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ORIGINAL ARTICLE

Accelerating Wound Healing of Traumatic Ulcer with Topical Application of Dental Pulp Mesenchymal Stem Cell Secretome and Robusta Green Coffee Bean Extract Combination *in vivo*

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ABSTRACT

Objective: To investigate the ability of a combination dental pulp mesenchymal stem cell secretome (DPMSCS), robusta green coffee bean extract (RGCBE), and Carboxymethylcellulose-Natrium (CMC-Na) in a Wistar rats model of traumatic ulcers. **Material and Methods:** Twenty-eight young, male, healthy Wistar rats (*Rattus norvegicus*) were divided into seven groups randomly: Group K_0, group K_1-3 (traumatic ulcer rats that received CMC-Na gel for three days), group K_1-7 (traumatic ulcer rats that received CMC-Na gel for seven days), group K_2-3 (traumatic ulcer rats that received RGCBE for three days), group K_2-7 (traumatic ulcer rats that received RGCBE for seven days), group K_3-3 (traumatic ulcer rats that received DPMSCS for three days), and group K_3-7 (traumatic ulcer rats that received DPMSCS for seven days). An ulcer was made with an amalgam stopper on the right buccal mucosa of the rats. DPMSCS 50% gel was applied to the ulcer on the left buccal mucosa. The ulcer diameter was measured on day 3 and day 7. **Results:** There was a significant difference in the diameter of the ulcer, the number of neutrophils, and fibroblasts in the treatment group compared to the control group on day 7. **Conclusion:** A combination of DPMSCS and RGCBE 50% accelerates traumatic ulcer wound healing by lowering ulcer diameter, decreasing neutrophil counts, and increasing fibroblast proliferation *in vivo*.

Keywords: Fibroblasts; Neutrophils; Stem Cells; Wound Healing; Medicine.

Introduction

Injuries are unpredictable events. One of the most common types of wounds is burns. Burns are injuries to the skin or organic surfaces caused by heat or radiation, radioactivity, electricity, or contact with chemicals. According to the World Health Organization (WHO) in 2018, 11 million cases of burns occur every year and often occur in lower-middle-income countries such as Africa and Southeast Asia [1,2]. Most patients with serious burns require prompt emergency treatment to reduce morbidity and mortality. The high fatality rate is not only due to hypovolemic shock and vascular leakage but also to abnormal body responses, including immunosuppression, excessive inflammation, and hypermetabolism. This response can lead to increased infection, sepsis, and multiple organ dysfunction syndrome (MODS), leading to patient death [2].

Therefore, fast and adequate wound closure is needed so as not to cause unwanted events. The wound model made in this study was traumatic ulcer due to burns in Wistar rats. Ulcers are lesions that form due to local damage to the epithelial tissue [3]. Traumatic ulcers are one of the most common lesions on the oral mucosa. These traumatic injuries can occur in the cheek mucosa, labial mucosa, palatal mucosa, and the edge of the tongue. Injuries to the oral mucosa occur due to physical, thermal, or chemical trauma [4]. Physical factors that can cause this include sharp tooth margins, use of orthodontic appliances, or bitten cheeks [5,6]. The prevalence of ulcers worldwide is 25% [5], while in Indonesia at the Oral Medicine Clinic of the Dental and Oral Hospital, Faculty of Dentistry, University of Jember, from March to November 2016, the prevalence of traumatic ulcers was 6.5% out of a total of 766 patients [7].

