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Original Article

Safety study of allogeneic mesenchymal stem cell therapy in animal model

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ABSTRACT

Intravenous (IV) infusion of mesenchymal stem cells (MSCs) from nascent tissues like Wharton's Jelly of the umbilical cord is reported to offer therapeutic effects against chronic diseases. However, toxicological data essential for the clinical application of these cells are limited. Thus, this study aimed to determine the safety of IV infusion of Wharton's Jelly derived MSCs (WJ-MSCs) in rats. Fifteen male Sprague–Dawley rats were randomised into the control or treatment group. Each group received an equal volume of saline or WJ-MSC (10×10^6 cell/kg) respectively. The animals were evaluated for physical, biochemical and haematological changes at Week 0, 2, 4, 8 and 12 during the 12-week study. Acute toxicity was performed during Week 2 and sub-chronic toxicity during Week 12. At the end of the study, the relative weight of organs was calculated and histology was performed for lung, liver, spleen and kidney. The findings from physical, serum biochemistry and complete blood count demonstrated no statistically significant differences between groups. However, pathological evaluation reported minor inflammation in the lungs for all groups, but visible healing and resolution of inflammation were observed in the treatment group only. Additionally, the histological images of the treatment group had significantly improved pulmonary structures compared to the control group. In summary, the IV administration of WJ-MSC was safe in the rats. Further studies are needed to determine the long-term safety of the WJ-MSC in both healthy and diseased animal models.

1. Introduction

Mesenchymal stem cells (MSCs) have been investigated extensively for their roles in tissue regeneration and response to inflammation since the 'stemness' of MSC was comparable to human embryonic stem cells (ESCs) [1]. Standardized by the International Society for Cellular Therapy (ISCT), these cells are identified from their expression of positive markers, CD73, CD90 and CD105, and negative markers, CD11b or CD14, CD19 or CD79a, CD34, CD45 and HLA-DR [2]. The cells must also be able to differentiate into the triad of cell lineages, i.e., osteocyte, chondrocyte and adipocyte [3]. MSC naturally select its differentiation pattern in response to direct cell-to-cell interaction or paracrine signaling from adjacent cells. They also secrete functional metabolites that participate in tissue regeneration, angiogenesis, immunoregulation, anti-fibrotic and programmed cell death. Several tissues were found to have a larger

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constitution of these cells, namely, the bone marrow, adipose tissue and umbilical cord [4–6].

MSC has already been credited for treating many chronic diseases, such as stroke, myocardial infarction, pulmonary artery hypertension, spinal cord injury and diabetes [4,7–9]. MSC infusion corrected hyperglycemia and hypoinsulinemia in the diabetic rodent model by differentiating into islet beta cells, subsequently improved insulin secretion [7]. Safety and functionality of WJ-MSC using bacterial sepsis mice model showed strong clearance of bacteria and a lower mortality rate compared to untreated mice [10]. In addition, MSC therapy has also been used on neonatal diseases, such as necrotizing enterocolitis, intraventricular hemorrhage and bronchopulmonary dysplasia [4]. Clinical trials using allogeneic MSCs showed equally restoration of impaired cardiac and pulmonary functions occurred with a low risk of arrhythmia [8,9]. Despite the increase in MSC applications, the safety of cell therapy remains debatable due to infrequent safety analysis and the lack of comprehensive tests.

One of the safety challenges is the stability of MSCs in large-scale production to fulfil clinical demand [5,11]. In 2009, Pal et al. [12] performed upscaling of MSC production in different culture mediums up to 25 passages and found minute variations during characterisation, population doubling time and differentiation potential at every 5th passage. Another issue of standardisation is the variations in MSCs from different sources. For example, the WJ-MSC was found to have a unique affinity for age-independent proliferation compared to BM-MSC [13,14]. The long-term storage of these cells through biobanking had minimal to no significant changes in secretion of growth factors, differentiation potential and surface markers after revival [15,16]. Cooper and Viswanathan (2011) reported that the revived Wharton's Jelly UC-MSC remained viable even after cryopreservation for 3 years [17]. All the MSC from five cords sustained cell viability above 95% from the initial culture and expressed markers of CD73 and CD105 consistently at 98%. In their clinical trial, the UC-MSC infused was safe and had corrected the perfusion defects from patients undergoing coronary bypass surgery [18].

Despite decades establishing the low immunogenicity and malignancy of MSC-based treatment, cases of adverse reaction have been reported [11,19–21]. MSCs do not express MHC-I surface protein [22] which allows the cells to safely migrate to the site of inflammation. However, the subsequent differentiation and the expression of MHC-I and MHC-II surface protein could lead to the possible eradication of these cells [21]. Additionally, MSCs share similar hallmarks and behaviors with cancer cells [1,15]. Both cells express prolonged lifespan, high proliferative capacity and resistance to apoptosis. These overlapping bio-characteristics are regulated by the same machinery, prompting concerns for cellular instability [23,24]. Rosland et al., 2009 [25] shared from their ex-vivo expansion of human BM-MSCs were prone to malignant transformation and had found that approximately 45.8% were affected during a study period of 106 weeks. Although this spontaneous transformation was caused by cross-contamination, the susceptibility of MSCs demands for a more stringent cultivation method. For example, incorporation of cell validation techniques would help to eliminate or minimize the tumorigenic and cellular impurities from the undifferentiated MSCs [26–28].

The objective of this study was to determine the safety of WJ-MSC administered through IV injection in rats. We hypothesise that the slow infusion of WJ-MSC by systemic route will have no significant impact on the physical, biochemical, haematology and histopathology of the animal model. Through this study, we verify the safety of this cell therapy to allow further progress into the potential treatment of chronic diseases. Eventually, this cell therapy is intended to serve as an alternative or substitution of traditional medicine, which relies on chemical drugs that carry known side effects.

2. Materials and methods

2.1. Research and animal ethics

The study protocol was approved by the Universiti Kebangsaan Malaysia Research Ethics Committee (JEP-2020-790) and the Animal Ethics Committee of the Faculty of Medicine, Universiti Kebangsaan Malaysia (TEC/FP/2020/YOGESWARAN/23-SEPT./1124-OCT.-2020-SEPT-2023).

2.2. Animals

Fifteen male Sprague Dawley rats at 12 weeks old weighing ~300 g were obtained from iPharm, National Institute of Biotechnology Malaysia (Penang, Malaysia). Rats were housed individually in ventilated polycarbonate cages (Allentown Inc., United States) at room temperature of 22 °C with a 12-h light 12-h dark cycle and acclimatised for two weeks prior to the experiment. The animals were fed with standard lab chow (Altromin 1314) (Lage, Germany) and autoclaved tap water *ad libitum*.

