

## Research Article



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# Acute Toxicity Studies on Secretome Derived from Umbilical Cord Blood Dendritic Cells in Rats by Different Dosaged Levels

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## ABSTRACT

Dendritic cells-derived umbilical cord blood monocytes were characterized by surface markers such as CD83, CD86, CD54, CD40, and HLA-DR, which are essential for T-cell activation. These cells produce a secretome that is rich in several types of proteins like cytokines, chemokines, and growth factors, showing potential therapeutic applications, especially in cancer treatment. To assess acute toxicity, a total of 30 female Sprague-Dawley rats (*Rattus norvegicus*) were randomly divided into six different groups (n = 5 rats per group) based on UCB-derived DCs Secretome concentrations (0.015%, 0.15%, 1%, 10%, 100%) and control group administered via intramuscular injection, followed by histopathological analysis of vital organs like liver, heart, spleen, kidney, and lungs was conducted in a 14-day period. The results revealed no mortality, adverse event signs, or significant pathological changes in any organs, indicating that the UCB-DCs secretome did not induce acute toxicity. This research concludes that UCB-derived DCs secretome is non-toxic at the tested dosage levels, affirming its safety for potential clinical use. These findings highlight the promise of UCB-DCs secretome as a therapeutic agent, particularly in oncology, and emphasize the need for further research, including randomized clinical trials, to explore its full potential in clinical settings.



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## 1. Introduction

Dendritic cells (DCs) are professional antigen presenting cells (APCs) that connect the innate immune system to the adaptive immune system which play a crucial role in initiating antigen-specific immune responses. Originating from a bone marrow pre-cursor, DCs act as surveillance cells, circulating in the blood in an immature form and activated upon

encountering tumor associated antigens (TAAs), DCs will move from non-lymphoid tissue to the lymph node to participate in the initiation of immune responses through CD8 T cells and their effector CTLs (cytotoxic T lymphocytes) to eliminate tumor cells Fu and Jiang (2018). There are over 249 clinical trials of autologous DCs for cancer, with only 56 trials bearing results. However, the clinical use of autologous DCs is still limited due to technical challenges in preparing DCs from each patient and the inability for widespread clinical practice Merrick *et al.* (2008). Another source for DCs can be used, such as umbilical cord blood

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(UCB), but it still has limitations during its production and stability throughout storage, hindering its potential in immunotherapy. Therefore, DCs secretomes, which includes cytokines and exosomes, may potentially become a platform for a more efficient production and delivery. These exosomes incorporate targeted peptides with MHC I/II on their surface and present 10 to 100-fold more TAA-MHC II complexes compared to DCs Nikfarjam *et al.* (2020). Unfortunately, there are a limited number of studies that have been done to evaluate the toxicity profiles and efficacy of secretome from UCB-derived DCs. Secretome of UCB-derived DCs holds great promise for therapeutic applications due to its immunomodulatory and anti-inflammatory properties. Determining the acute toxicity of UCB-derived DCs Secretome is essential to establish safe dosage ranges and guide further clinical investigations. This study investigates the acute toxicity of UCB-derived DCs Secretome in rat using different dosage levels according to Indonesian Food and Drug Authority Regulation for Acute Toxicity Testing *In Vivo*.

## 2. Materials and Methods

### 2.1. Ethics and Registrations

This study involving animals (rats) was approved by the Animal Ethics Committee of the School of Veterinary Medicine and Biomedical Science, IPB University, under approval number 156/KEH/SKE/I/2024 (January 10, 2024). All experimental procedures were conducted in accordance with relevant institutional and national ethical guidelines for animal research, and complied with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guideline.

### 2.2. Donor Eligibility

Umbilical cord blood was obtained from a healthy female donor after negative screening for infectious diseases such as HIV, hepatitis B and C viruses, cytomegalovirus, toxoplasma, and syphilis according to established standards. This study was approved by the appropriate Medical Ethical Review Board and written informed consent was obtained from the donor prior to the procedure.

### 2.3. UCB-derived DCs Isolation and Secretome Preparation

UCB-derived DCs were manufactured in a laboratory with Good Manufacturing Practice (GMP) standards. A total of minimum 40 mL of umbilical cord blood was

isolated from mononuclear cells (MNC) using Ficoll and Sepax 2 (Cytiva, USA). Mononuclear cells (MNC) were cultured in basal media for two hours and replaced with differentiation media containing GM-CSF (50 ng/mL) and IL-4 (20 ng/mL) (Miltenyi Biotec, Germany) for five days to obtain immature DCs. These immature DCs were matured with a cytokine cocktail including GM-CSF (50 ng/mL), IL-4 (20 ng/mL), TNF- $\alpha$  (10 ng/mL), IL-1 $\beta$  (10 ng/mL), and CD40-L (10 ng/mL) (Miltenyi Biotec, Germany) for 2 days. On day 7, mature DCs were introduced with EBV-specific antigens for 4-6 hours. UCB-derived DCs were separated from conditioned medium and centrifuged at 2,000 g for 15 minutes to remove cell debris and filtered using 0.22  $\mu$ m to remove bacterial contamination. UCB-derived DCs were tested for surface markers. The conditioned medium which called as secretomes tested for sterility, endotoxin, mycoplasma, cytokine, total protein before it is stored at -80°C for future use.

### 2.4. Quality Control of UCB-derived DCs Secretome

To ensure mature DCs production, cells were detected using antibody CD40, CD83, CD86, CD54, and HLA-DR (Miltenyi Biotec, Germany). The cells were stained with the antibodies mentioned above and incubated at room temperature in the dark for 30 min, washed with buffer, and analyzed by using a flow cytometer BD FACSCanto II (BD Bioscience, USA). The secretome was tested for cytokine expression and concentration using BD FACSCanto II (BD Bioscience, USA). The kit used is MACSplex Cytokine 12 kit (Miltenyi Biotec, USA) to detect IFN- $\alpha$ , IFN- $\gamma$ , IL-6, IL-10, IL-12p70, TNF- $\alpha$ . The work was carried out based on the protocol of the MACSplex Cytokine 12 kit (Miltenyi Biotec, USA). Total protein was calculated using BCA Assay (Abcam, UK) according to manufacturer protocol and read in ELISA reader. Sterility was done by direct inoculation onto TSA and incubated for 14 days at standard temperature according to regulation. Endotoxin and mycoplasma were tested using Pyrosate kit (Associates of Cape Cod Inc, USA) and MycoAlert detection kit (Lonza, Switzerland), respectively.