The clinical picture for this injury shows signs of acute inflammation, such as oval shape, redness, swelling, and pain. The ulcer is lined with a yellowish-white fibrinous exudate and surrounded by an erythematous halo [6]. Treatment for traumatic ulcers is often topical corticosteroids such as topical triamcinolone acetonide. However, this drug can cause side effects such as itching, burning, irritation, dry skin, headaches, and dizziness. In addition, long-term use can cause immune system resistance and epithelial cell atrophy [7]. From this description, it is necessary to use alternative ingredients from herbal sources which have the advantages of anti-inflammatory properties and fewer side effects. The use of synthetic drugs also causes antibiotic resistance. The alternative currently being developed is to combine two ingredients, namely dental pulp mesenchymal stem cell secretome (DPMSCS) and robusta green coffee bean extract (RGCBE), so that they can complement each other. RGCBE contains caffeine, phenolic compounds, trigonellines, and chlorogenic acid, which have antibacterial and anti-inflammatory properties [8,9]. RGCBE is preferred because it has the most chlorogenic acid phenolic compounds compared to other coffee beans [10]. This water-soluble chlorogenic acid is a polyphenol with antimicrobial, antioxidant, anti-carcinogenic, antipyretic, analgesic, and anti-inflammatory functions in vitro and in vivo experiments in animal models [11-13].

Nowadays, stem cells are the main attraction in medical practice due to their regenerative abilities. The DPMSCS surrounding these stem cells can secrete growth factors, cytokines, chemokines, angiogenic factors, and exosomes that have the beneficial function of regenerating damaged tissues through a paracrine effect that stimulates endogenous stem cells [14-16]. Therefore, the combination of DPMSCS and RGCBE is expected to be an alternative for wound treatment. This study hypothesizes that DPMSCS and RGCBE gel combination can decrease neutrophil count and enhance wound healing of traumatic ulcers by decreasing the traumatic ulcer diameter. Furthermore, this study aims to investigate the ability of DPMSCS and RGCBE on wound healing in a Wistar rats (*Rattus novergicus*) model of traumatic ulcer.

Material and Methods

Ethical approval for this study (Ref. Number 999/UN25.8/KEPK/DL/2020) was provided by the Ethical Committee of Medical Research at the Faculty of Dentistry at Jember University, Jember, on September 10, 2020. This was laboratory experimental research with a randomized control group post-test only design.

Dental Pulp Stem Cells Secretome Processing Procedure

Dental pulp was taken from human tooth stock cultured in the research stem cell and development center at Universitas Airlangga Surabaya. The culture media was αMEM (α- Minimum Essential Medium) Eagle (Sigma-Aldrich, UK) Fetal Bovine Serum (Biolegend, China), Penicillin-streptomycin (Biolegend, China). After 4-5 rounds of processing and reaching 80% confluence, cultures were separated with a trypsin solution (Sigma-Aldrich, UK) and centrifuged to obtain a supernatant called the DPMSCS.

Robusta Coffee Bean Extract Manufacturing Process

RGCBE was obtained from the Indonesian Coffee and Cocoa Research Center located on Jalan Renteng, Gebang, Nogosari, Rambi Puji District, Jember Regency, East Java. The coffee beans were identified at Jember State Polytechnic. RGCBE is made using the maceration method. One kilogram of robusta green coffee beans was ground into small flakes and sifted into a fine powder, weighed 346.30 grams and then macerated with a 96% ethanol solution (1:5 ratio) for three days. Stirring was done twice a day, in the morning and evening. The maceration results were filtered using filter paper and then concentrated using a rotary evaporator at a temperature of 40°C so that preparation with a concentration of 100% was obtained. Then, DPMSCS and RGCBE with concentrations of 50% were combined and made into a gel.

Preparation of the Gel DPMSCS – RGCBE

The gel was made of 2 grams of CMC-Na 2% developed in 98 mL of hot water at 70ºC in a mortar for 15 minutes until it swelled, then stirred until a clear preparation formed. The robusta coffee bean extract was then added and stirred until homogeneous in the mortar. To get a gel combination of DPMSCS and RGCBE with a concentration of 50%, the combination of DPMSCS and RGCBE was mixed with 2.5 grams of CMC-Na. RGCBE and a combination of RGCBE and DPMSCS along with the results of the (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) test were obtained from previous research results [17].