2.3. Animal treatment

Animals were randomly divided into two groups. The control group (n = 6) received saline and the treatment group (n = 9) received a high dose WJ-MSC (10×10^6 cells/kg body weight) suspended in saline (0.5 mL) via IV injection of the lateral tail vein once at the beginning of the study. Anaesthesia was performed via intraperitoneal (i.p.) injection of ketamine-xylazine cocktail (ratio 1:10) at 1 mL/kg (Troy Laboratories, Australia). The animals were observed and evaluated for a study period of 12 weeks. During the study period, physical measurement was recorded with minimal restrain. The fasting blood samples were collected from the periorbital sinus under anaesthesia (K-X cocktail, 1 mL/kg, i.p. route). The blood was analysed to determine complete blood count and serum biochemistry. These parameters were recorded at Week 0, 2, 4, 8 and 12. At Week 2, three animals were randomly selected from the treatment group to determine the acute toxicity of WJ-MSC infusion. Animals were sacrificed by pentobarbital sodium (Vetoquinol, France) overdose via i.p. route. Necropsy and harvest of organs were performed for each animal. The remaining animals were evaluated for sub-chronic toxicity at the end of 12 weeks.

2.4. Wharton's Jelly MSC protocol

The human umbilical cord-derived Wharton's Jelly mesenchymal stem cells (WJ-MSCs) Passage 5 was used for this study under the approval of Human Primary Cell Banking (UKM 1.5.3.5/244/FF-2015-376), Centre for Tissue Engineering and Regenerative Medicine (CTERM), Universiti Kebangsaan Malaysia Medical Centre. The isolation, characterisation and culture of WJ-MSCs were established is previous studies [29]. A general instruction and results for the WJ-MSC used in this study is provided below while preparation of the manuscript is in progress.

2.4.1. Isolation and culture of WJ-MSC

The umbilical cord was procured with consent from maternal volunteers via elective caesarean section at Universiti Kebangsaan Malaysia Medical Centre. After collection, the umbilical cord was transferred in sterile conditions to the laboratory. The local arteries and veins were carefully excised and the remaining white parenchyma was shredded into thin strips (0.5–1.0 mm^2). The tissue was

digested in 0.6% Collagenase Type 1 (Sigma–Aldrich, Germany) and incubated in a 37 °C shaker incubator for 1–2 h. Complete medium of Dulbecco's Modified Eagle Medium-Low Glucose (Gibco, Germany) and 10% human platelet lysate (HPL) (prepared in-house) were added in equal volume to neutralise the collagenase activity. HPL was prepared using expired platelet concentrates as described previously [30–32]. Following centrifugation at 5000 rpm for 5 min, the supernatant was discarded and resuspended in a complete medium. The cells were seeded at an initial density of 3000 cells/cm^2 and maintained in a 37 °C incubator with 5% CO_2. The media change was performed after the first 24 h and thereafter, every three days. At 90% confluency, the cells were harvested by 0.05% Trypsin-Ethylene Diamine Tetra Acetic acid (Gibco, Germany) and subcultured. By passage 5, the cells were enumerated and cryopreserved at 2 million cells per cryovial.

2.4.2. Characterisation of WJ-MSC

The WJ-MSCs were identified by high expression of positive markers (CD44, CD73, CD90 and CD105) and low expression of negative markers (CD11b, CD19, CD34, CD45 and HLA-DR) using Human MSC Analysis Kit (BD Biosciences, United States) following the manufacturer's protocols. Trilineage differentiation of WJ-MSC for osteogenesis, adipogenesis and chondrogenesis was confirmed using StemPro™ [Osteogenesis/Chondrogenesis/Adipogenesis] Differentiation Kit (Gibco, Germany) following manufacturer's protocol. The WJ-MSCs achieved a >98% viability rate and a population doubling time of approximately 24 h. Furthermore, the immunosuppression assay conducted had shown the suppression of the co-cultured PBMCs by the WJ-MSC [33]. The tumorigenic potential was determined by cell cycle assay via BD Cycletest Plus DNA Kit (BD Biosciences, United States) following manufacturers protocol and tested in-vivo via subcutaneous injection in mice. There were no significant tumorigenicity reported.

2.4.3. Preparation of cell suspensions for transplantation

The WJ-MSC was thawed from cryo-storage and added with a complete medium. WJ-MSC was allowed to adhere to flasks for 24 h prior to infusion. The cells were enumerated and suspended in 0.5 mL of 0.9% saline (Ain Medicare Sdn. Bhd., Malaysia) at a concentration of 10×10^6 cells/kg body weight for IV injection. The WJ-MSC dosage was estimated using the human equivalence dose, HED (mg/kg) = Animal NOAEL (mg/kg) x $(\text{weight}_{\text{animal}}\ [\text{kg}]/(\text{weight}_{\text{human}}\ [\text{kg}])^{(1-0.67)}$ adopted from Nair and Jacob (2016) [34]. The HED was selected from a clinical trial at 1×10^8 total cells [35] and the translated dose (10×10^6 cells/kg body weight in rodents) in preclinical studies [36,37] that were effective as treatment.

2.5. Observed parameters for animal study

2.5.1. Physical observation

The animals were restrained and measured weekly for body weight, body length (nose to anus) and abdominal circumference. Body mass index (BMI) was calculated by the ratio of body weight to body length (squared). Food was measured by weight (g) consumed per week. Water intake was calculated as the volume (mL) consumed per week from the weight (g) change of the bottled water.

2.5.2. Blood analysis

Rodents were bled at periods of Week 0, 2, 4, 8 and 12 from the tail vein under anaesthesia using K-X cocktail via i.p. route. Blood was collected in clot-activator tubes (BD Biosciences, United States), allowed to clot at room temperature and centrifuged at 3000 rpm for 10 min to obtain serum. Complete blood count and biochemistry analysis were performed at Haematology Laboratory in Veterinary Laboratory Service Unit (VLSU), Universiti Putra Malaysia. The complete blood count consisted of red blood cell (RBC), hemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), white blood cell (WBC); neutrophil differential count (Band N), neutrophil count (NEUTRO), lymphocyte count (LYMPH), monocyte count (MONO), eosinophil count (EOSIN), basophil count (BASO) and platelet count (PLT). The blood biochemistry test consisted of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), cholesterol (CHOL), amylase (AMY), creatinine (CREAT) and lactate acid dehydrogenase (LDH).

2.5.3. Necropsy

The major metabolic organs harvested were the lungs, liver, spleen and kidneys. Other organs, such as the heart, gut, bone (femur), and muscle (biceps femoris), were included to identify any adverse effects previously unreported for the WJ-MSC therapy. The pathological evaluation was conducted and the relative weight of organs was calculated as the percentage (% of body weight) by dividing organ weight with body weight and multiplied by 100.