### 2.5. Animal Study in Rat

The animals were housed individually in cages measuring 625.5 cm<sup>2</sup> in area and 18.7 cm in height, constructed from autoclavable PVC. The cages were

equipped with an air handling unit operating at an air exchange rate of 60 ACH, and temperature and humidity were controlled using an air conditioner to maintain a range of  $22\pm3^{\circ}\text{C}$ . The bedding consisted of dust-free wood shavings, which were replaced every 3 days. Humidity was maintained at 55-68%. The lighting cycle was set to 12 hours of light and 12 hours of darkness, following standard rodent husbandry practices. The animals were fed a standard rodent basal metabolic diet containing 18% crude protein and 5.7% fat, as per the standard rodent feed formula.

A total of 30 female Sprague-Dawley rats (*Rattus norvegicus*) were randomly divided into six different groups (n = 5 rats per group) based on UCB-derived DCs Secretome concentrations (0.015%, 0.15%, 1%, 10%, 100%) and control group according to Indonesian Food and Drug Authority Regulation for Acute Toxicity Testing *In Vivo*. Random numbers were generated using the standard = RAND() function in Microsoft Excel. Healthy adult female Sprague-Dawley rats (*Rattus norvegicus*), weighing 180-220 g and aged 8-10 weeks, were included in the study. Only animal with no visible sign of disease, injury, or abnormal behavior upon physical examination were selected. Rats showing any sign of illness, injury, abnormal body weight (<180 g or >220 g), pregnancy, or lactation during the acclimatization period were excluded from the study. Any animal that developed severe distress or moribund condition during the experimental period was humanely euthanized and excluded from data analysis.

A total of 1-mL UCB-derived DCs Secretome was administered to each group. The test animals were individually observed for the first 24 hours at hours 0, 4, 8, 12, and 24, and then every 24 hours for a 14-day observation period. Observations included daily monitoring of toxic and clinical signs, as well as daily body weight. Daily observations of toxic and clinical signs included changes in skin, fur, eyes, mucous membranes, secretions, excretions, and appetite; changes in gait, abnormal behaviors (such as walking backward), convulsions, tremors, and hypersalivation. At the end of the study, all animals were euthanized by anesthesia with Ketamine 100 mg/kg body weight and Xylazine 3 mg/kg body weight, dissolved in 0.5 mL saline and administered intraperitoneally. After the animals were anesthetized, the thoracic cavity was opened and maximum intracardiac blood was collected until the animals died. Samples collected after the animals died included organ samples for macroscopic and histopathological analysis.

Macroscopic observations of major internal organs such as the liver, kidneys, heart, lungs, and spleen included changes in color, size, and weight, and absolute weight calculations. Monitoring of the health of the test animals was conducted daily until the end of the test. Monitoring was conducted by a designated veterinarian. Health monitoring included body weight, food and water intake, and daily activity. Observations included the skin, fur, eyes, mucous membranes, as well as the respiratory system, autonomic nervous system, central nervous system, somatomotor activity, and behavior. Additionally, observations were made of conditions such as trembling, convulsions, salivation, diarrhea, weakness, sleep, and coma.

Data analysis was performed using the ANOVA method to evaluate differences between the normal control group and the experimental groups for the parameter of relative organ weight.

### 3. Results

#### 3.1. Quality Control of UCB-Derived DCs Secretome

To ensure the safety of UCB-derived DCs secretome, sterility, endotoxin, and mycoplasma were conducted before injection into rat. All parameters are negative and safe to be used. Cell morphology was observed visually using a microscope and the data was presented at 10x magnification. Monocytes were isolated based on the principle of density separation of mononuclear cells, erythrocytes, and plasma. After 2 hours of culturing on the surface, monocytes adhered and formed intact spheres (Figure 1A). After the addition of GM-CSF and IL-4, monocytes differentiated into immature DCs with the ability to uptake pro-inflammatory antigens in their surroundings and process them into surface markers characteristic of mature DCs in T cell activation. Various receptor proteins for T cell activation are shown in the form of dendrites on mature DCs. In DCs from umbilical cord blood, a dendrites shape as a single cells or clusters are observed (Figure 1C).

To activate cytotoxic T cells, DCs express several essential surface markers, including CD83, CD86, CD54, CD40, and HLA-DR (Figure 2A). Our results demonstrate that all of these markers are positively expressed on mature DCs. After confirming the mature DCs, we analyzed the cytokines and total protein in UCB-derived DCs secretome. Our UCB-derived DCs