Establishment of Traumatic Ulcer Animal Model

Young, male, healthy Wistar rats (Rattus norvegicus) (±200-300 g) were obtained from the dentistry faculty at Jember University. The animals were acclimatized in the laboratory for one week. All animals were allowed free access to water and fed with standard rat pellets. The Wistar rats were divided into seven groups randomly, with each group consisting of four rats: Group K_0 (normal group), group K_1-3 (traumatic ulcer rats that received CMC-Na gel for three days), group K_1-7 (traumatic ulcer rats administered CMC-Na gel for seven days), group K_2-3 (traumatic ulcer rats administered with RGCBE for three days), group K_2-7 (traumatic ulcer rats administered with RGCBE for seven days), group K_3-3 (traumatic ulcer rats administered with a combination of DPMSCS and RGCBE for three days), and group K_3-7 (traumatic ulcer rats administered with combination DPMSCS and RGCBE for seven days). Ketamine (0.4 mL/kg) and xylazine (0.2 mL/kg) were administered for general anesthesia via the intramuscular route. A traumatic ulcer model was made from an amalgam stopper with a diameter of 3mm. This was heated for 30 seconds on a spirit burner and lightly touched for one second to the left buccal mucosa of the rats. Mice waited for 2 x 24 hours for the ulcer formation process.

The ulcer diameter was measured before gel application using a UNC-15 periodontal probe (Osung, Korea) along the ulcer [18]. All samples were sacrificed with ketamine and xylazine in a lethal dose.

After being sacrificed, cleaned buccal mucosa was placed in a 10% neutral buffered formalin (HT501128, Sigma Aldrich Corp., St. Louis, MO, USA) for at least eight hours. After fixation, dehydration was carried out with 95%, 80%, 70%, and absolute alcohol for 60 minutes each, clearing by inserting the tissue into xylol three times for 60 minutes at each stage. A paraffin block was attached to the holder and then the buccal mucosa was cut vertically to 6µm with a microtome and put into slides. The paraffin was removed and left on a slide warmer at a temperature of 30°C-35°C for 12 hours.

The deparaffinization stage was carried out by immersing the sample in xylol for 2-3 minutes. Samples were dehydrated with alcohol from high to low concentrations (absolute and 95%) for 3 minutes and then rinsed with running water. The slides were stained using hematoxylin and eosin (HE) (MHS1, Sigma Aldrich Corp., St. Louis, MO, USA) for 15 minutes. The sample was stained with eosin for 1-2 minutes and then dehydrated with 95% and absolute alcohol for 2-3 minutes. The clearing was carried out with xylol for three minutes each step, and mounting was carried out for five minutes. The result of this staining shows that the cytoplasm is pink and the nucleus is blue/black. Neutrophils or polymorphonuclear neutrophils are seen with the presence of granulocytes and have multi-lobed nuclei consisting of 3-5 segments that are continuous with thin strands or isthmuses [19]. Fibroblasts have elongated spindle or stellate formations with a multitude of cytoplasmic projections [20]. Cells were counted using a light microscope (Olympus, Tokyo, Japan) at 400x magnification. The counting area consisted of three visual fields in the area around the wound in the form of a V pattern for each sample and was calculated using a counter [21]. Then, the number of fibroblasts and neutrophils was counted in each sample [22,23].

Statistical Analysis

The data were analyzed using Statistical Package for the Social Sciences 20.0 software (SPSS for Windows; SPSS, Chicago, IL, USA). Data obtained were analyzed with a normality test using the Shapiro-Wilk test to determine whether the data were normally distributed or not, then continued with the variant homogeneity test to examine population variations employing the Levene test ($p>0.05$). Finally, the Kruskall-Wallis test and the Mann-Whitney test were performed to determine the significance of differences between groups ($p<0.05$).

Results

The diameter of ulcers of Wistar rats on the left buccal mucosa was carried out on day 3 and day 7. The clinical presentation of traumatic ulcer can be seen in Figure 1 (A-F). The mean and standard deviations of ulcer diameter, both from the control group and the treatment groups, were tabulated and presented in Figure 2.

