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Preclinical Safety Evaluation of WJMSCs and Their Secretome

(Penilaian Keselamatan Praklinikal WJMSC dan Sekretomnya)

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ABSTRACT

Mesenchymal stem cells (MSC) are a promising therapy in regenerative medicine due to their unique ability to differentiate into other cells. Among established sources of MSC, MSC derived from Wharton's jelly (WJSC) of an umbilical cord is a popular source since it involves a painless procedure to obtain the cord with an exceptional proliferation rate compared to other sources. However, the safety and efficacy of MSC must be confirmed through preclinical studies before clinical trials in humans. A study was designed to achieve the maximum tolerable dose (MTD) and safety and toxicity effects of WJSC and its secretomes in animal models. The MTD was achieved through acute toxicity testing on healthy female Sprague Dawley (SD) rats while the safety was assessed using a subchronic toxicity study on healthy male and female SD rats divided into four groups (control, low dose, medium dose and high dose). The safety assessments were then evaluated through biochemical, haematological, and histopathological analyses where the data obtained were analysed using a one-way ANOVA followed by Tukey's test. Statistical analysis confirmed no significant differences in all tests performed in the study groups. At the study's termination, neither cells nor secretomes injected rats were found to be deceased and no toxic or severe adverse effects were discovered. Thus, both WJSC and their secretomes applications in humans could be considered harmless for medical purposes.

Keywords: Acute toxicity; safety and efficacy; subchronic toxicity; Wharton's jelly stem cells

ABSTRAK

Sel stem mesenkimal (MSC) merupakan terapi yang meyakinkan dalam perubatan regeneratif kerana keupayaan uniknya untuk membeza menjadi sel lain. Antara sumber MSC yang ada pada masa ini adalah MSC daripada jeli Wharton (WJSC) tali pusat yang merupakan sumber popular kerana mempunyai kadar percambahan yang lebih banyak berbanding sumber lain dan tidak melibatkan prosedur yang menyakitkan. Walau bagaimanapun, keselamatan dan keberkesanan MSC mesti disahkan melalui kajian praklinikal sebelum ujian klinikal pada manusia. Satu kajian telah direka untuk mencapai dos maksimum yang boleh diterima (MTD) dan kesan keselamatan dan ketoksikan WJSC dan sekretomnya dalam model haiwan. MTD dicapai melalui ujian toksisiti akut ke atas tikus Sprague Dawley (SD) betina yang sihat manakala keselamatan dinilai menggunakan kajian toksisiti subkronik ke atas tikus SD jantan dan betina sihat yang dibahagikan kepada empat kumpulan (kawalan, dos rendah, dos sederhana dan dos tinggi). Penilaian keselamatan kemudiannya dinilai melalui analisis biokimia, hematologi dan histopatologi dan data yang diperoleh dianalisis menggunakan ANOVA sehala diikuti dengan ujian Tukey. Analisis statistik mengesahkan tiada perbezaan yang signifikan dalam semua ujian yang dijalankan dalam kumpulan kajian. Pada pengakhiran kajian, tiada tikus yang disuntik dengan sel dan sekretom ditemui mati dan tiada kesan toksik atau kesan sampingan yang teruk ditemui. Oleh itu, kedua-dua aplikasi WJSC dan sekretom untuk manusia boleh dianggap tidak berbahaya untuk tujuan perubatan.

Kata kunci: Keselamatan dan keberkesanan; ketoksikan akut; ketoksikan subkronik; sel stem jeli Wharton

INTRODUCTION

Stem cell therapy involves the introduction of viable cells into the human body to repair, replace or restore diseased, damaged or missing tissues. These cells have an innate ability to multiply and regenerate making them useful in the medical field. They can naturally identify and attract

inflammation and damaged cells to provide permanent healing. Stem cell therapies offer benefits for physical health and well-being by reversing inflammation, regulating the immune system, replacing damaged cells and regenerating normal blood flow (Aurora & Olson 2014).

Over the years, multi-potential mesenchymal stem cells (MSCs) have become an emerging therapeutic solution for certain diseases due to their unique characteristics. These cells can be found in various tissues like bone marrow, adipose tissue, synovium fluid, skeletal muscle, liver, cord blood, umbilical cord, and peripheral blood (Jiang et al. 2002). Moreover, under controlled *in vitro* conditions MSCs can be differentiated into osteoblasts, chondroblasts, adipocytes and myoblasts which contribute to the formation of mesenchymal tissues such as bone, cartilage, muscle, marrow stroma, ligament, tendon, fat, dermis, and connective tissue (Xu et al. 2004).

To specify, MSCs isolated from the human umbilical cord prove to promote tissue renewal by directly contributing to new tissue and stimulating repair through the paracrine effect. These cells gain more attention for tissue regeneration because it is easy and painless to collect umbilical cord, low cost, convenient isolation, high cell proliferation, enhanced gene transfection efficiency, and have low immunogenicity (Hoffmann et al. 2017). In a study by Conconi et al. (2011), MSCs were isolated from various parts of UC such as the subendothelial layer, perivascular zone, Wharton's jelly, umbilical cord lining and whole umbilical cord. However, according to the study it was found that MSCs from Wharton's jelly (WJSC) possess greater clinical potential than those from other parts of the cord.