2.5.4. Histological analysis

The organs were preserved in 4% paraformaldehyde solution before being embedded in paraffin. Sections were cut with a microtome, deparaffined with xylene and stained with haematoxylin and eosin staining. The stained section was observed under a light microscope to check for pathological changes by a blinded histopathologist. The pathological conditions were scored as healthy, minor, moderate or severely affected.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.4.3 (GraphPad Software, California, USA). All quantitative variables were presented as mean ± standard error mean (SEM). Comparisons between treatment and control groups for physical measurement, serum biochemistry and whole blood profile test were conducted through mixed-effects repeated measures Analysis of Variance (ANOVA) with Geisser-Greenhouse correction. The time-point pairwise intergroup analysis was calculated using Sidak's post-hoc test, while the intra-group analysis used Tukey's post-hoc test. The multiple comparisons were indicated by the least significant difference when there was a significant difference between both groups. The end-point comparison (i.e., the relative weight of organs) was calculated using independent t-test. A difference at $p < 0.05$ was considered statistically significant.

3. Results

3.1. WJ-MSCs do not exert adverse effects in rats

Body weight, body length, abdominal circumference, food intake, water intake and BMI levels were not statistically significantly different between the control and treatment groups throughout the study in Fig. 1 (A). The body weight and body length of the control group increased ($p < 0.05$) beginning from Week 8 and Week 4, respectively, compared to the baseline value. The treatment group experienced increased body length from Week 8–12 ($p < 0.05$). The resulting value of BMI decreased significantly in both groups during Week 8 and 12 ($p < 0.05$). Food intake showed a trend of reduction throughout the study, but it was only statistically significant during Week 8 and 12 in both groups ($p < 0.05$). Conversely, water intake had a decreasing trend ($p < 0.05$) from Week 2 and remained constant until the end of the study.

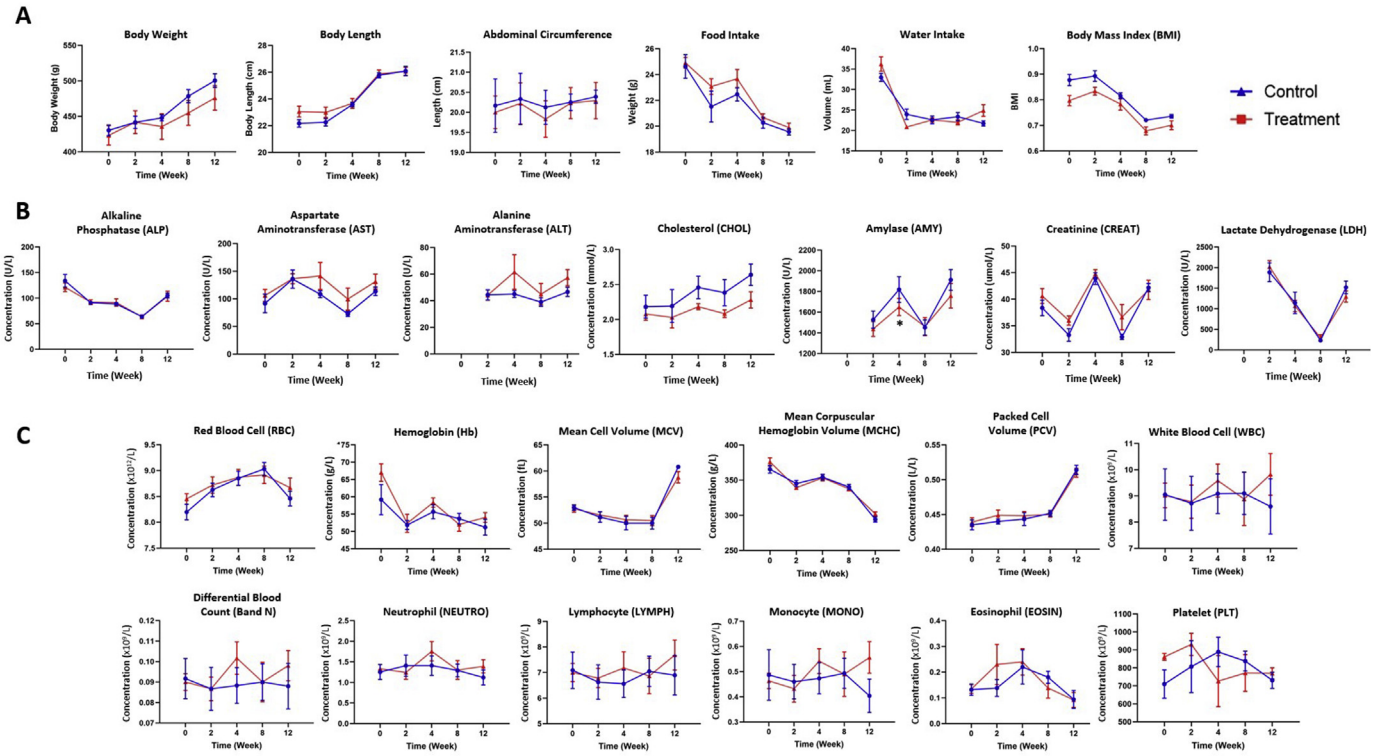

Fig. 1. (from left to right) [A] Physical measurements of Body Weight, Body Length, Abdominal Circumference, Food Intake, Water Intake and BMI. [B] Serum biochemistry of ALP, AST, ALT, CHOL, AMY, CREAT and LDH. [C] Whole blood profile of RBC, Hb, MCV, MCHC, PCV, WBC, BAND N, NEUTRO, LYMPH, MONO, EOSIN and PLT. Data was presented as mean ± SEM (n = 6 rodent per group) for the control and treatment group for a study period 0, 2, 4, 8 and 12 weeks. Superscripts indicate significant differences of data: *$p < 0.05$ to earliest or baseline value; # ($p < 0.05$) between groups at the same time period.

Similar to the physical parameters, serum chemistry in Fig. 1 (B) of ALP, AST, ALT, CHOL, AMY, CREAT and LDH did not reveal a statistically significant difference between the control and treatment groups throughout the study ($p < 0.05$. In both groups, ALP decreased significantly ($p < 0.05$) from Week 2 to Week 8 but increased again by Week 12. AMY decreased significantly ($p < 0.05$) for the treatment group at Week 4 but increased significantly ($p < 0.05$) for the control group at Week 12 compared to the baseline. Lastly, LDH was showed a significant decrease ($p < 0.05$) for both groups at Week 4 and 8 followed by a significant increase ($p < 0.05$) at Week 12.