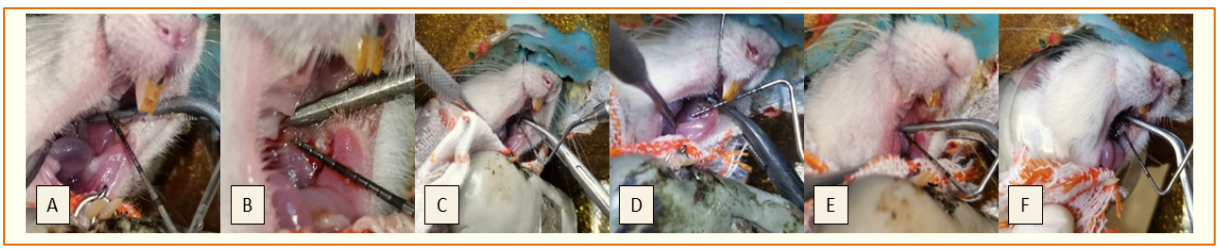

Figure 1. Traumatic ulcer on the left buccal mucosa of rats, measured with the UNC-15 periodontal probe. A) K_1-3; B) K_1-7; C) K_2-3; D) K_2-7; E) K_3-3; F) K_3-7.

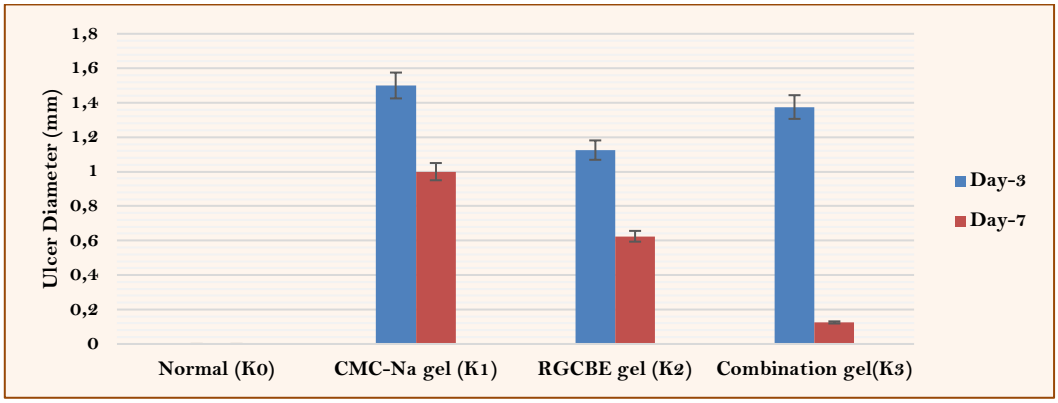

Figure 2. The mean and standard deviation of traumatic ulcer's diameter (mm) in all groups on day 3 and day 7.

Observations revealed that ulcers in the normal group are 0mm because no ulcer was made. In control group K_1-3, the ulcer has a healing diameter of 1.5±0.40mm, the K_1-7 control group has a diameter of 1±0.40mm, the K_2-3 RGCBE gel treatment group has a diameter of 1.125±0.63mm, the RGCBE gel treatment group K_2-7 has a diameter of 0.625 ± 0.25 mm, the K_3-3 combination gel treatment group has a diameter of 1.375±0.25mm, and the K_3-7 combination gel treatment group has a diameter of 0.125 ± 0.25 mm (Figure 2). The Kruskal-Wallis test result revealed a significant difference in the wound healing process on day 7 (p=0.026); however, no difference was found in the day 3 group. The Mann-Whitney test recorded a significant difference in ulcer diameter of the treatment group on day 7 (Table 1).

Table 1. Mann Whitney test results of diameter ulcer at day-7.

Group	K_1-7	K_2-7	K_3-7
K_1-7	-	0.155	0.025*
K_2-7	-	-	0.040*
K_3-7	-	-	-

*Statistically Significant.

Histopathological observation on the left buccal mucosa was carried out on day 3 and day 7. The mean and standard deviation neutrophils and fibroblasts, from both the control group and treatment group, were presented in Figures 3 and 4. There were increased neutrophils in all treatment groups on day 3 and increased fibroblasts on day 7. The fluctuation is described in Figures 3 and 4 and the histopathological image of neutrophils and fibroblasts is in Figure 5.