Besides the cells, cell secretion from cultured media also contains a lot of immunoregulatory properties. The secreted factors from the cells are referred to as secretomes and could be divided into functional families based on the immunological responses including cytokines, interleukins, growth factors, chemokines, microvesicles and exosomes (Kim, Choi & Kim 2013). These secretions are small proteins, glycoprotein or peptide molecules produced by the cells through cell signalling. Wharton's Jelly-derived MSC secretomes contain a diverse mixture of bioactive compounds that provide a paracrine mechanism in the therapeutic field. However, few studies demonstrate that WJSC secretomes differ drastically from other sources of MSCs. For instance, WJSC secreted a minimum level of proangiogenic factors such as VEGF-A, angiogenin and PLGF and produced a high level of antiangiogenic factors including thrombospondin-2 and endostatin when compared to other sources of secretomes by bone marrow and adipose tissue-derived MSCs (Amable et al. 2014; Kuchroo et al. 2015). Besides that, WJSC secretomes also contain higher levels of interleukins (IL-1α, IL-6, IL-8, and IL-11) (Friedman et al. 2007), chemokines (CXCL1, CXCL, CXCL5, CXCL6, and CXCL8), growth factors (HGF, bFGF, NGF, VEGF-D, PDGF-AA, G-CSF, and TGF-β2) (Balasubramanian et al. 2012) and neurotrophic factors (NT3, NT4, and GDNF) (Balasubramanian et al. 2013).

Due to their unique qualities, MSCs and their secretomes hold great promise for future regenerative medicine especially in tissue engineering (Barry & Murphy 2004). As a result, studies on cell and gene therapy products are moving forward from basic science research to clinical trial practice. To establish product development from preclinical stages to clinical trials, definitive studies and steps are required to produce safety data. A preclinical study expresses the safety and efficacy of an investigational cell-based product for future clinical use. It is essential to identify the survival, migration, phenotype, function, and potential toxicities of the invented product in animals before it can be introduced to humans. An effective preclinical study should establish the activity, effective dose, dosing schedule and route of administration of a therapeutic product, providing evidence that it is practically safe to conduct a proposed clinical investigation with a specific product (Frey-Vasconcells et al. 2012).

Most toxicity experiments often use laboratory animals such as rodents to determine the maximum tolerable doses of new drugs and their possible adverse effects on humans. This preference is due to their clearly defined genetic constitution, control over exposure and practicality in harvesting tissues and organs for detailed examination (Arome & Chinedu 2013). Similarly, the safety and efficacy of transplanted mesenchymal stem cells in clinical setting are crucial for developing cell-based therapies for various human diseases. It is essential to evaluate the safety of MSC and validate the proof of concept in animal models to predict potential problems that might arise in clinical applications (Joers & Emborg 2010). This aligns with criteria set by the FDA for the registration of stem cell-based therapies which include the safety of donor, risk of contamination of processed cells or tissue, types of cells and the safety of cells in the final product (Halme & Kessler 2006).

MATERIALS AND METHODS

ISOLATION AND EXPANSION OF WHARTON'S JELLY TISSUES

Human umbilical cords were obtained from a healthy donor and processed in a GMP-accredited laboratory to isolate WJSC using standard enzymatic digestion method. The cord was cut into 3-5 cm long pieces, rinsed in a washing solution containing Dulbecco Phosphate-buffered saline (DPBS) and 1% antibiotic to remove blood traces. The washed cord was split apart and all the blood vessels were removed. Wharton's jelly tissues were collected, rinsed and transferred into tubes containing 0.2% Collagenase Type IV dissolved in Knockout Dulbecco's Modified Eagle Medium (KO-DMEM). The cord tissue was incubated at 37 °C in a 5% humidified CO_2 incubator overnight. An equal volume of complete culture media ((CCM; comprising

Knock-out-Dulbecco's Modified Eagle Medium, Fetal Bovine Serum, L- glutamine aminoglycoside, antibiotic and Basic Fibro-blast Growth Factor) was added to neutralize Collagenase Type IV activity and centrifuged at 1800 rpm for 15 min. The supernatant was discarded and the digested jellies were cultured in CCM. The cells were dissociated with detachment solution TrypLE Select to proceed with the subculture process. The cells were subcultured and cryopreserved in cryogenic vials containing approximately 5×10^6 cells per mL of ready-to-use freezing media until further use.

ISOLATION OF SECRETOMES FROM MESENCHYMAL STEM CELLS CONDITION MEDIUM

The process of preparing MSCs secretomes involves cultured WJSC at SC2 at a seeding density of 5000 cells/cm^2 in a humidified CO_2 incubator. After reaching 40% confluency, CCM was completely removed, rinsed with DPBS and replaced with Dulbecco's Modified Eagle's Medium (DMEM) high glucose without phenol red containing 5% glutamax for three days. The conditioned media is collected, centrifuged to remove cell debris and stored at -20 °C for long-term storage.

QUALITY CONTROL TESTING FOR WJSC

Cell morphology analysis of WJSC and secretome-cultured WJSC was performed using an inverted microscope and the images were captured using cellSens imaging software. Characterization was done through immunophenotyping and the surface markers were analysed using Human MSC Analysis Kit antibodies. Cells were stained with hMSC Positive Cocktail (CD73, CD90 and CD105) and hMSC Negative Cocktail (CD45, CD38, CD19, CD11b and HLA-DR) and analysed using BD Flow Cytometry System FACS Calibur.