For complete blood count in Fig. 1 (C), RBC, Hb, PCV, MCV, MCHC, WBC, BAND N, NEUTRO, LYMPH, MONO, EOSIN and PLT did not show statistically significant differences between the control and treatment groups. Within-group comparison showed increased MCV ($p < 0.05$) at Week 12; decreased MCHC ($p < 0.05$) at Week 12 and increased PCV ($p < 0.05$) at Week 8 and 12 compared to the previous weeks in the control groups. Meanwhile, the treatment group showed increased RBC ($p < 0.05$) at Week 2; decreased Hb ($p < 0.05$) at Week 2 and 8; increased MCV ($p < 0.05$) at Week 12; decreased MCHC ($p < 0.05$) at Week 2, 8 and 12; and increased PCV ($p < 0.05$) at Week 12 compared to the previous week. The BASO count was not plotted because it was below the detection limit.

3.2. The relative weight of organs was unaffected but histopathology of lungs improved after WJ-MSC administration over time

At the end of the study, the relative weight of the harvested organs of the treatment group was not significantly different compared to the control group as shown in Fig. 2 (A). However, the results of the histopathology had stark comparisons compared to the former parameters. In Fig. 2 (B), the bronchiole region in the lungs (left image) for the control group at Week 12 showed ciliated epithelial cell shrinkage, structural loss of pseudostratified epithelial layer and localized hemorrhage. Signs of deterioration were observed in the alveolar region (right image), which had collapsed, forming large masses of tissue. In both regions, a darker contrast of nucleic mass (mononuclear cells) had *en masse* around the bronchiole, invaded the blood vessel endothelium and occupied the alveolar space. By Week 2, the treatment group showed a reduction in mononuclear cell activities, allowing the regeneration of the bronchial structure. Additionally, the alveolar region appeared to be more defined in the treatment group compared to the control. At Week 12, the bronchiole and alveoli of the treatment group were found in a convalescent state as the walls of the bronchiole and blood vessels were denser and the concentration of mononuclear cells in the lungs had significantly reduced. The liver was found unaffected by the treatment of MSCs. All groups maintained their structural integrity with identifiable key components, such as the central vein of the centrilobular structure (left image) and periportal triad (right image). The kidneys appeared to be normal for both groups. The cortical region (left image) and glomerulus (right image) were consistent and identified easily. Lastly, the spleens also appeared normal. The white pulp (left image) maintained an oval-like circumference with a clear depiction of the three layers; germinal, mantle and marginal zones. The splenic follicle (right image) was made of central white pulp with the surrounding parenchyma of the red pulp.

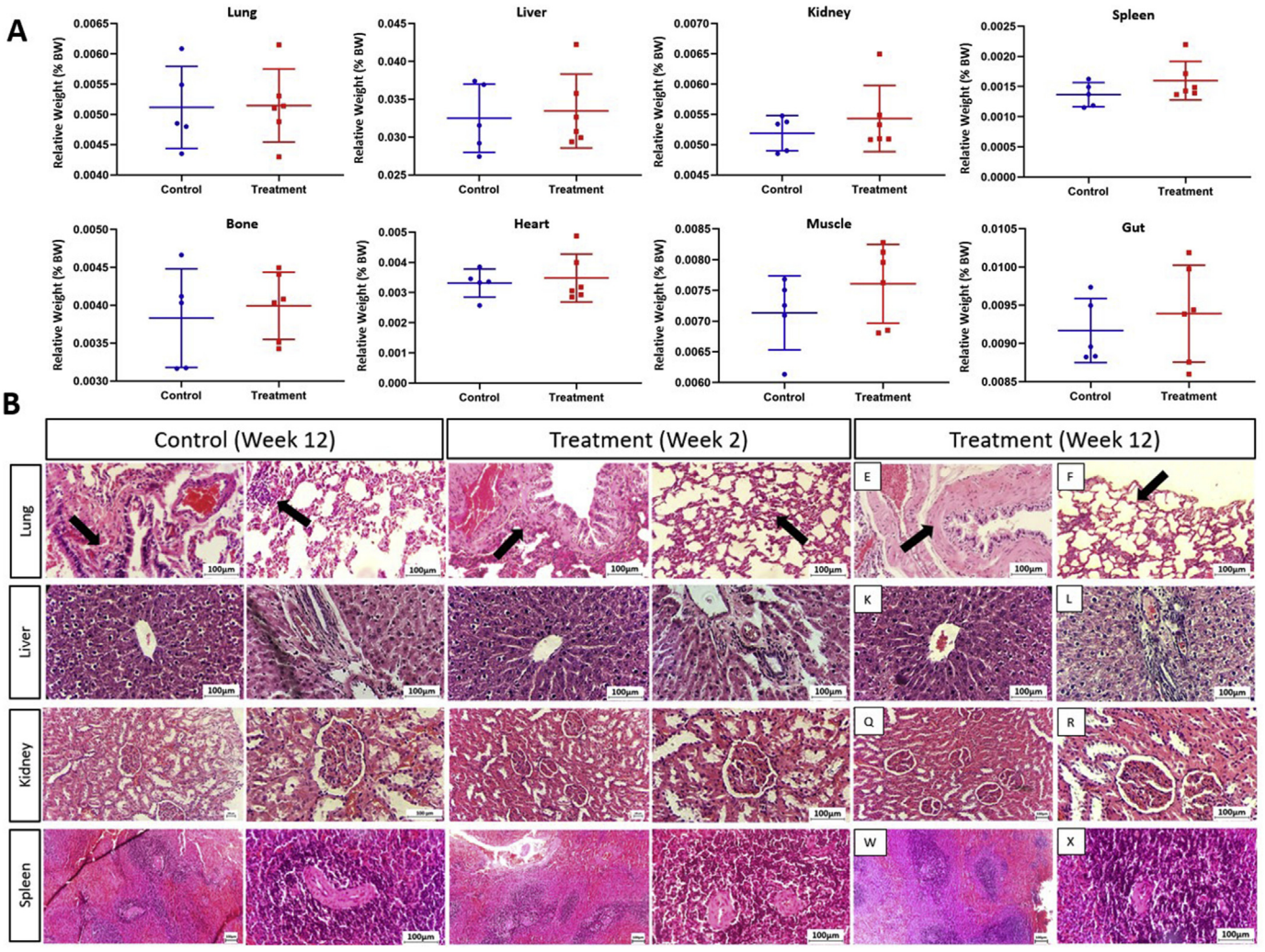

Fig. 2. Post-mortem assessment of control and treatment animals after euthanasia (from left to right). [A] Relative weight of lungs, liver, kidneys, spleen, bone, heart, muscle and gut; Data was presented as mean ± SEM. Superscripts indicate significant differences of data: *p < 0.05 between groups. [B] H&E-stained lung, liver, kidney and spleen for control (Week 12) and treatment group for acute toxicity (Week 2) and sub-chronic toxicity (Week 12). Black arrows indicate the specific region for lung pathological condition. Images were captured at magnification of 10x or 40x with scale bar, 100 μm.