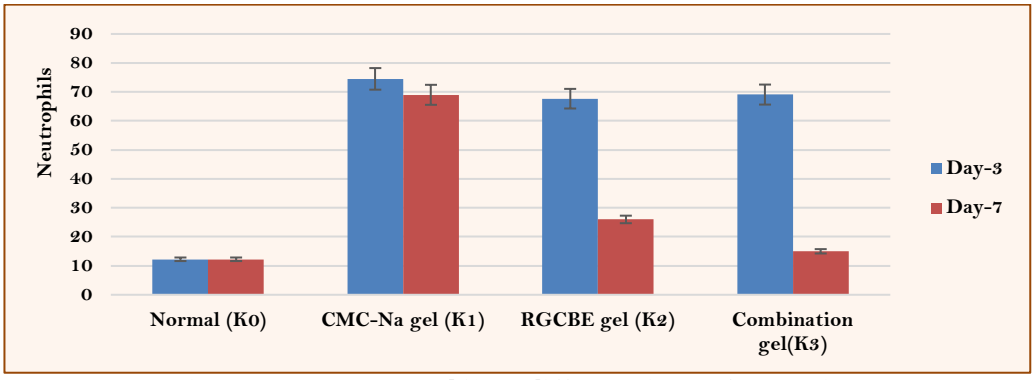

Figure 3. Total neutrophils at different times of evaluation.

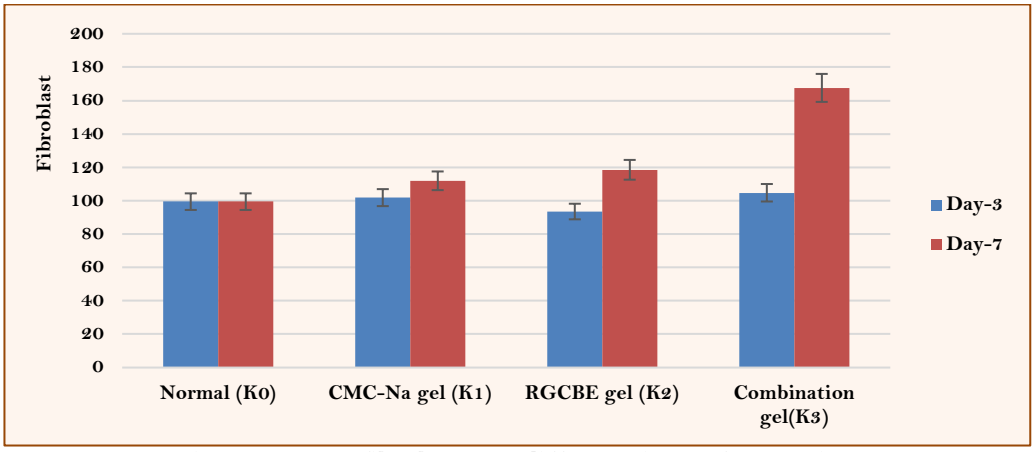

Figure 4. Total fibroblasts at different times of evaluation.