PRECLINICAL STUDY

A series of toxicology studies were performed in healthy adult Sprague Dawley (SD) rats using WJSC and WJSC-derived secretomes. These studies were conducted in Central Animal House, AIMST University, Malaysia with approval from the Animal Research Review Panel guidelines of the AIMST University Human and Animal Ethics Committee (Ref No.: AUAEC/FAS/2018/01). Prior to the study, the rats were kept in polyacrylic cages (W 460 × L 300 × H 160 mm) in an environmentally controlled room at 20-25 °C with 12 h light and 12 h dark cycle with 60-65% relative humidity. All the rats were provided with common laboratory diets (*ad libitum* pellet) and an unlimited supply of drinking water. These rats were allowed to adapt to the laboratory conditions for at least five to seven days before initiating the safety study.

Acute toxicity testing was carried out on female SD rats to determine the maximum tolerable dose of WJSC and secretomes that can be injected into the rats. Following the Organization for Economic Co-operation and Development (OECD), revised draft guidelines 423 (OECD 2002), the cells were administered in varying doses from a low number of cells ranging from 1×10^6, 2×10^6, 3×10^6, 5×10^6 to a high number of cells around 10×10^6. Whereas, the secretomes were administered from a low volume of 1 mL, 2 mL to a high volume of 3 mL. Each dose was administered to three female SD rats and monitored for 14 days in terms of their behavioural, neurological, autonomic profiles and mortality. If any animal shows mortality after drug administration, the possible organs were collected and subjected to histopathological investigation.

Based on acute toxicity testing, sub-chronic toxicity studies were conducted on SD rats using three different doses of cells and secretomes as per the guidelines set by OECD, revised draft guidelines 407 (OECD 2008). The rats were divided into four groups, each consisting of 6 males and 6 females; control (no treatment at all), low dose (1×10^6 cells), medium dose (2×10^6 cells) and high dose (3×10^6 cells) while for secretomes the low dose was (0.5 mL secretomes), medium dose (1.0 mL secretomes) and high dose (2.0 mL secretomes). The cells and secretomes were administered at the tail vein via intravenous (IV) at intervals of 7 days for 42 days. The injection site was cleaned and disinfected with 70% ethanol before the administration procedure. The syringe was filled with 1 mL of WJSC suspended in saline solution and a 27G needle was inserted at a shallow angle (15-30 degrees) into the vein. After injection, slight pressure was applied to the injection site to prevent hematoma formation. Animals were monitored for behavioural alterations, biochemical changes and other abnormalities during this period. Their regular body weight changes, food intake and water intake were also observed and blood samples were collected using retro-orbital sinus procedures on days 2, 23, and 42 for biochemical (renal and liver function test) and haematological (full blood count) analysis. In case of any mortality observed during the study, possible organs were collected and subjected to histopathological analysis. At the end of the study, all the animals were sacrificed and observed for specific toxicity with organs such as the brain, lung, heart, liver, kidney, spleen, and gonadal being examined for histopathological analysis.

PRECLINICAL STUDIES ANALYSIS

The study involved analysing the body and organ weight of rats from various experimental groups for 42 days. Using an analytical lab balance, the regular bodyweight of each SD rats from all experimental groups were weighed and recorded every week for 42 days. On day 42, the rats were sacrificed and organs such as brain, lung, heart, liver, kidney, spleen, and gonadal (ovaries and testes) were harvested. The absolute weights of harvested organs were measured and recorded.

Approximately 1 mL of blood samples were collected in a vacutainer blood collection tube from each group on days 2, 23, and 42 under general anaesthesia (diethyl ether) using a retro-orbital sinus procedure. The collected blood samples were sent to Clinipath Sdn. Bhd. Laboratory, Malaysia for biochemical and haematological analysis. Biochemical comprises of liver function test (aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin, globulin and bilirubin) and renal function test (urea, creatinine, sodium, chloride, and potassium) were analysed using cobas® 6000 analyzer series from Roche. Whereas, haematological analysis or full blood count test includes haemoglobin, red cell count (RBC), white cell count (WBC), platelets, packed cell volume (PCV), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV) and red cell distribution width (RDW) were analysed using CELL-DYN Sapphire Hematology Analyzer from Abbott.

For histopathological tests, harvested organs such as brain, lung, heart, liver, kidney, spleen, and gonadal (ovaries and testes) were preserved in 10% formalin and sent to the Clinipath Sdn. Bhd. Laboratory, Malaysia. The tissues were processed, embedded, sectioned and stained using Sakura Tissue-Tek stainer (haematoxylin and eosin method) and mounted on a slide. The prepared slides were then observed under a microscope and interpreted by a pathologist.

STATISTICAL ANALYSIS

Data obtained were calculated as mean ± standard error of the mean (SEM) for each group and analysed using one-way ANOVA followed by Tukey's test using GraphPad software. Analytical results that give $p < 0.05$ were considered statistically significant.