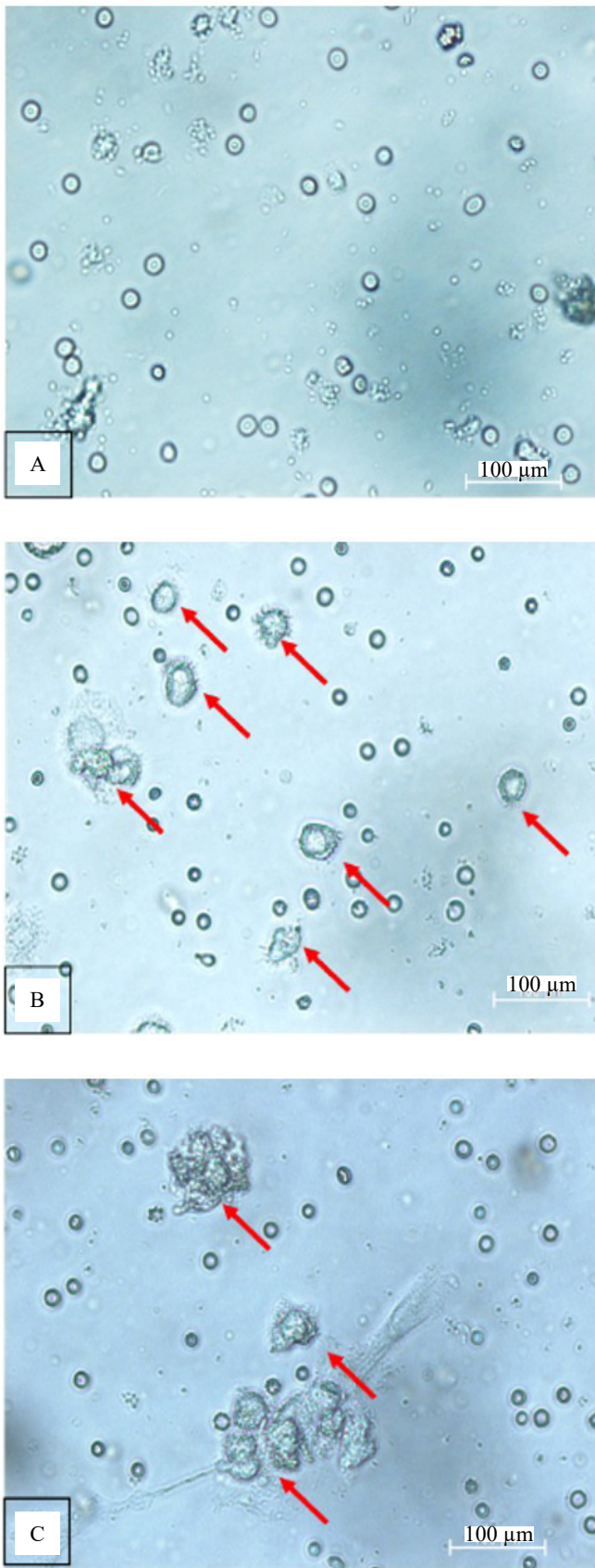


Figure 1. Differentiation of dendritic cells from (A) monocytes, (B) immature dendritic cells, and (C) mature dendritic cells

secretome consists of approximately 205 µg/mL total protein and several key cytokines such as interleukins (IL-6, IL-10, IL-12p70), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IFN- $\alpha$ , and IFN- $\gamma$ , as shown in Figure 2B. DCs secretome contains IL-10 ( $103\pm18$  pg/mL), IL-12p70 ( $299\pm31$  pg/mL), TNF- $\alpha$  ( $199\pm15$  pg/mL), IFN- $\gamma$  ( $204\pm35$  pg/mL), IFN- $\alpha$  ( $61\pm21$  pg/mL), and IL-6 ( $655\pm118$  pg/mL).

3.2. Body Weight Analysis after Secretome Administration

The treated animals were not adversely affected during the entire period of investigation (14 days). Figure 3 illustrates the continuous weight gain of rats injected with 1 mL of UCB-derived DCs secretome at different concentrations until they were euthanized after 14 days of observation.

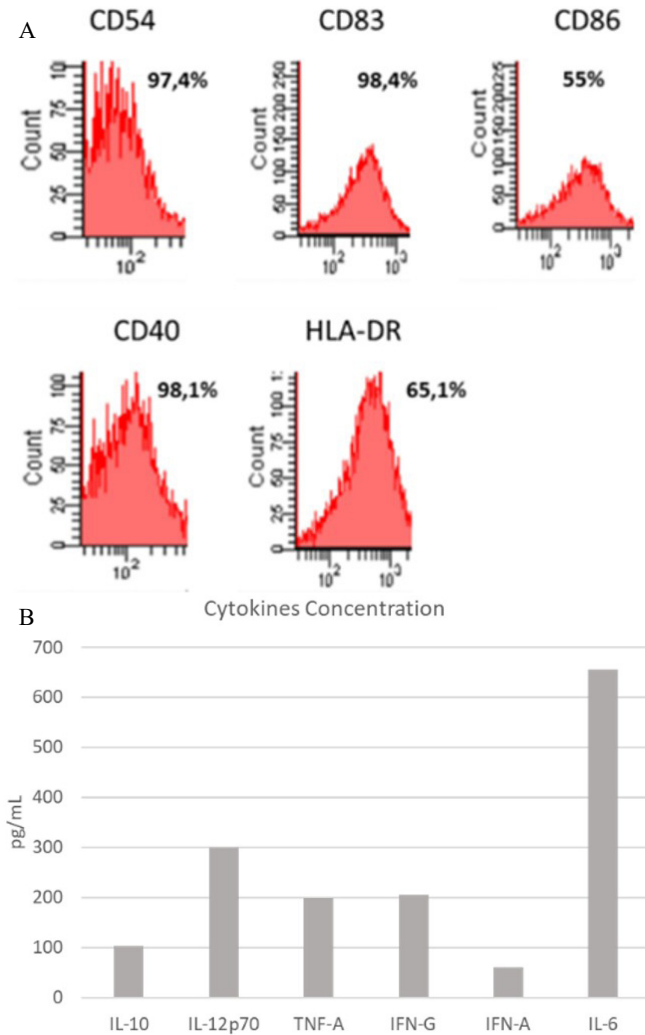


Figure 2. (A) Surface Markers of UCB-derived DCs and (B) cytokine of UCB-derived DCs secretome