3.3. The mortality and morbidity of IV administration of WJ-MSC in rats were not significant

Generally, the physical and behavioral assessment of rodents did not present any significant outcomes throughout the study as shown in Table 1. However, a single rodent from the control group was euthanised by pentobarbital overdose (i.p. route) per the veterinarian consultation at Week 11. Two days prior, the rat had abnormally halted food and water intake, which prompted further observation. The rat displayed signs of weight loss, hyperventilation, lethargic movement, poor response to stimulus, and arched posterior. Since minor inflammation of the lungs was observed from acute toxicity during Week 2 for all rats (n = 3), this response was taken to avoid the spread of a potential infection. The carcass was preserved in −20 °C freezer for the subsequent necropsy by a veterinarian. Post-mortem evaluation of that individual animal reported severely inflamed lungs, hypertrophied heart, hypertrophied and degenerated liver, enlarged mesenteric lymph nodes and pooled blood in the lower abdomen from suspected hemorrhaged colon and urinary tract. The exact cause of death of the rat could not be determined but was deduced to be unrelated to the treatment given. The remaining rats did not show any signs of morbidity or mortality until the end of the study.

4. Discussion

Wharton's Jelly of the umbilical cord, previously deemed a medical waste of newborn deliveries, is becoming a preferred source of MSCs [38–40]. Since the discovery of WJ-MSC by Friedenstein (1996) [41], repurposing the cord for tissue engineering and regenerative medicine has become a unique research interest. The Malaysia National Healthcare Establishments and Workforce Survey [42] reported a total of 487,957 deliveries within 2019. This supersedes the concern for the scarcity of MSCs and long intervals between available tissue samples, which are a present challenge for bone marrow and adipose tissue. Since many factors influence the efficacy of WJ-MSC therapy, such as the donor's age, family history of diseases, lifestyle choices and more, we conducted a toxicology study of WJ-MSCs following the guidelines of 'The Joint Scientific Committee for International Harmonization of Clinical Pathology Testing [43].

Our study revealed that WJ-MSC did not cause any adverse changes to the physical outcomes of the rats. Body weight and body length were indicators of the growth pattern of the rats. Abnormal growth patterns have been associated with the disruption in the growth hormone cycle and tumorigenicity [11,19,20]. Throughout this study, the growth rate of rats was similar between control and treatment groups, implying negligible effects of WJ-MSC towards

Table 1
Morbidity and Mortality of animals throughout study followed by necropsy for acute and sub-chronic toxicity post-euthanasia.

Symptoms	Study Period						Necropsy		
	Acclimatisation	Week 0	Week 2	Week 4	Week 8	Week 12	Control (Week 12)	Treatment (Week 4)	Treatment (Week 12)
Anorexia, weight loss and/or dehydration (related to food and water intake)	–	–	–	–	Control group only (n = 1)	–	Minor inflammation of the lungs (n = 5) Minor degeneration of the liver (n = 1) Animal euthanised before completion of study (n = 1)	Minor inflammation of the lungs with signs of recovery (n = 3)	Minor inflammation of the lungs with signs of recovery (n = 6)
Dyspnea (labored breathing, hyperventilation, abdominal distension)	–	–	–	–	Control group only (n = 1)	–			
Prolonged hypothermia or hyperthermia (palpable temperature)	–	–	–	–	Control group only (n = 1)	–			
Stress and/or poor grooming (rough or stained coat and porphyrin built around nose and eyes)	–	–	–	–	Control group only (n = 1)	–			
Lethargy, hunched posture and inability to rise or ambulate	–	–	–	–	Control group only (n = 1)	–			
Poor reflex or irresponsiveness to external stimuli	–	–	–	–	Control group only (n = 1)	–			
Tumor growth	–	–	–	–	–	–			

*Symbols: (–) = no symptoms.

growth functions. Our results aligned with the previously established safety of human MSC in rodent models. Aithal et al. (2017) reported no significant effect in their safety assessment of intravenous MSC therapy in Wistar rats [36]. They concluded their results with no alterations in the feeding behaviour of the animals and body weight. However, they noticed the intra-group differences ($p > 0.01$) of the body weight at the end of the study compared to the beginning, which closely matches our findings. Secondly, complete blood count and serum biochemical analysis also did not report significant differences between each group despite the fluctuating results in several tests. Vital functions such as the oxygen-carrying capacity, immunoreactivity and clot functions were unaffected by the infusion of WJ-MSC. Zhao et al. (2019) designed a wide comprehensive array of safety parameters using rodent (mouse, rat), leporid (rabbit), canine (beagles), and rhesus (monkey) models [44]. Their study showed that human gingiva-derived MSC (GMSC) transplantation did not induce any significant outcomes in the biochemistry, hematology and histopathology in the treated animals. Moreover, further studies on two autoimmune disease models reported similar outcomes to the healthy rodents. Thus, ensuring that the intended effects from cell therapy did not elicit an adverse effect due to the unfavorable microenvironment of chronic diseases.

The health status of the rats can be assessed through various signs such as fluctuation in weight loss, hyperventilation, lethargic movement, poor response to stimulus, arched posterior, stress-induced nose bleeds, and more [45–48]. This is a crucial assessment since stem cell therapy has been associated with transient fever, nausea and loss of appetite in humans [49,50]. In a pilot clinical study, Liu et al. (2014) reported that patients receiving intravenous WJ-MSC showed reduced diabetic complications [51]. However, minor incidences of mild to moderate fever recovered spontaneously and nausea with vomiting subsided within the week were reported. In their subsequent clinical trial, similar results were observed without any acute or chronic adverse effects [52]. In the multi-species safety study established by Zhao et al., 2019, they reported no significant effects among the various doses of BM-MSC and in either healthy or diseased animal models [44]. In spite of the conflicting reports, our study found that the wellbeing and mortality of animals were unaffected by the WJ-MSC infusion.

Histological examination of metabolic organs, such as the liver, kidney and spleen, was carried out and did not reveal any significant alterations. While only a handful of studies incorporate the observation of multiple organs, most practice one or two target organs to track the disease progression [53,54]. We performed the necropsy on the major metabolic organs including several accessory organs, followed up with histopathology of the metabolic organs only. Thus, strengthening the safety assessment and ensuring a significant coverage of potential novel effects from the administered WJ-MSC. From our results, we confirmed our hypothesis that the transplanted cells had no effect on the liver, spleen and kidney [44].