RESULTS

CELL MORPHOLOGY AND CHARACTERIZATION

Figure 1(A) shows the cell's growth transformation from subculture 0 (SC0) to subculture 3 (SC3). The cells were able to maintain the fibroblast spindle-shaped morphology in all the passages. On the other hand, WJSC cultured for secretomes collections also exhibits fibroblast spindle-shaped morphology despite being cultured in high glucose media (Figure 1(B)). Human MSC Analysis Kit antibodies which consist of positive markers (CD73, CD90, and CD105) and negative markers (CD45, CD38, CD19, CD11b, and HLA-DR) were used to characterize the immunophenotypes of isolated WJSC. The expressions of all markers for WJSC at SC3 were 98.10% for CD73, 99.70% for CD90, 94.50% for CD105 and 0.60% for the negative markers. The immunophenotypes of WJSC were analysed using flow cytometry and the results are presented in Figure 1(C).

ACUTE TOXICITY STUDY OF WJSC AND SECRETOMES

Female SD rats received an intravenous (IV) dosage of Wharton's Jelly derived mesenchymal stem cells (WJSC) and its secretomes. By administering several different dosages to the SD rats, the maximum tolerated dose (MTD) of WJSC and secretomes could be determined. Based on the findings, the maximum number of WJSC the SD rats could tolerate was 3×10^6 cells. Consequently, the MTD for this investigation was determined to be 18×10^6 cells/kg body weight (BW) based on the rat's approximate body weight of 165 g. This gives a sufficient safety margin for toxicological investigations. On the other hand, it was shown that the safe and nontoxic MTD of WJSC secretomes is 2 mL.

SUBCHRONIC TOXICITY STUDY OF WJSC AND SECRETOMES

In both male and female rats given WJSC and secretomes during the subchronic toxicity testing, the analysis showed that there were no mortality, abnormal clinical symptoms or significant changes in body weight that happened ($p > 0.05$) between the control and experimental groups (Figure 2). Similarly, the absolute organ weights of the male and female rats' brain, lungs, liver, heart, kidney, spleen, testis and uterus also did not differ significantly between the control and experimental groups (Data not reported).

BIOCHEMICAL AND HAEMATOLOGICAL ANALYSIS OF SD RATS ADMINISTERED WITH WJSC AND SECRETOMES

For both male and female rats administered with WJSC and secretomes, there were few significant changes observed in the biochemical parameters such as total protein, AST, ALT, ALP, albumin, globulin, urea, sodium, chloride, and potassium levels in the experimental groups compared to the control. Regarding rats given WJSC on day 23, there were notable alterations ($p < 0.01$) in the chloride levels of female rats in the medium and high dose experimental groups and on day 42, there were notable alterations ($p < 0.01$, $p < 0.05$) in the urea level of male rats in the experimental groups in comparison to the control group (Figure 3(a)-3(b)). On the other hand, when rats were given secretomes, there were significant ($p < 0.001$) alterations in the ALP level in male rats and for the female rat significant changes ($p < 0.01$, $p < 0.05$) were observed in ALP and potassium levels (Figure 3(c)-3(d)).

For haematological analysis for both male and female rats given WJSC and secretomes on day 2, all the parameters including haemoglobin, RCC, WCC, platelet, MCH, MCHC, and RDW were within the normal ranges and did not differ significantly ($p > 0.05$) from the values obtained in the experimental group animals compared to the control group. On day 23, the haemoglobin level of female rats given WJSC in high dose shows some significant

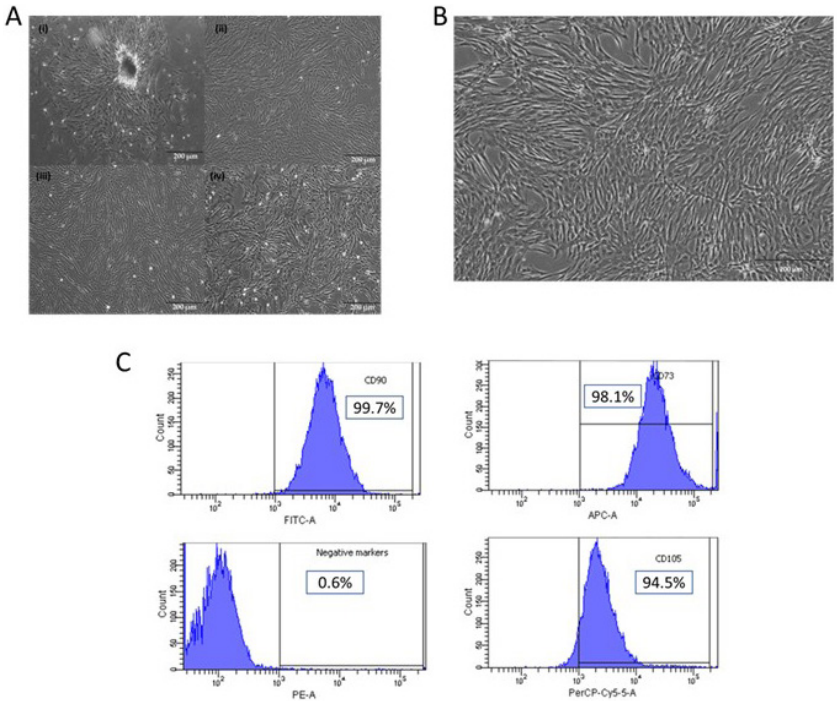


FIGURE 1. Morphology and characterization of WJSC. (A) Morphology of WJSC displaying fibroblast spindle shape morphology culture at (i) SC0 cells began to form colonies and became confluent, (ii) SC1, (iii) SC2, and (iv) SC3; (B) Morphology of isolated WJSC for secretomes at SC3. All the images were taken at 4x magnification. (C) Immunophenotyping of isolated WJSC at SC3 labelled with antibodies CD90, CD73, CD105 and Negative markers