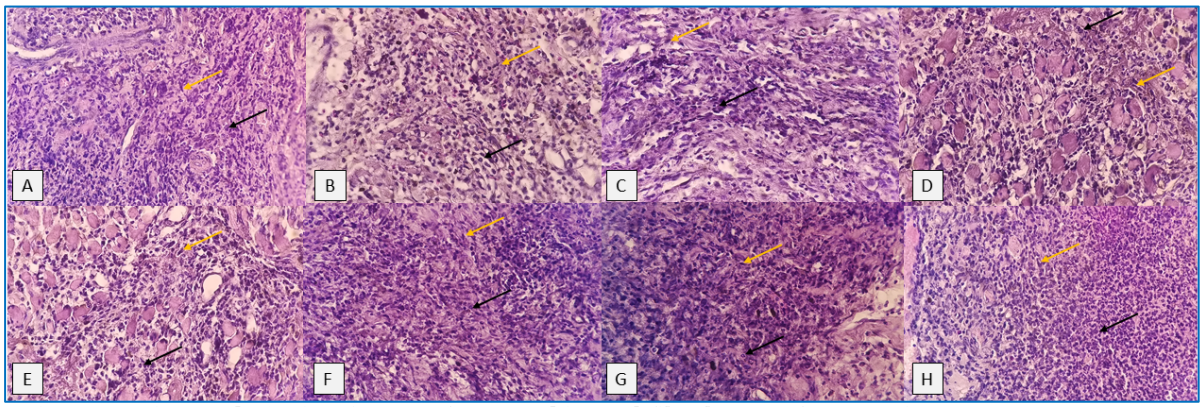

Figure 5. Histopathological image of neutrophils and fibroblasts of traumatic ulcers. Yellow arrows indicate fibroblasts and black arrows indicate neutrophils using HE staining with 400x magnification with a light microscope. A) K_0-3; B) K_0-7; C) K_1-3; D) K_1-7; E) K_2-3; F) K_2-7; G) K_3-3; H) K_3-7.

The Kruskal-Wallis test result revealed a significant difference in neutrophils (p=0.007) and fibroblasts (p=0.028) on day 7. The Mann-Whitney test recorded a significant difference in neutrophils of the treatment group on day 7. There was no significant difference (p>0.05) found in neutrophils and fibroblasts on day 3 with the Kruskal-Wallis test (Table 2).

Table 2. Mann-Whitney test results of neutrophils and fibroblast.

	Neutrophils				Fibroblast			
Group	K_0	K_1-7	K_2-7	K_3-7	K_0	K_1-7	K_2-7	K_3-7
K_0	-	0.773	0.564	0.043*	-	1.000	0.564	0.043*
K_1-7	-	-	0.885	0.043*	-	-	0.309	0.021*
K_2-7	-	-	-	0.043*	-	-	-	0.043*
K_3-7	-	-	-	-	-	-	-	-

*Statistically Significant.

Discussion

This traumatic ulcer model uses Wistar rats because they have a similar mucosal tissue structure to humans, with both having epithelial, basal layer, and connective tissue structures [24]. After the treatment of traumatic ulcers in rats, ulcer diameter decreased more rapidly in the DPMSCS and RGCBE combination gel group. This examination found that DPMSCS can accelerate wound closure by decreasing the number of

neutrophil cells and increasing the number of fibroblasts, functions that accelerate wound healing. In addition, the ulcer diameter after administration of RGCBE and DPMSCS combination gel group decreased more rapidly. This is caused by several factors, including that the active substance contained in the DPMSCS gel contains extracellular proteins, growth factors, cytokines, chemokines, and several molecules that can affect the microenvironment and directly modulate endogenous mesenchymal stem cells (MSCs) to proliferate. Furthermore, the presence of growth factors in the DPMSCS can accelerate wound healing by inducing inflammatory mediators such as interleukin-1 (IL-1), Interleukin-6 (IL-6), and prostaglandin E2 (PGE2) *in vitro*, reducing the initial inflammatory reaction *in vivo* [25,26].

RGCBE contains acidic compounds that contain more chlorogenic acid (CGA) than arabica coffee and other plants. CGA has been investigated for its antibacterial, antioxidant, and anti-inflammatory activity. RGCBE makes the wound environment acidic [27]. When an injury occurs, pH greatly affects the reactions of the biochemical healing process, such as increasing protease activity, oxygen release, and reducing bacterial (antibacterial) toxicity. RGCBE can control infection and inflammation because it contains CGA, caffeine, trigonelline, carbohydrates, fats, and amino acids. CGA contained in RGCBE gel can shorten the inflammatory phase by inhibiting the release of IL-6. The combination of these ingredients accelerates the ulcer healing process. This was in line with a previous study that showed MSCs' secretome has paracrine effects that are important in modulating regeneration after therapy. Secretome has various therapeutic effects, such as tissue regeneration, angiogenesis, anti-apoptosis, and immunoregulation [28]. The secretome mesenchymal stem cell group was shown to be able to reduce IL-1β compared to other groups with a significance level of 0.0001 ($p<0.05$) [29]. In addition, it is also known that DPMSCS can increase the expression of OCN and ALP in terms of increasing bone growth *in vivo* [30].

