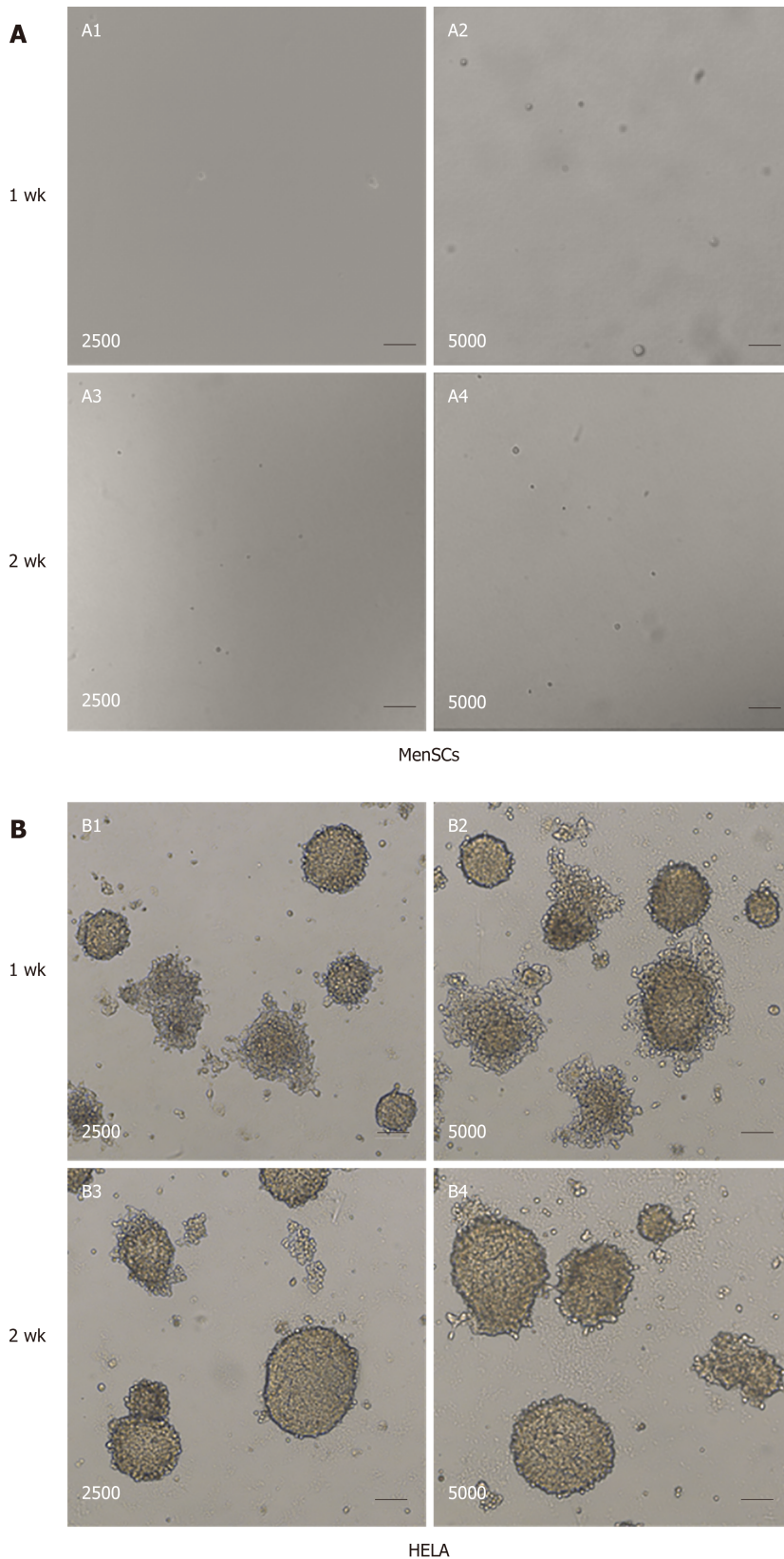
**Table 4** Selected biochemistry analyses of rats in toxicity study