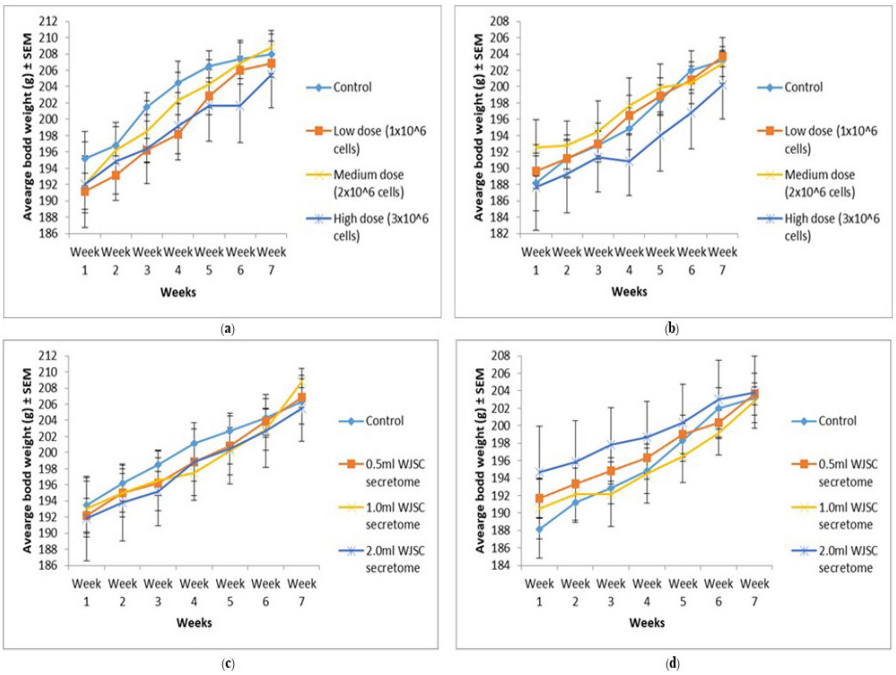


FIGURE 2. Average body weight of male SD rats and female SD rats administered with three different repeated doses of WJSC (a,b) and secretomes (c,d). The values are mean ± SEM (n = 6) for all (a) to (d)

differences ($p < 0.05$). On the other hand, when rats were given secretomes, there were notable variations ($p < 0.05$) observed in MCHC and MCH values of male rats on day 42 in comparison to the control group (Figure 4(a)-4(d)).

HISTOPATHOLOGY RESULTS FOR SD RATS ADMINISTERED WITH WJSC AND SECRETOMES

Both male and female rats' organ biopsies for the WJSC and secretomes control group showed normal histopathological structures. All organs showed normal histological tissue structures in rats given low doses of WJSC except for interstitial thickening and lung congestion in both male and female rats. Both male and female rats from the medium and high dosage groups showed a similar pattern of lung congestion and slight localised degeneration of the kidney tubules, while male rats in the medium dose group showed mild feathery degeneration of the liver (Table 1). On the other hand, all groups that received secretomes showed signs of minor pneumocytic hyperplasia of the lung. In addition, mild hydropic degeneration of the kidney tubules in the male rats and uterus endometrium eosinophilic infiltration were detected in the medium dose group female rats (Table 2).

DISCUSSION

Toxicity testing is an *in vivo* safety assessment study of newly developed drugs or therapeutic agents to determine their possible toxic effects and characterise their mechanism of action before being utilised for clinical purposes. Based on the methodology used toxicity studies can be categorised into acute, sub-acute and chronic toxicity studies. These studies include monitoring physiologic and biochemical abnormalities, terms of drug delivery and animal species selection. Based on biochemical, haematological, histological and statistical analyses all of these studies are validated (Arome & Chinedu 2013).

The umbilical cord was viewed by many as a waste product that lacked the same scientific value as cord blood. But when McElreavey et al. (1991) effectively identified and characterised fibroblast-like cells from Wharton's jelly tissues in 1991, this perspective was drastically altered. These fibroblast-like cells isolated from Wharton's jelly got more attention among stem cell researchers when they were proven to be MSCs as they were able to express CD29, CD44, CD51, CD73, and CD105 while lacking the expression of CD34 and CD45. In addition to that, they could also develop into osteogenic and adipogenic cell lineages (Wang et al. 2004).