Based on histological observations, there was a significant difference in the decrease in the number of neutrophil cells on day 7 and an increase in the number of fibroblasts on day 7 in the combination DPMSCS and RGCBE gel group compared to the normal group. This test proves that DPMSCS can accelerate wound closure by decreasing the number of neutrophil cells and increasing the number of fibroblasts, all of which function in accelerating wound healing [31]. It can be seen in the study that DPMSCS can reduce neutrophils to nearly normal groups on day 7. Similar studies with 50% RGCBE gel were also shown to reduce tumor necrosis factor-α (TNF-α) expression. TNF-α is known as an inflammatory cytokine that is often used as an indicator of cellular oxidative stress, apoptosis, or necrosis. TNF- α levels will increase when inflammation occurs [32]. When ulcers occur, pH greatly affects the reactions of biochemical healing processes, including increasing protease activity and oxygen release, reducing bacterial toxicity (antibacterial), triggering angiogenesis, and increasing fibroblast activity [32,33].

The results of this study showed that the ulcer diameter healing process was faster in the combination of DPMSCS and RGCBE group than in the RGCBE-only group, proving that if only one ingredient is given, the healing process is less than optimal because only the compounds contained in RGCBE that have antibacterial and anti-inflammatory activities but do not contain growth factors function to accelerate the healing process. Processing coffee beans can reduce the ability of these compounds [34]. Previous study have shown that the toxicity test of caffeine with keratinocytes is not toxic at various doses [35].

Research on MSCs *in vivo* has shifted the paradigm from MSCs as tissue replacement to becoming a medical signaling cell because of their ability *in vivo* to express the secretome by secreting soluble factor materials in areas of injury and areas of inflammation. MSCs are believed to have a role in repairing tissue damage. The presence of tissue injury is always associated with the activity of the immune system or inflammatory cells, not

only macrophages or neutrophils but also adaptive immune cells, including clusters of differentiation (CD) CD4+T cells, CD8+T cells, and B cells. Inflammatory mediators such as TNF-α, IL-1β, free radicals, chemokines, and leukotrienes are produced by phagocytic cells in response to cell damage. The interaction between MSCs and inflammatory cells is very complex. MSCs can either secrete cytokines or inhibit the action of cytokines. For example, MSCs secrete transforming growth factor-β (TGF-β) and other factors, increasing the induction of T cells and macrophages. The paracrine effects of MSCs are critical for maintaining epithelial cell integrity and promoting angiogenesis through their ability to regulate cell proliferation and differentiation [25,36-38]. This study's results were only limited to traumatic ulcer animal model using Wistar rats *(R. novergicus*). In addition, the biomarker of traumatic ulcer wound healing and examination method in this study is limited, other biomarkers examination regarding traumatic ulcer wound healing is necessary with various methods.

Conclusion

A combination of DPMSCS and RGCBE 50% accelerates traumatic ulcer wound healing by lowering ulcer diameter, decreasing neutrophils counts and increasing fibroblast proliferation *in vivo*. Further research is needed on combining stem cell secretomes with robusta coffee bean extract to heal ulcers on a combination of days and other dosage forms and cure other dental diseases.

Authors' Contributions

EI	https://orcid.org/0000-0002-4270-8205	Conceptualization, Methodology, Formal Analysis, Investigation, Resources, Writing - Original Draft and Writing - Review and Editing.
IET	https://orcid.org/0000-0002-0590-5170	Conceptualization, Methodology, Formal Analysis, Investigation, Resources, Writing - Original Draft and Writing - Review and Editing.
DSS	https://orcid.org/0000-0002-4801-7229	Conceptualization, Methodology, Formal Analysis, Investigation, Writing - Original Draft, Writing - Review and Editing and Supervision.

All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

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None.

Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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