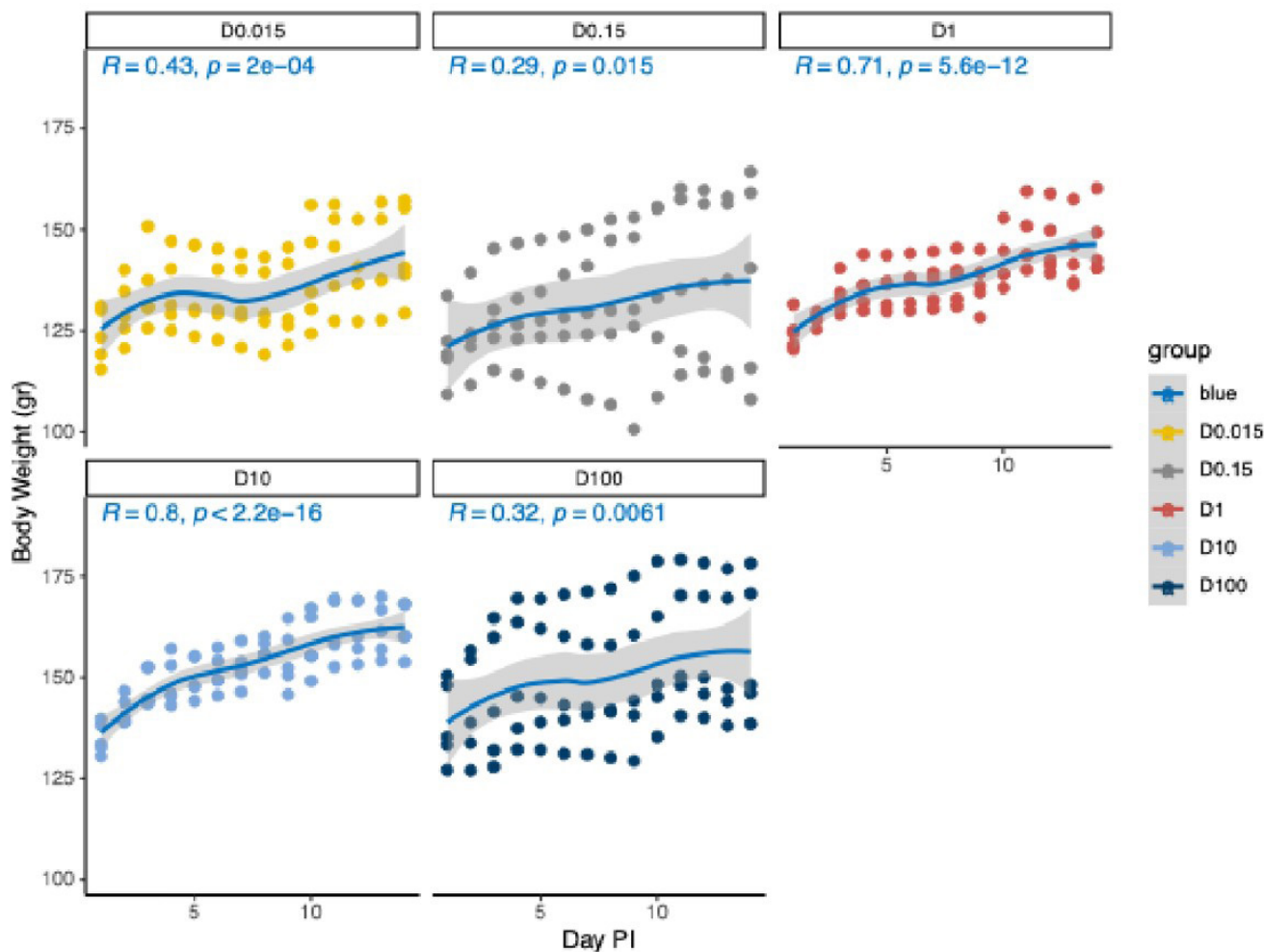


Figure 3. Graphical representation of body weight development of test animals in dendritic secretome groups

3.3. Clinical Signs and Mortality

Observations of toxicity symptoms in the test animals (n = 30) after treatment with UCB-derived DCs secretome did not reveal any clinical signs related to toxicity or mortality during the 14-day observation period as summarized in Table 1.

2.4. Organ Histopathology Observations

Histopathological analysis (Figure 4-8) showed no signs of acute toxicity in the liver, heart, spleen, kidney, or lungs of the rats injected with UCB-derived DCs secretome at various concentrations (n = 30). The rats displayed no significant clinical toxicity or abnormalities in laboratory and histopathological data across all concentrations.

4. Discussion

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that connect the innate immune

Table 1. Summary of mortality in studied groups

| Secreted proteins                   | Absolute mortality (n = 5) | % Mortality |
|-------------------------------------|----------------------------|-------------|
| 1                                   |                            |             |
| (0.015% UCB-derived DCs Secretomes) | 0/5                        | 0           |
| 2                                   |                            |             |
| (0.15% UCB-derived DCs Secretomes)  | 0/5                        | 0           |
| 3                                   |                            |             |
| (1% UCB-derived DCs Secretomes)     | 0/5                        | 0           |
| 4                                   |                            |             |
| (10% UCB-derived DCs Secretomes)    | 0/5                        | 0           |
| 5                                   |                            |             |
| (100% UCB-derived DCs Secretomes)   | 0/5                        | 0           |
| 6                                   |                            |             |
| Control Group                       | 0/5                        | 0           |