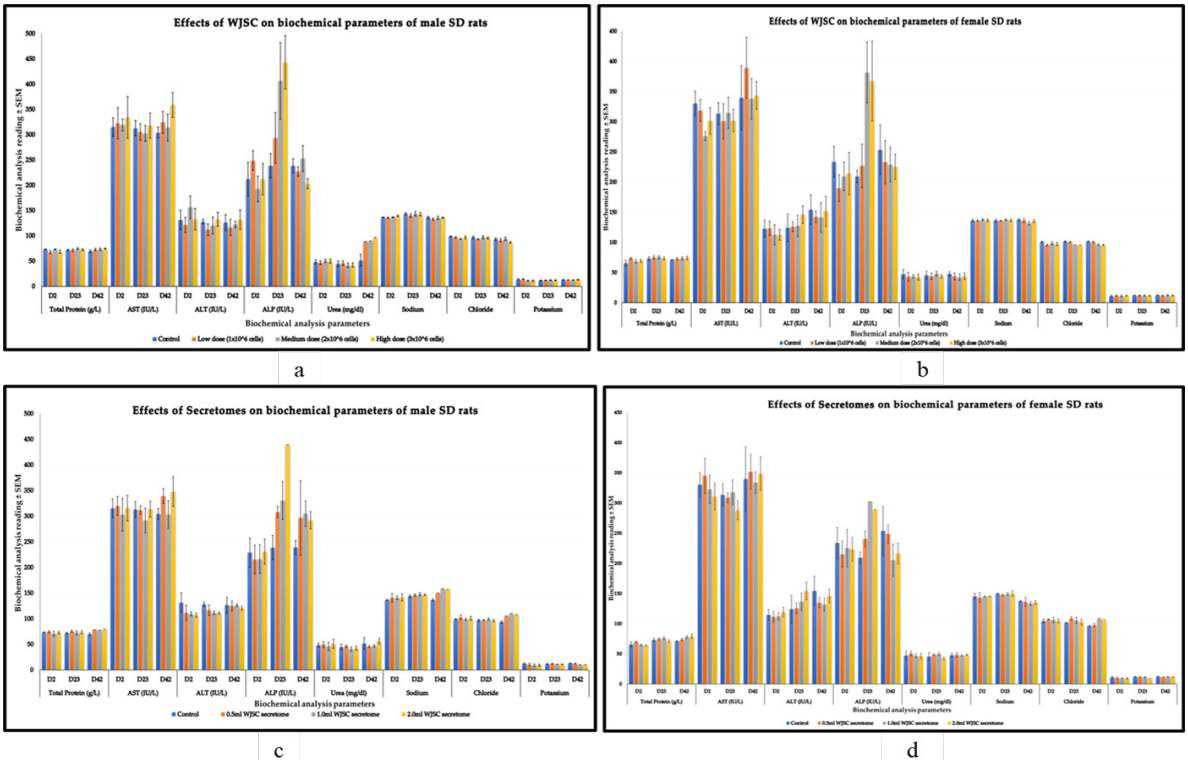
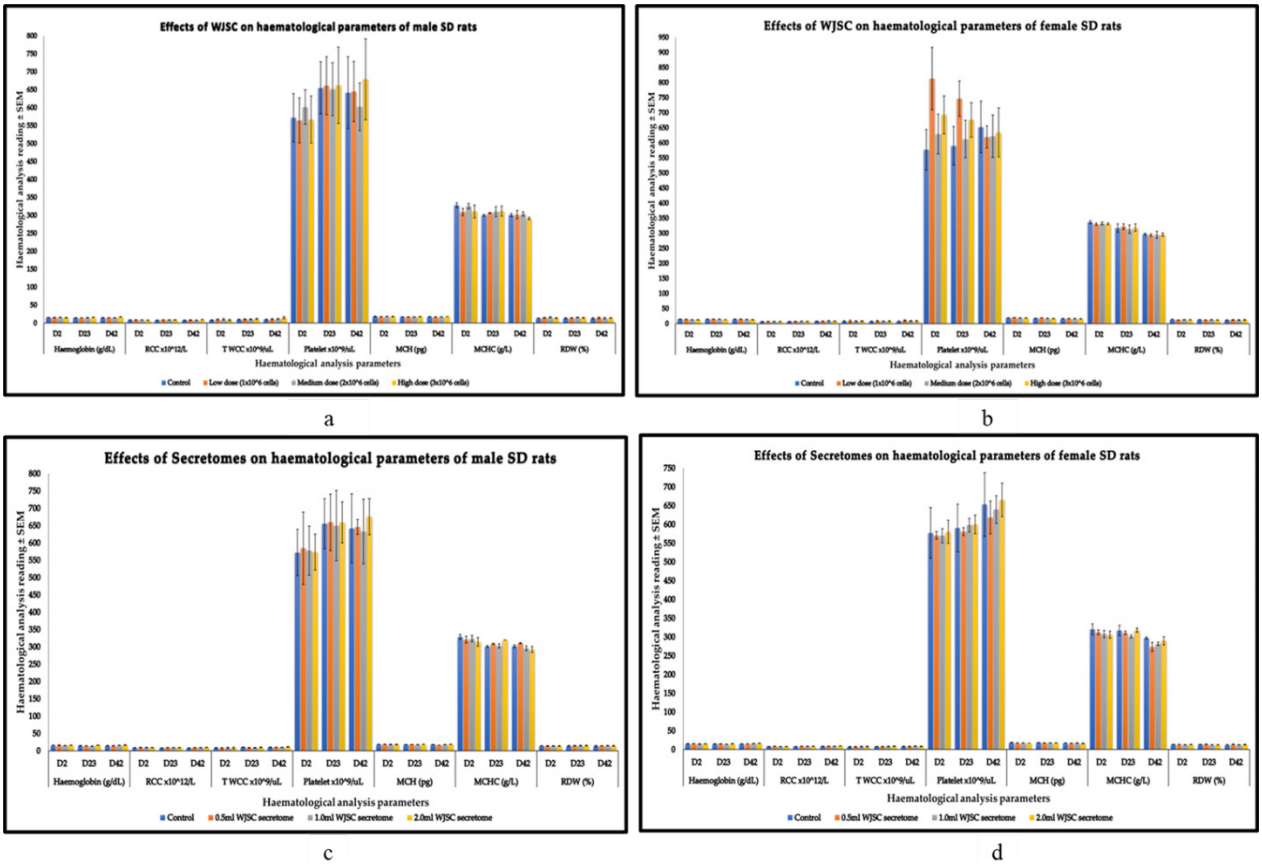