Surprisingly, necropsy reported all rodents with signs of minor inflammation and pulmonary blebs in the lungs. Regrettably, we did not incorporate any specific biochemical markers to monitor the minor inflammation upon identifying lung injury in the rats sacrificed during Week 2. Hence, the source for the pulmonary damage could not be determined. The lungs of the control group animals were suspected for the onset of bronchopneumonia [55,56]. Hence, increased activity of pulmonary lymphocytes was suspected to result in the lesion of pulmonary interstitium as well as rupture of local blood vessels. It was worth noting that the treatment group had significant improvements in the lungs as compared to the control. These rats had visible tissue resolution, indicative of the healing process and lesser pulmonary blebs.

However, the inference that WJ-MSC improved the pulmonary conditions was inconclusive as not all rats from the treatment group made a similar recovery.

In spite of that, this unexpected event presented an opportunity to evaluate the potential of WJ-MSC in reversing respiratory damage. The histology of organs was conducted to probe deeper and to visualise the degree of cellular damage and the subsequent recovery from infused WJ-MSC [57]. We report that the treatment group during acute and chronic toxicity had successfully recovered its pulmonary structures compared to the control group. The alveolus was regenerated as pictured by the thin connective tissue of Type 1 pneumocytes. These improvements could be contributed by the immunomodulatory and regenerative effects of WJ-MSC for mild bronchopneumonia. Previous studies on MSCs in murine and leporid models showed significant immunomodulatory effects of the cells by suppressing pro-inflammatory cytokines, marked by a significant decrease of plasma IFN-γ, IL-6, IL-8, IL-17, CRP and TNF-α [56,58–60]. It is speculated that such improvement will be of therapeutic interest in managing acute respiratory syndrome (ARS), a fatal cause amongst severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) patients [61]. The current medication used to treat this disease had often led to irreversible damage to the lungs [62].

There were several limitations while conducting this study. A significant delay occurred between whole blood collection and haematological analysis. Some samples have issues of haemolysis and might affect the reading of serum biochemical analysis. Additionally, the occurrence of the lung infection was neither intentionally induced nor predicted to happen and the exact cause could not be determined. Fortunately, the veterinarian assessment deduced that the pulmonary conditions were minor and unrelated to the treatment, thus it did not impede the progress of the study.

5. Conclusion

In conclusion, administration of WJ-MSC through the IV route was safe, without causing any marked changes in physical, haematological, biochemical and histological changes in the treated rats. The WJ-MSC could improve lung recovery following an inflammatory event of unknown cause, probably through its immunomodulatory and regenerative properties. Further studies should be conducted to assess the health beneficial effects of systemic infusion of WJ-MSCs in appropriate disease models.

Disclosure of potential conflicts of interest

All authors have declared no conflict of interest to report.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author contributions

A.M.H.N., M.H.M.Y., R.H.I., J.X.L., M.D.Y., K-Y.C., and Y.L.: concept and design; S.A.S., R.M.Y., R.A.R., M.A.M.A., B.K., and Y.L.: administrative support; M.N.F.H. and Y.L.: provision of study material; S.N.N. and Y.L.: financial support; A.M.L.C. and R.M.Y.: collection of data; A.M.L.C, A.M.H.N., J.X.L., K-Y.C., and Y.L.: data analysis and interpretation, manuscript writing; ALL AUTHORS.: final approval of manuscript.

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References

[1] Bongso A, Fong CY, Gauthaman K. Taking stem cells to the clinic: major challenges. J Cell Biochem 2008;105(6):1352–60. https://doi.org/10.1002/jcb.21957.
[2] Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8(4):315–7. https://doi.org/10.1080/14653240600855905.
[3] Marino L, Castaldi MA, Rosamilio R, et al. Mesenchymal stem cells from the wharton's jelly of the human umbilical cord: biological properties and therapeutic potential. Int J Stem Cells 2019;12(2):218–26. https://doi.org/10.15283/ijsc18034.
[4] Liau LL, Al-Masawa ME, Koh B, et al. The potential of mesenchymal stromal cell as therapy in neonatal diseases. Front Pediatr 2020;8:591693. https://doi.org/10.3389/fped.2020.591693.
[5] Hassan MNFB, Yazid MD, Yunus MHM, et al. Large-scale expansion of human mesenchymal stem cells. Stem Cell Int 2020;2020:9529465. https://doi.org/10.1155/2020/9529465.
[6] Looi QH, Eng SP, Liau LL, et al. Mesenchymal stem cell therapy for sports injuries – from research to clinical practice. Sains Malays 2020;49(4):825–38. https://doi.org/10.17576/jsm-2020-4904-12.
[7] Gu LH, Zhang TT, Li Y, Yan HJ, Qi H, Li FR. Immunogenicity of allogeneic mesenchymal stem cells transplanted via different routes in diabetic rats. Cell Mol Immunol 2015;12(4):444–55. https://doi.org/10.1038/cmi.2014.70.
[8] Mocini D, Colivicchi F, Santini M. Stem cell therapy for cardiac arrhythmias. Ital Heart J 2005;6(3):267–71.
[9] Almeida SO, Skelton RJ, Adigopula S, Ardehali R. Arrhythmia in stem cell transplantation. Card Electrophysiol Clin 2015;7(2):357–70. https://doi.org/10.1016/j.ccep.2015.03.012.
[10] Laroye C, Boufenzer A, Jolly L, et al. Bone marrow vs Wharton's jelly mesenchymal stem cells in experimental sepsis: a comparative study. Stem Cell Res Ther 2019;10(1):192. https://doi.org/10.1186/s13287-019-1295-9.
[11] Lukomska B, Stanaszek L, Zuba-Surma E, Legosz P, Sarzynska S, Drela K. Challenges and controversies in human mesenchymal stem cell therapy. Stem Cell Int 2019;2019:9628536. https://doi.org/10.1155/2019/9628536.
[12] Pal R, Hanwate M, Jan M, Totey S. Phenotypic and functional comparison of optimum culture conditions for upscaling of bone marrow-derived mesenchymal stem cells. J Tissue Eng Regen Med 2009;3(3):163–74. https://doi.org/10.1002/term.143.
[13] Peng L, Jia Z, Yin X, et al. Comparative analysis of mesenchymal stem cells from bone marrow, cartilage, and adipose tissue. Stem Cell Dev 2008;17(4):761–73. https://doi.org/10.1089/scd.2007.0217.
[14] Hu Y, Liang J, Cui H, et al. Wharton's jelly mesenchymal stem cells differentiate into retinal progenitor cells. Neural Regen Res 2013;8(19):1783–92. https://doi.org/10.3969/j.issn.1673-5374.2013.19.006.
[15] Wang Y, Han ZB, Song YP, Han ZC. Safety of mesenchymal stem cells for clinical application. Stem Cell Int 2012;2012:652034. https://doi.org/10.1155/2012/652034.
[16] Thirumala S, Goebel WS, Woods EJ. Clinical grade adult stem cell banking. Organogenesis 2009;5(3):143–54. https://doi.org/10.4161/org.5.3.9811.
[17] Cooper K, Viswanathan C. Establishment of a mesenchymal stem cell bank. Stem Cell Int 2011;2011:905621. https://doi.org/10.4061/2011/905621.
[18] Viswanathan C, Davidson Y, Cooper K, Tipnis S, Pujari G, Kurian VM. Transplantation of autologous bone marrow derived mesenchymal stem cells transepicardially in patients undergoing coronary bypass surgery. Indian Heart J 2010;62(1):43–8.
[19] Moll G, Rasmusson-Duprez I, von Bahr L, et al. Are therapeutic human mesenchymal stromal cells compatible with human blood? Stem Cell 2012;30(7):1565–74. https://doi.org/10.1002/stem.1111.
[20] Amer MH, Rose FRAJ, Shakesheff KM, Modo M, White LJ. Translational considerations in injectable cell-based therapeutics for neurological applications: concepts, progress and challenges. NPJ Regen Med 2017;2:23. https://doi.org/10.1038/s41536-017-0028-x.
[21] Huang XP, Sun Z, Miyagi Y, et al. Differentiation of allogeneic mesenchymal stem cells induces immunogenicity and limits their long-term benefits for myocardial repair. Circulation 2010;122(23):2419–29. https://doi.org/10.1161/CIRCULATIONAHA.110.955971.
[22] Musiał-Wysocka A, Kot M, Majka M. The pros and cons of mesenchymal stem cell-based therapies. Cell Transplant 2019;28(7):801–12. https://doi.org/10.1177/0963689719837897.