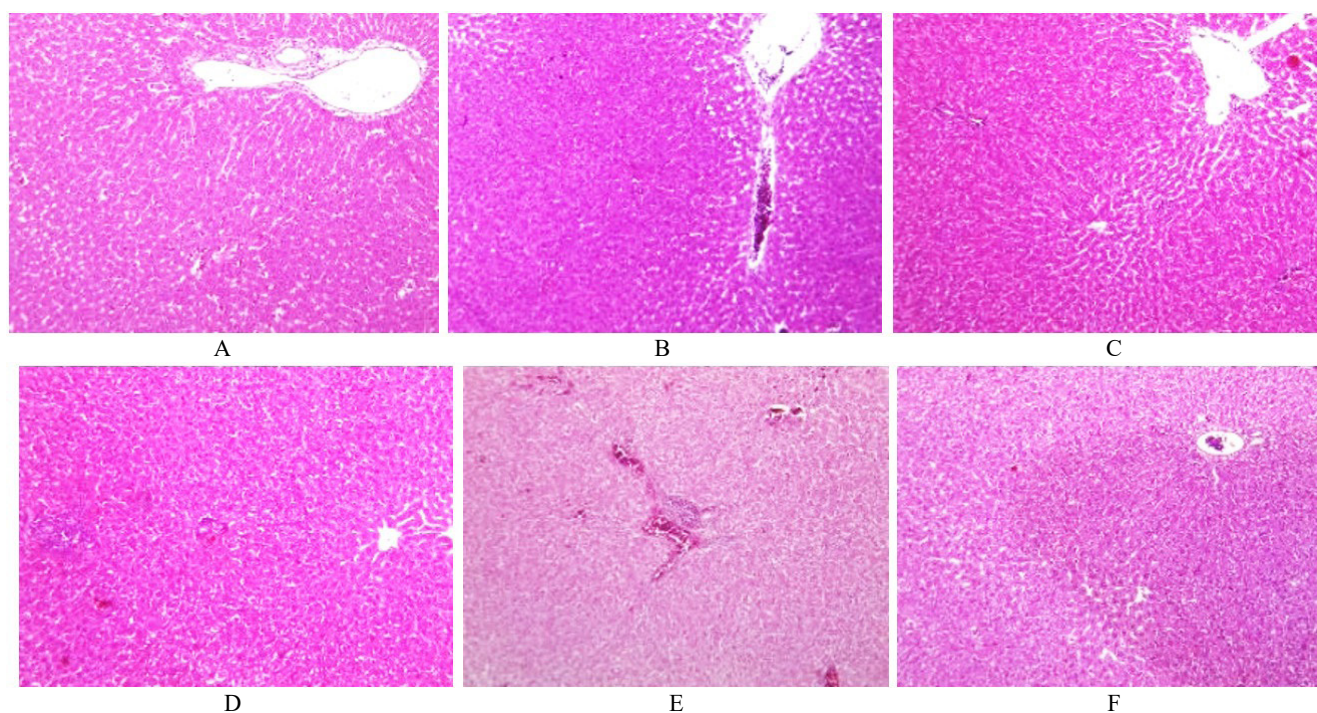


Figure 4. Histopathological examination of the liver following the injection of UCB-derived DCs Secretome in Sprague-Dawley rats to assess the impact of acute toxicity (n = 5 per group). Representative images of the liver section from (A) control group, (B) secretome 0.015%, (C) secretome 0.15%, (D) Secretome 1%, (E) secretome 10%, and (F) secretome 100%

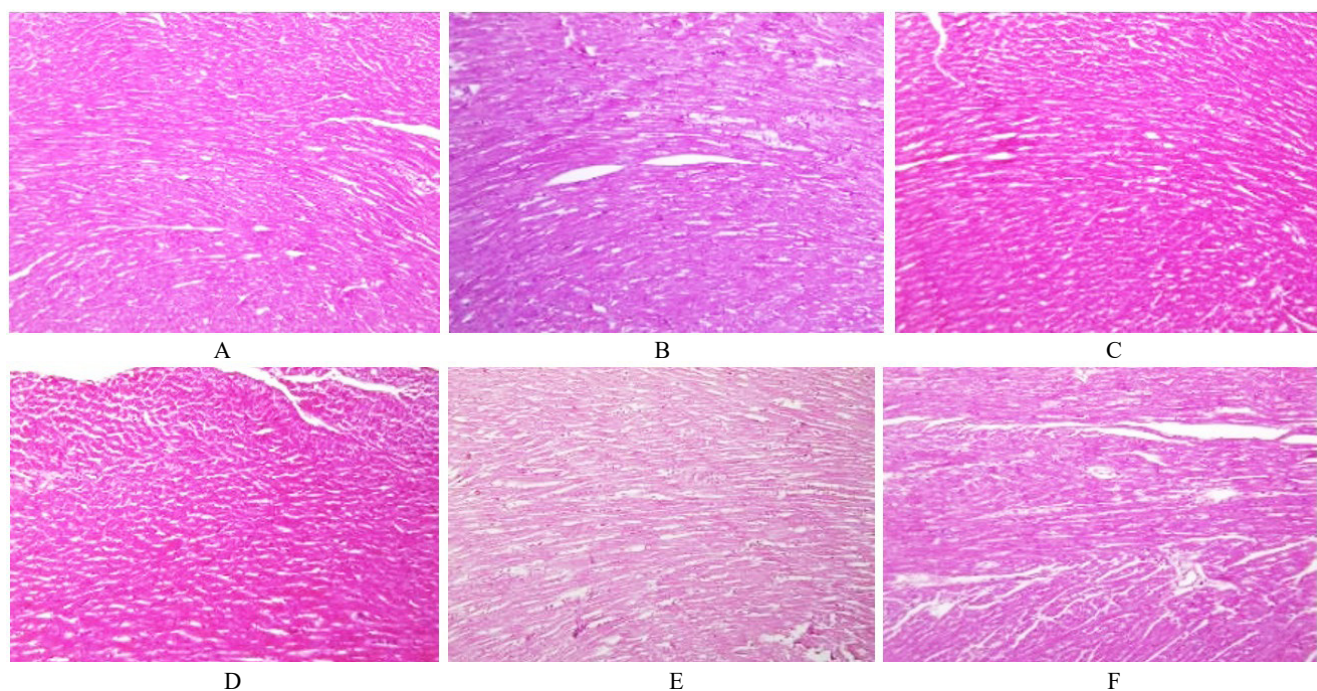


Figure 5. Histopathological examination of the heart following the injection of UCB-derived DCs Secretome in Sprague-Dawley rats to assess the impact of acute toxicity (n = 5 per group). Representative images of the liver section from (A) control group, (B) secretome 0.015%, (C) secretome 0.15%, (D) Secretome 1%, (E) secretome 10%, and (F) secretome 100%