FIGURE 3. Comparison chart of biochemical parameters study of male SD rats and female SD rats administered with WJSC (a-b) and secretomes (c,d) on days 2, 23 and 42 for all the four groups analysed using One-way ANOVA followed by Tukey's test. All the values are mean ± SEM (n = 6). Abbreviations: AST- aspartate aminotransferase; ALT- alanine aminotransferase; ALP- alkaline phosphatase



RCC- red cell count; WCC- white cell count; MCH- mean corpuscular haemoglobin; MCHC- mean corpuscular haemoglobin concentration; RDW- red cell distribution width

FIGURE 4. Comparison chart of haematological parameters study of male SD rats and female SD rats administered with WJSC (a-b) and secretomes (c,d) on days 2, 23 and 42 for all the four groups analysed using One-way ANOVA followed by Tukey's test. All the values are mean ± SEM (n = 6).

TABLE 1. Biopsy results summary of SD rat's organ administered with WJSC

Group	Organs	Interpretation	
		Male	Female
Control	Brain	Normal	Normal
	Spleen	Normal	Normal
	Lung	Normal	Normal
	Liver	Normal	Normal
	Heart	Normal	Normal
	Kidney	Normal	Normal
	Testis	Normal	
	Uterus		Normal

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Low dose (1×10^6 cells)	Brain	Normal	Normal
	Spleen	Normal	Normal
	Lung	Marked interstitial thickening with an inflammatory component	Marked interstitial thickening and congestion with an inflammatory component
	Liver	Normal	Normal
	Heart	Normal	Normal
	Kidney	Normal	Normal
	Testis	Normal	
	Uterus		Normal
Medium dose (2×10^6 cells)	Brain	Normal	Normal
	Spleen	Normal	Normal
	Lung	Focal emphysematous changes with interstitial thickening and congestion	Focal emphysematous changes with interstitial thickening and congestion
	Liver	Mild feathery degeneration	Normal
	Heart	Normal	Normal
	Kidney	Focal degenerative changes of the tubules	Focal mild nephrotoxicity
	Testis	Normal	
	Uterus		Normal
High dose (3×10^6 cells)	Brain	Normal	Normal
	Spleen	Normal	Normal
	Lung	Mild interstitial thickening	Florid emphysematous changes with interstitial thickening and congestion
	Liver	Normal	Normal
	Heart	Normal	Normal
	Kidney	Focal degenerative changes of tubules (mild toxic effect)	Mild focal degenerative changes tubules (mild toxic changes)
	Testis	Normal	
	Uterus		Normal

TABLE 2. Biopsy results summary of SD rat's organ administered with WJSC secretomes

Group	Organs	Interpretation	
		Male	Female
Control	Brain	Normal	Normal
	Spleen	Normal	Normal
	Lung	Normal	Normal
	Liver	Normal	Normal
	Heart	Normal	Normal
	Kidney	Normal	Normal
	Testis	Normal	
	Uterus		Normal
0.5 mL WJSC secretome	Brain	Normal	Normal
	Spleen	Normal	Normal
	Lung	Normal	Mild pneumocytic hyperplasia
	Liver	Normal	Normal
	Heart	Normal	Normal
	Kidney	Normal	Normal
	Testis	Normal	
	Uterus		Normal
1.0 mL WJSC secretome	Brain	Normal	Normal
	Spleen	Normal	Normal
	Lung	Hyperplastic pneumocytes	Marked pneumocytic hyperplasia
	Liver	Normal	Normal
	Heart	Normal	Normal
	Kidney	Mild hydropic degeneration of renal tubules	Normal
	Testis	Normal	
	Uterus		Eosinophilic infiltration in the endometrium
2.0 mL WJSC secretome	Brain	Normal	Normal
	Spleen	Normal	Normal
	Lung	Marked pneumocytic hyperplasia	Mild pneumocytic hyperplasia with bronchopneumonia
	Liver	Normal	Normal
	Heart	Normal	Normal
	Kidney	Normal	Normal
	Testis	Normal	
	Uterus		Normal

In this study, Wharton's Jelly derived mesenchymal stem cells were successfully isolated from an umbilical cord and cultured up to third subculture. The homogenous monolayer of WJSC attached to the plastic flask was able to proliferate and achieve 80-90% confluency in 7-9 days. Under an inverted microscopic view, the WJSC displayed a typical fibroblast-like spindle-shaped morphology. A similar observation of spindle-shaped fibroblast cells isolated from WJSC was observed to develop and proliferate from small spindle-shaped fibroblasts to elaborated shapes in later subcultures (Ranjbaran et al. 2018). Furthermore, it has been proven that WJSC did not show any sign of senescence for 9 passages (Jo et al. 2008). On the other hand, WJSC cultured for secretomes collections also exhibits fibroblast spindle-shaped morphology despite being cultured in high glucose media. Research has verified that high glucose concentrations in culture conditions for cells do not impact their ability to proliferate and differentiate (Al-Qarakhli et al. 2019; Weil et al. 2009). Besides that, based on the analysed marker expression WJSC shows high expression of stromal specific markers and low expression of endothelial and hematopoietic markers which indicates pure characteristics of the MSC population as per ISCT guidelines (Dominici et al. 2006).