[23] Barkholt L, Flory E, Jekerle V, et al. Risk of tumorigenicity in mesenchymal stromal cell-based therapies–bridging scientific observations and regulatory viewpoints. Cytotherapy 2013;15(7):753–9. https://doi.org/10.1016/j.jcyt.2013.03.005.
[24] Liu G, Zhou H, Li Y, et al. Evaluation of the viability and osteogenic differentiation of cryopreserved human adipose-derived stem cells. Cryobiology 2008;57(1):18–24. https://doi.org/10.1016/j.cryobiol.2008.04.002.
[25] Røsland GV, Svendsen A, Torsvik A, et al. Long-term cultures of bone marrow-derived human mesenchymal stem cells frequently undergo spontaneous malignant transformation. Cancer Res 2009;69(13):5331–9. https://doi.org/10.1158/0008-5472.CAN-08-4630.
[26] Kusakawa S, Yasuda S, Kuroda T, Kawamata S, Sato Y. Ultra-sensitive detection of tumorigenic cellular impurities in human cell-processed therapeutic products by digital analysis of soft agar colony formation. Sci Rep 2015 Dec 8;5:17892. https://doi.org/10.1038/srep17892.
[27] Kusakawa S, Machida K, Yasuda S, Takada N, Kuroda T, Sawada R, et al. Characterization of in vivo tumorigenicity tests using severe immunodeficient NOD/Shi-scid IL2Rγnull mice for detection of tumorigenic cellular impurities in human cell-processed therapeutic products. Regen Ther 2015;1:30–7. https://doi.org/10.1016/j.reth.2014.12.001.
[28] Kono K, Takada N, Yasuda S, Sawada R, Niimi S, Matsuyama A, et al. Characterization of the cell growth analysis for detection of immortal cellular impurities in human mesenchymal stem cells43; 2015. p. 146–9. https://doi.org/10.1016/j.biologicals.2014.11.007 (2).
[29] Lim J, Razi ZR, Law J, Nawi AM, Idrus RB, Ng MH. MSCs can be differentially isolated from maternal, middle and fetal segments of the human umbilical cord. Cytotherapy 2016;18(12):1493–502. https://doi.org/10.1016/j.jcyt.2016.08.003.
[30] Budi Harto PH, Mahmud MH, Othman AH, et al. Human platelet lysate promotes proliferation but fails to maintain chondrogenic markers of chondrocytes. Sains Malays 2019;48(10):2169–76. https://doi.org/10.17576/jsm-2019-4810-12.
[31] Hassan MN, Yap ZY, Tang YL, Ng MH, Law JX. Expired platelet concentrate as a source of human platelet lysate for Xenogeneic-free culture of human dermal fibroblasts. Sains Malays 2021;50(8):2355–65. https://doi.org/10.17576/jsm-2021-5008-18.
[32] Liau LL, Hassan MNFB, Tang YL, Ng MH, Law JX. Feasibility of human platelet lysate as an alternative to foetal bovine serum for in vitro expansion of chondrocytes. Int J Mol Sci 2021;22(3):1269. https://doi.org/10.3390/ijms22031269. Published 2021 Jan 28.
[33] Lim J, Eng SP, Yeoh WY, et al. Immunomodulatory properties of Wharton's jelly-derived mesenchymal stem cells from three anatomical segments of umbilical cord. Sains Malays 2021;50(6):1715–26. https://doi.org/10.17576/jsm-2021-5006-18.
[34] Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm 2016;7(2):27–31. https://doi.org/10.4103/0976-0105.177703.
[35] Florea V, Rieger AC, DiFede DL, et al. Dose comparison study of allogeneic mesenchymal stem cells in patients with ischemic cardiomyopathy (the TRIDENT study). Circ Res 2017;121(11):1279–90. https://doi.org/10.1161/CIRCRESAHA.117.311827.
[36] Aithal AP, Bairy LK, Seetharam RN. Safety assessment of human bone marrow-derived mesenchymal stromal cells transplantation in wistar rats. J Clin Diagn Res 2017;11(9):FF01–3. https://doi.org/10.7860/JCDR/2017/29515.10534.
[37] Idriss NK, Sayyed HG, Osama A, Sabry D. Treatment efficiency of different routes of bone marrow-derived mesenchymal stem cell injection in rat liver fibrosis model. Cell Physiol Biochem 2018;48(5):2161–71. https://doi.org/10.1159/000492558.
[38] Bańkowski E, Sobolewski K, Romanowicz L, Chyczewski L, Jaworski S. Collagen and glycosaminoglycans of Wharton's jelly and their alterations in EPH-gestosis. Eur J Obstet Gynecol Reprod Biol 1996;66(2):109–17. https://doi.org/10.1016/0301-2115(96)02390-1.
[39] Nagamura-Inoue T, He H. Umbilical cord-derived mesenchymal stem cells: their advantages and potential clinical utility. World J Stem Cell 2014;6(2):195–202. https://doi.org/10.4252/wjsc.v6.i2.195.
[40] Liau LL, Ruszymah BHI, Ng MH, Law JX. Characteristics and clinical applications of Wharton's Jelly-derived mesenchymal stromal cells. Curr Res Translat Med 2020;68(1):5–16. https://doi.org/10.1016/j.retram.2019.09.001.
[41] Ala Fridenshteĭn. Stvolovye osteogennye kletki kostnogo mozga [Osteogenic stem cells of the bone marrow]. Ontogenez 1991;22(2):189–97.
[42] Mahidin MU. Vital statistics, Malaysia. 2020 [online] Dosm.gov.my. Available at: https://www.dosm.gov.my/v1/index.php?r=column%2FcthemeByCat&cat=165&bul_id=T3Q0SENtcWxWdTFJa05QcHJiN0t1UT09&menu_id=L0pheU43NWJwRWVSZklWdzQ4TlhUUT09. [Accessed 1 April 2021].