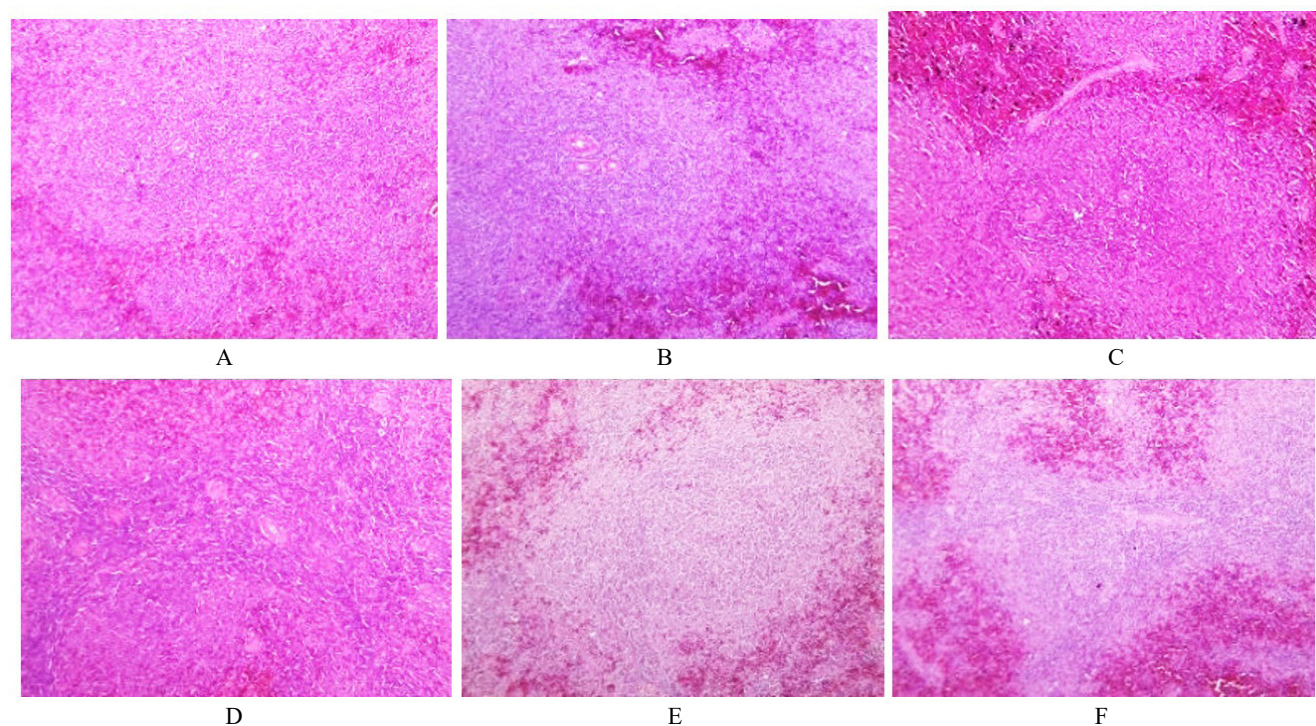


Figure 6. Histopathological examination of the spleen following the injection of UCB-derived DCs Secretome in Sprague-Dawley rats to assess the impact of acute toxicity (n = 5 per group). Representative images of the liver section from (A) control group, (B) secretome 0.015%, (C) secretome 0.15%, (D) Secretome 1%, (E) secretome 10%, and (F) secretome 100%

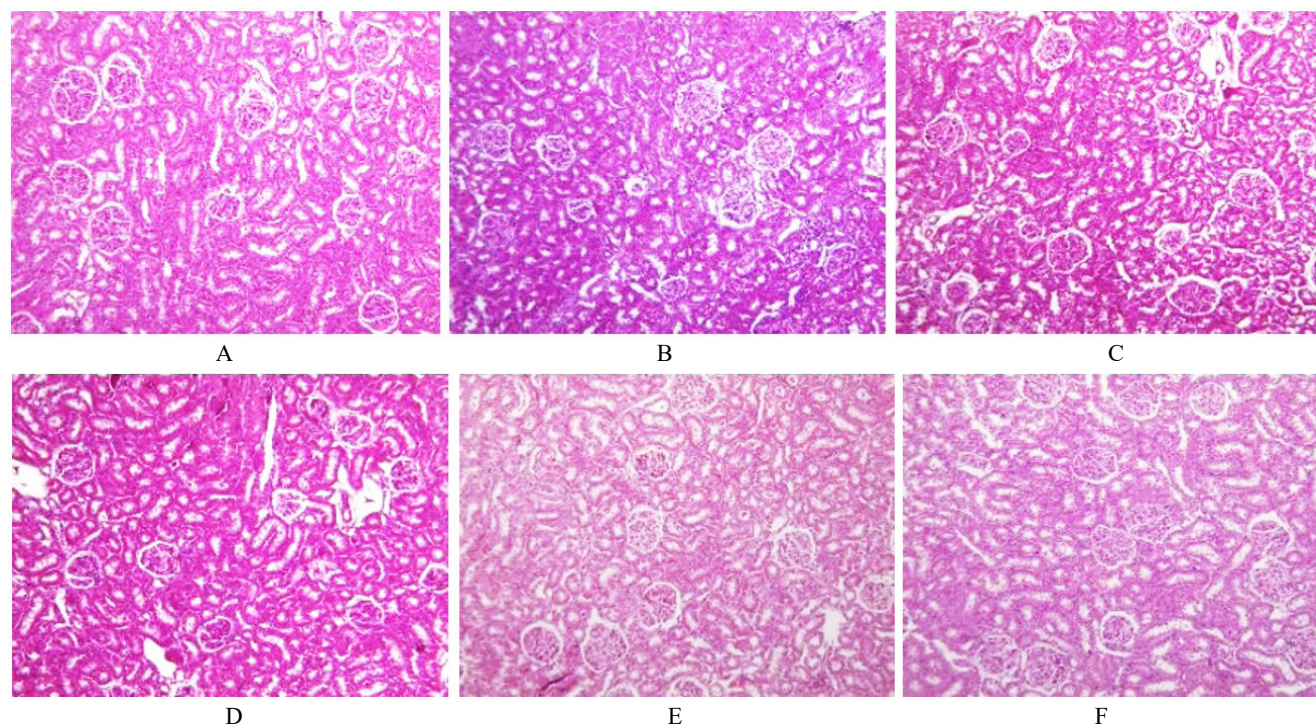


Figure 7. Histopathological examination of the kidney following the injection of UCB-derived DCs Secretome in Sprague-Dawley rats to assess the impact of acute toxicity (n = 5 per group). Representative images of the liver section from (A) control group, (B) secretome 0.015%, (C) secretome 0.15%, (D) Secretome 1%, (E) secretome 10%, and (F) secretome 100%