The MTD of WJSC to be used in this investigation was examined by the data obtained from the acute toxicity study. From the test analysis, the maximum dose for the subsequent subchronic study has been designed to be 18×10^6 cells/kg BW and 2 mL of secretomes per rat. This dose was pretty similar to the dosage of 10×10^6 cells/kg BW that has been studied in Swiss albino mice and it has been estimated that the acute IV toxicity of WJSC can exceed 10 million cells per BW (Kannaiyan et al. 2017). Apart from that, repeated intravenous infusions of umbilical cord mesenchymal stem cells (mUC-MSCs) in Cynomolgus monkeys have been observed to provide a similar dosage of 10×10^6 cells/kg (He et al. 2017).

A body weight assessment is one of the physical observations that indicate the adverse effects of any treatment. The growth hormone cycle will be disrupted by undesirable foreign particles in the body which will obliquely cause an aberrant growth pattern (Chan et al. 2022). As a result, the study's findings support the theory that repeated IV injections of WJSC did not impact their body and organ weight as it does not affect the rat's daily diet and initiates physical systemic toxicity. A similar occurrence of insignificant effects of intravenous MSC therapy on the growth progression of rodent models has also been reported in a few studies related to MSC safety. The studies have established comparable growth rates between control and experiment groups which describe the feeding behaviour, body weight and mortality rate of the animals (Aithal, Bairy & Seetharam 2017; Chan et al. 2022).

Overall, the investigation showed no appreciable variations between the control and experimental groups of biochemical and haematological analyses for either male or female rats. Thus, it could be concluded that repeated IV administration of WJSC and secretomes did not alter the immune related biochemical and haematological parameters of cell and secretomes treated rats measured on days 2, 23 and 42. Comparable research by He et al. (2017) and Wang et al. (2012) has demonstrated that repeated IV administration of umbilical cord derived MSC in cynomolgus monkeys does not exhibit any MSC-related toxicity. Similarly, WJ-MSC infusion in SD rats did not significantly affect the complete blood count, serum biochemical analysis and important functions like oxygen-carrying capacity, immunoreactivity or clot function according (Chan et al. 2022). In comparison to the control group neither male nor female rats showed any signs of aberrant histological organ biopsies. It only exhibited mild thickening, congestion, and degeneration of organs such as the lung, kidney, and liver. In one of the studies, it has been demonstrated that IV administration of human UC-MSCs could migrate into organs such as the brain, heart, lung, liver, quadriceps, and iliac marrow of cynomolgus monkeys (Wang et al. 2012). Thus, it is possible to conclude that the repeated IV injection of WJSC in this investigation moved towards the organs of the SD rats resulting in modest pathological alterations to some of those organs. However, the outcomes of the secretomes study could not be supported by any other studies as references since there were limited safety studies conducted on MSC secretomes. Thus, the findings achieved from this study could be used as potential references for future preclinical studies performed on MSC secretomes.

CONCLUSIONS

This study assessed the safety and efficacy of WJSC and WJSC secretomes in SD rats to evaluate their toxicity level. The isolated MSC from WJ exhibited fibroblast-like cell morphology displaying all MSC properties such as plastic adherence and expressed MSC phenotype markers. These cells were used to conduct a comprehensive preclinical safety study to evaluate the safety and tolerability of WJSC and secretomes. The MTD of cells was calculated to be 18×10^6 cells/kg body weight (BW), and for secretomes, it was 2 mL/per rat. Multiple IV injections of WJSC were proven safe and did not cause systemic toxic effects or severe adverse effects in animal models during the observation period. This data significantly contributes to unmet medical needs and could be a promising regenerative therapeutic for treating various incurable diseases.

PROSPECTIVE FOR FUTURE WORK

To enhance the reliability and applicability of future stem cell safety studies, several improvements can be made in the study design, observation duration and technological approaches. First, refining experimental design is critical. Increasing sample sizes and incorporating diverse populations with varied genetic, age and health profiles can improve statistical power and generalizability. Extending

the duration of observation is another vital consideration. Long-term monitoring can capture delayed effects, such as tumorigenicity or chronic immune responses, while periodic assessments at different intervals provide insights into both acute and chronic outcomes. Other than that, quantification of secretomes using a liquid chromatographic mass spectrometer (LCMS) or ProcartaPlex Analyst using ready to use Human Cytokine/Chemokine/Growth Factor Panel Elisa kit could be considered to quantify the content of secretomes secreted by WJSC. This will facilitate to develop the perceptive of the secreted factor profile and provide information about its function and regulation for clinical use. By addressing these areas, future studies can overcome current challenges, yielding more robust and actionable insights for the safe and effective use of stem cell therapies.

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