	Group 1	Group 2	Group3	Group 4	Group 5	Group 6
Urea	6.220 $\pm$ 0.512	6.260 $\pm$ 0.657	5.860 $\pm$ 0.493	6.19 $\pm$ 0.659	5.840 $\pm$ 0.270	6.380 $\pm$ 0.570
CREA	27.60 $\pm$ 5.128	32.30 $\pm$ 6.430	28.40 $\pm$ 4.722	26.40 $\pm$ 2.011	31.00 $\pm$ 6.557	31.10 $\pm$ 6.367
BUN/CREA	55.80 $\pm$ 11.71	48.90 $\pm$ 7.279	55.20 $\pm$ 8.899	58 $\pm$ 8.679	56.60 $\pm$ 5.771	57.80 $\pm$ 3.967
TP	60.8 $\pm$ 4.087	62.20 $\pm$ 5.673	61.80 $\pm$ 7.014	57.40 $\pm$ 2.914	60.20 $\pm$ 3.962	60.30 $\pm$ 5.165
ALB	31.80 $\pm$ 2.683	32.4 $\pm$ 4.060	36.80 $\pm$ 3.899	30.2 $\pm$ 3.584	30.00 $\pm$ 2.739	32.90 $\pm$ 3.695
GLOB	29.40 $\pm$ 1.817	29.8 $\pm$ 2.300	25.90 $\pm$ 3.194	27.4 $\pm$ 1.350	26.00 $\pm$ 3.674	27.80 $\pm$ 2.201
ALT	35.00 $\pm$ 6.042	34.7 $\pm$ 5.539	31.40 $\pm$ 8.355	38.30 $\pm$ 3.889	34.60 $\pm$ 7.162	36.30 $\pm$ 6.897
AST	73.40 $\pm$ 11.84	66.6 $\pm$ 14.010	70.20 $\pm$ 7.662	75.2 $\pm$ 15.050	64.20 $\pm$ 16.68	61.4 $\pm$ 13.16
ALKP	108 $\pm$ 25.03	121.7 $\pm$ 32.840	107.0 $\pm$ 37.36	100.3 $\pm$ 11.910	108.4 $\pm$ 23.39	95.20 $\pm$ 11.70
Na	142.6 $\pm$ 1.949	141.2 $\pm$ 2.348	143.2 $\pm$ 1.483	141.7 $\pm$ 2.627	142.2 $\pm$ 1.643	144.1 $\pm$ 2.234
K	5.180 $\pm$ 0.148	5.230 $\pm$ 0.457	4.820 $\pm$ 0.601	5.01 $\pm$ 0.213	4.700 $\pm$ 0.245	5.96 $\pm$ 0.390
CL	103.8 $\pm$ 2.387	106.2 $\pm$ 3.293	104.2 $\pm$ 2.387	106.6 $\pm$ 3.084	103.8 $\pm$ 2.775	105.4 $\pm$ 5.719

Note: Measurements are given as the mean  $\pm$  SD. CREA: Creatinine; TP: Total protein; ALB: Albumin; GLOB: Globulin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALKP: Alkaline phosphatase; BUN: Blood urea nitrogen.



**Figure 2** Tumorigenicity analysis of menstrual-derived stromal stem cells on nude mice 4 wk after subcutaneous injection. Representative photographs show the cell proliferation of menstrual-derived stromal stem cells (MenSCs) on nude mice 4 wk after subcutaneous injection. Fluorescent expression, which represented the MenSCs, gradually decreased over time. No metastatic or proliferative fluorescent signals were detected in other parts of the body.



**Figure 3 Tumorigenicity analyses of menstrual-derived stromal stem cells and HeLa cells in soft-agar colony formation assay.** A: Representative photomicrographs show the cell malignant proliferation of 2500 and 5000 menstrual-derived stromal stem cells (MenSCs) after 1 and 2 wk of culturing. No cell colony was formed in the MenSCs group. B: Representative photomicrographs show the cell malignant proliferation of 2500 and 5000 HELA cells after 1 and 2 wk of culturing. Scale bar = 100  $\mu$ m. MenSCs: Menstrual-derived stromal stem cells.

## ARTICLE HIGHLIGHTS

**Research background**

Intrauterine adhesion (IUA) can cause serious damage to women's reproductive health. In our previous studies, we demonstrated that menstrual-derived stromal stem cells (MenSCs), with high proliferative capacity and self-renewal ability, have a powerful therapeutic effect in patients with severe IUA.

**Research motivation**

Safety assessment of MenSCs transplantation is essential for its further application in patients with severe IUA.

**Research objectives**

The purpose of this study was to evaluate the short-, medium-, and long-term biosafety of MenSCs *via* intrauterine transplantation in a rat model of IUA, with a focus on toxicity and tumorigenicity.

**Research methods**

MenSCs were injected into the sub-serosal layer of the uterus in an IUA rat model, for 3 d, 3 mo, and 6 mo separately, to monitor the corresponding acute, sub-chronic, and chronic effects. Healthy rats of the same age served as negative controls. Toxicity effects were evaluated by body weight, organ weight, histopathology, hematology, and biochemistry tests. Tumorigenicity of MenSCs was investigated in Balb/c-nu mice *in vivo* and by colony formation assays *in vitro*.

**Research results**

Compared with the same week-old control group, all of the IUA rats receiving MenSC transplantation demonstrated no obvious changes in body weight, main organ weight, or blood cell composition during the acute, sub-chronic, and chronic observation periods. At the same time, serum biochemical tests showed no adverse effects on metabolism or liver and kidney function. After 4 wk of subcutaneous injection of MenSCs in Balb/c-nu nude mice, no tumor formation or cell metastasis was observed. Moreover, there was no tumor colony formation of MenSCs during soft agar culture *in vitro*.

**Research conclusions**

There was no acute, sub-chronic, or chronic poisoning, infection, tumorigenesis, or endometriosis in rats with intrauterine adhesions after MenSC transplantation. The above results suggested that intrauterine transplantation of MenSCs is safe for endometrial treatment.

**Research perspectives**

MenSCs are not only safe but also a promise source of cells for treating IUA and other types of endometrial damage. In addition, it is necessary to conduct longer follow-up studies on patients to fully ensure the safety of MenSC application.

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