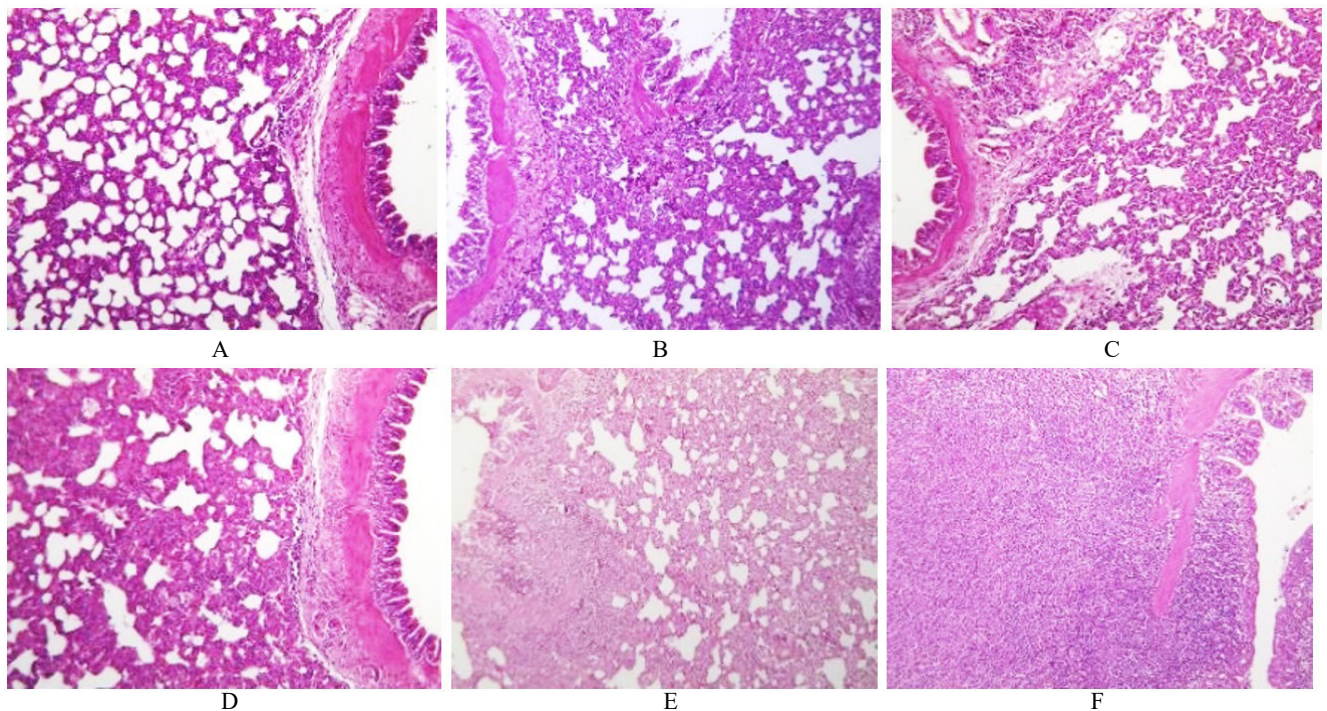


Figure 8. Histopathological examination of the lungs following the injection of UCB-derived DCs Secretome in Sprague-Dawley rats to assess the impact of acute toxicity (n = 5 per group). Representative images of the liver section from (A) control group, (B) secretome 0.015%, (C) secretome 0.15%, (D) Secretome 1%, (E) secretome 10%, and (F) secretome 100%

system to the adaptive immune system by presenting antigen peptides on major histocompatibility complexes (MHC I and MHC II). In this study, DCs were successfully obtained from monocytes isolated from UCB after 7 days of culture (Figure 1). Monocytes were characterized as circular and round cells, while immature DCs were larger, irregular in shape, and expressed surface proteins that bind to T cells.

The process of DCs activating T cells requires at least three signals. The first signal is through MHC-I and MHC-II carrying specific antigens that will interact with the TCR. Next, co-stimulatory molecules such as CD80/86, CD83, and HLA-DR function to strengthen the first signal. Lastly, the secretion of pro- and anti-inflammatory cytokines activates effector T cells, both CD4+ and CD8+. High expression of CD40 and high secretion of chemoattractant by cells activated using a maturation cocktail is an example of an "all-out" immune response, which can only be controlled by anti-inflammatory cytokines like IL-10 Marin *et al.* (2018). Therefore, a balance between pro- and anti-inflammatory cytokines is necessary in the use of DCs vaccines. Monocytes initially expressed positive surface markers such as CD14 and CD54, while negative markers included CD80, CD83, CD86, HLA-DR, and CD40. During differentiation into immature DCs, CD14

decreased, while CD83, CD80, HLA-DR, CD40, and CD86 increased. There is no official global standard for the minimum target of surface markers and cytokines produced. However, some producers of DCs for cancer use a minimum positive surface marker specification of 50-70% for CD80, CD83, CD86, HLA-DR, while a maximum of 20% for negative surface markers such as CD14, obtained from internal validation data (Nava *et al.* 2015; Boudousquié *et al.* 2020). Our results showed mature DCs with similar morphology description from several studies.

Umbilical cord blood (UCB) is considered a promising source of DCs. UCB-DCs induce less proliferation of CD3+ T lymphocytes than adult peripheral blood dendritic cells (APB-DCs) but produce higher levels of several cytokines than APB-DCs Kumar *et al.* (2015). Activated immunogenic DCs release inflammatory cytokines such as IL-12p70, IL-6, IFN- $\alpha$ , Tumor Necrosis Factor (TNF- $\alpha$ ), IFN- $\gamma$ , and IL-10 Nava *et al.* (2015). The potential functions of these cytokines in cancer treatment are summarized in Table 2. IL-12p70, IL-6, IFN- $\alpha$ , TNF- $\alpha$ , and IFN- $\gamma$  can be categorized as pro-inflammatory cytokines. Meanwhile, IL-10 can be categorized as an anti-inflammatory cytokine. IL-6 has both pro- and anti-inflammatory properties. Interleukin-12 (IL-12) p70 is a heterodimeric cytokine composed of