[43] Weingand K, Brown G, Hall R, et al. Harmonization of animal clinical pathology testing in toxicity and safety studies. The Joint scientific committee for international harmonization of clinical Pathology testing. Fund Appl Toxicol 1996;29(2):198–201.
[44] Zhao J, Wang J, Dang J, et al. A preclinical study-systemic evaluation of safety on mesenchymal stem cells derived from human gingiva tissue. Stem Cell Res Ther 2019;10(1):165. https://doi.org/10.1186/s13287-019-1262-5.
[45] Grossberg AJ, Scarlett JM, Marks DL. Hypothalamic mechanisms in cachexia. Physiol Behav 2010;100(5):478–89. https://doi.org/10.1016/j.physbeh.2010.03.011.
[46] Ray MA, Johnston NA, Verhulst S, Trammell RA, Toth LA. Identification of markers for imminent death in mice used in longevity and aging research. J Am Assoc Lab Anim Sci 2010;49(3):282–8.
[47] Morton GJ, Meek TH, Schwartz MW. Neurobiology of food intake in health and disease. Nat Rev Neurosci 2014;15(6):367–78. https://doi.org/10.1038/nrn3745.
[48] Snyder JM, Ward JM, Treuting PM. Cause-of-Death analysis in rodent aging studies. Vet Pathol 2016;53(2):233–43. https://doi.org/10.1177/0300985815610391.
[49] Stiff PJ, Fox-Geiman MP, Kiley K, et al. Prevention of nausea and vomiting associated with stem cell transplant: results of a prospective, randomized trial of aprepitant used with highly emetogenic preparative regimens. Biol Blood Marrow Transplant 2013;19(1):49–55. https://doi.org/10.1016/j.bbmt.2012.07.019. e1.
[50] Truong TH, Moorjani R, Dewey D, Guilcher GM, Prokopishyn NL, Lewis VA. Adverse reactions during stem cell infusion in children treated with autologous and allogeneic stem cell transplantation. Bone Marrow Transplant 2016;51(5):680–6. https://doi.org/10.1038/bmt.2015.331.
[51] Liu X, Zheng P, Wang X, et al. A preliminary evaluation of efficacy and safety of Wharton's jelly mesenchymal stem cell transplantation in patients with type 2 diabetes mellitus. Stem Cell Res Ther 2014;5(2):57. https://doi.org/10.1186/scrt446.
[52] Hu J, Wang Y, Gong H, et al. Long term effect and safety of Wharton's jelly-derived mesenchymal stem cells on type 2 diabetes. Exp Ther Med 2016;12(3):1857–66. https://doi.org/10.3892/etm.2016.3544.
[53] Zhou Y, Hu Q, Chen F, et al. Human umbilical cord matrix-derived stem cells exert trophic effects on β-cell survival in diabetic rats and isolated islets. Dis Model Mech 2015;8(12):1625–33. https://doi.org/10.1242/dmm.021857.
[54] Xiao N, Zhao X, Luo P, et al. Co-transplantation of mesenchymal stromal cells and cord blood cells in treatment of diabetes. Cytotherapy 2013;15(11):1374–84. https://doi.org/10.1016/j.jcyt.2013.06.013.
[55] Terrabuio Junior AA, Parra ER, Farhat C, Capelozzi VL. Autopsy-proven causes of death in lungs of patients immunocompromised by secondary interstitial pneumonia. Clinics 2007;62(1):69–76. https://doi.org/10.1590/s1807-59322007000100011.
[56] Kong D, Liu X, Li X, et al. Mesenchymal stem cells significantly improved treatment effects of Linezolid on severe pneumonia in a rabbit model. Biosci Rep 2019;39(9):BSR20182455. https://doi.org/10.1042/BSR20182455.
[57] Sasseville VG, Mansfield KG, Brees DJ. Safety biomarkers in preclinical development: translational potential. Vet Pathol 2014;51(1):281–91. https://doi.org/10.1177/0300985813505117.
[58] Hackstein H, Lippitsch A, Krug P, et al. Prospectively defined murine mesenchymal stem cells inhibit Klebsiella pneumoniae-induced acute lung injury and improve pneumonia survival. Respir Res 2015;16:123. https://doi.org/10.1186/s12931-015-0288-1.
[59] Liu D, Kong F, Yuan Y, et al. Decorin-modified umbilical cord mesenchymal stem cells (MSCs) attenuate radiation-induced lung injuries via regulating inflammation, fibrotic factors, and immune responses. Int J Radiat Oncol Biol Phys 2018;101(4):945–56. https://doi.org/10.1016/j.ijrobp.2018.04.007.
[60] Huang ZW, Liu N, Li D, et al. Angiopoietin-1 modified human umbilical cord mesenchymal stem cell therapy for endotoxin-induced acute lung injury in rats. Yonsei Med J 2017;58(1):206–16. https://doi.org/10.3349/ymj.2017.58.1.206.
[61] Durand N, Mallea J, Zubair AC. Insights into the use of mesenchymal stem cells in COVID-19 mediated acute respiratory failure. NPJ Regen Med 2020;5(1):17. https://doi.org/10.1038/s41536-020-00105-z.
[62] Harrell CR, Sadikot R, Pascual J, et al. Mesenchymal stem cell-based therapy of inflammatory lung diseases: current understanding and future perspectives. Stem Cell Int 2019;2019:4236973. https://doi.org/10.1155/2019/4236973.