Table 2. Summary of secreted cytokines from UCB-derived DCs secretome and its potential functions in cancer treatment

| Secreted cytokines                           | Function                                                                                                                                                                                                                                                                                                          | References                    |
|----------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|
| Interleukin 6                                | Regulator of anti-cancer immune responses                                                                                                                                                                                                                                                                         | Bent <i>et al.</i> 2021       |
| Interleukin 10                               | Immune activation, induce elevation of granzyme and IFN $\gamma$ , and well tolerated in solid tumors                                                                                                                                                                                                             | Ni <i>et al.</i> 2020         |
| Interleukin 12p70                            | Up-regulates NK cell perforin and granzyme, enhancing activated NK-cell and Th1 cells, releasing IFN- $\gamma$ to enhance the function of cytotoxic T cells, inhibiting angiogenesis in cancer cells                                                                                                              | Verma <i>et al.</i> 2014      |
| Tumor necrosis factor-alpha (TNF- $\alpha$ ) | Involved in inflammation associated carcinogenesis and inducing cancer cell death                                                                                                                                                                                                                                 | Wang and Lin 2008             |
| IFN- $\alpha$                                | Inhibit the proliferation of normal and tumor cells <i>in vitro</i> and <i>in vivo</i> , down regulate oncogene expression, induction of tumor suppressor gene, enhance immune recognition, stimulates the development and activation of NK and DC cells, activates Th1 cells, and induces B cell differentiation | Ferrantini <i>et al.</i> 2007 |
| IFN- $\gamma$                                | Mediating antitumor effects of radiation therapy, decreases tumor growth by inducing tumor cell cycle arrest                                                                                                                                                                                                      | Ni and Lu 2018                |

two covalently linked chains, p40 and p35. These chains are encoded by genes on different chromosomes and their production can be independently regulated. Pro-inflammatory cytokines like IL-12p70 and TNF- $\alpha$  function in activating NK cells and Th1 cells, which subsequently release IFN- $\gamma$  to enhance the function of cytotoxic T cells Fu and Jiang (2018). IL-12p70 also has anti-tumor effects by inhibiting angiogenesis in cancer cells (Baldin *et al.* 2020; Hussein *et al.* 2021). IFN- $\alpha$  can directly inhibit the proliferation of normal and tumor cells both *in vitro* and *in vivo*, and can have other direct effects on tumor cells. These effects include downregulating the expression of oncogenes and inducing tumor suppressor genes. In addition to direct effects on tumor cells, IFN- $\alpha$  has several effects on immune cells that can play a central role in the overall antitumor response. In early infection, IFN- $\alpha$  increases the expression of MHC class I and II on monocytes, stimulates the development and activation of NK and DC cells, activates Th1 cells, and induces B cell differentiation Boudousquie *et al.* (2020). Furthermore, IFN- $\alpha$  induces or increases the expression of restriction factors, which inhibit the integration and release of viruses after replicating in infected CD4+ T cells. IFN- $\gamma$  is essential for activating anti-tumor immune responses. A multifunctional cytokine with potent anti-tumor effects. It activates macrophages, enhances antigen presentation by DCs, and directly inhibits tumor cell growth. A pro-inflammatory cytokine with cytotoxic effects on tumor cells. It can also stimulate the immune response by activating macrophages and T cells. All these contents of UCB-derived DCs secretome can be used as adjuvant therapy in carcinoma.

As APCs, DCs can present tumoral antigens to T cells, stimulating anti-tumor responses, which has led to the development of DC-based cancer treatments

Ghorbaninezhad *et al.* (2022). A study identified 4,666 proteins produced after DC maturation, which mostly important for MHC I presentation Arya *et al.* (2019). Various secreted proteins after DC maturation using different stimulation methods have been identified, including cytokines, chemokines, growth factors, proteases, protease inhibitors, acute phase proteins, extracellular matrix proteins, marker proteins, stem cell growth factors, vasorin, plasma protein complement factor B, leucine rich alpha-2-glycoprotein, and zinc-alpha-2-glycoprotein. Unfortunately, our study did not include a protein analysis component.

From the histopathological examination of the liver, no mild, moderate, or severe degeneration or necrosis was found for all concentrations. Similarly, in the kidney, there was no mild to severe degeneration or necrosis in the proximal tubules. In the heart, histopathology of the heart muscle did not show any mild to severe inflammation or necrosis. There was no mild to severe depletion of the white pulp in the isolated spleen. Additionally, no damage was observed in the alveolar walls of the lungs of all test animals. The calculation of the LD<sub>50</sub> value would initially use probit/linear regression, but since there were no deaths in all groups, the probit formula could not be used in this study. Therefore, for the LD<sub>50</sub> in this acute toxicity test, the Loomis and Hayes (1996) method of classifying LD<sub>50</sub> dose ranges (up to 100% concentration) was used, falling into the category of practically non-toxic.

The use of UCB-derived DCs secretome as cancer therapy has been intensively studied due to its lower risks of transmitting infections by latent viruses, easy accessibility, can be cryopreserved for extended periods, and is collected through a non-invasive process. However, there have been a limited number of clinical trials and

preclinical studies that have been done to evaluate the toxicity profiles and efficacy of UCB-derived DCs secretome. As far as we know, this is the first toxicity study of UCB-derived DCs secretome in animal models. The optimal balance of pro- and anti-inflammatory cytokines in DCs secretome is critical for effective cancer therapy. Too much inflammation can lead to tissue damage, while insufficient inflammation may not be enough to eliminate tumor cells.

In conclusion, our findings demonstrate that the UCB-derived DCs secretome is well-tolerated by rats at the tested doses ( $n = 30$ ), without causing significant toxicity, mortality, or adverse effects. The DCs were derived from umbilical cord blood with morphological and phenotypic characteristics comparable to those of peripheral blood-derived DCs. These results suggest a favorable safety profile for the secretome, supporting its potential for further exploration as a therapeutic agent in various applications. The  $LD_{50}$  of the acute toxicity test of 100% UCB-derived DCs secretome is categorized as practically non-toxic. Randomized clinical studies are needed to evaluate the efficacy and safety of UCB-derived DCs secretome for cancer therapy.

## Conflicts of Interest

The authors declare no conflicts of interest.

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