ORIGINAL ARTICLE

A novel wound healing accelerator: Effect of vitreous gel of cow eyeball on a chronic wound model

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ABSTRACT

Introduction: Several studies have reported the disturbance in the process of wound healing after administration of mitomycin-C, which inhibits granulation tissue formation and collagen synthesis, resulting in chronic wounds. The vitreous gel of cow eyeballs contains a high level of hyaluronic acid, which has a role in inflammation, granulation, re-epithelialization, and remodelling. This study aims to understand the effect of 1% povidone iodine and vitreous gel of cow eyeballs on wound healing after administration of mitomycin-C.

Methods: This was an *in vivo* study with quasi-experimental methods on 32 Wistar mice. Full-thickness wounds were made and then treated with mitomicyn-C. The mice were divided into 4 groups: a control group with NaCl 0.9% vitreous gel of cow eyeball (VGCE), 1% povidone-iodine, and a combination of VGCE and 1% povidone-iodine groups. Macroscopic and microscopic observations of the process of wound healing were performed on days 3, 7, and 14.

Results: Vitreous gel administration produced significant wound healing rates within the first three days, and histological analysis revealed an increased number of fibroblasts and polymorphonuclear cells. However, the povidone iodine group and the combination group with vitreous gel did not produce significant results.

Conclusion: The single administration of VGCE can accelerate the wound healing process, increase the number of fibroblasts, and reduce inflammation in a chronic wound model.

KEYWORDS:
Vitreous Gel of Cow Eyeballs, Wound Healing, Fibroblasts, Hyaluronic Acid, Re-Epithelialization, Mitomycin, Inflammation

INTRODUCTION

The process of wound healing is a complex cellular and biochemical cascade that restores tissue function and cell integrity. Normally, it is a complex and dynamic process that includes various phases, such as haemostasis, inflammation, re-epithelialization, and remodeling.[1]

The failure of the migration process from one phase to the next causes an imbalance in the physiologic process of acute wound healing, resulting in a delay in healing and eventually turning into chronic wounds. The changes are ischemia, tissue hypoxia, and bacterial infections, causing prolonged inflammation.[2]

Currently, no theories can unify to answer the question of why chronic wounds fail to heal. However, chronic inflammation and bacterial infection are the major factors in chronic wound persistence.[3-5] Chronic nature can change the cell or local network on the wound area, which prolongs the inflammation phase and slows the healing phase. With aging tissue, vascularization is disrupted,[6] and composition dysregulation or replacement of the extracellular matrix (ECM) occurs,[7,8] as well as disruption of the function and response of fibroblasts. The latest evidence shows that failure to complete the inflammation process in chronic wounds is the result of fibroblast dysfunction. Fibroblasts play a major role in wound healing by forming components of the ECM, such as collagen, elastin, and proteoglycan, and producing mitogen for keratinocytes, fibroblasts, and endothelial cells.[9] Fibroblasts in chronic wounds have a decreased ability to react to growth factors that normally stimulate a mitogenic response. Research shows that a decline in response to basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF) are associated with dysfunction in intracellular signal delivery.[10] Older fibroblasts have a decreased ability to proliferate[11] and lack the prophylaxis of stimulation from transforming growth factor β1 (TGF- β1)[12,13] due to the decreased expression of the receptor gene.[14,15]

Studies on older fibroblasts showed that the improvement of the mechanical strength and fibroblast material with an injection of hyaluronic acid (HA) can restore and help fibroblast function in the proliferation of cells and the synthesis of ECM.[16] This finding was supported by a study on chronic wounds treated with a hyaluronic degenerative matrix.[17] Increased mechanical strength and structural support on the ECM cause changes in the morphology and lengthening of fibroblasts, which is associated with increased signal transmission line TGF- β and culminates in the target connective tissue growth factor (CTGF) and procollagen type

This article was accepted: 06 October 2020
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1. Additionally, fibroblast stimulation can be mediated by the direct bond between HA material and cellular receptors.[18] The addition of exogen monomeric HA to fibroblasts can stimulate signal delivery of TGF-β and collagen production.[19]

Topical mitomycin-C (MMC) is produced by the fungus Streptomyces caespitosus. Originally, it was introduced as a type of antibiotic and acts as an agent of chemotherapy. MMC upgrades cross-link deoxyribonucleic acid (DNA) and reduces or stops transcription. Enzyme inhibition and fibroblast proliferation are among the famous effects of MMC.[20] This effect can explain how it functions to reduce the risk of scar tissue formation.[21]

A model of chronic wounds can be made in experimental studies with rats using mitomycin C given shortly after the creation of acute injury to inhibit the proliferation of cells and fibroblasts to disrupt the wound healing process.[22]

Povidone iodine 10% (PVP-I) is a topical antiseptic with a wide spectrum effect that may be used in the liquid form. As an iodophor, PVP-I is formed from iodine compounds with carrier substances, usually pyrolidone soluble polymers that contain colloids and are highly osmotic. Povidone iodine can be used at full concentration (10%) or as needed.[23] Berkelman proposed that it is more effective when used in combination with electrolytes to reduce the concentration to 0.1-5%.[24] Most *in vivo* studies on the use of povidone-iodine in experimental studies or surgery were conducted on acute wounds. Successful application on chronic wounds has not been proven.[23]

Vitreous fluid in the eyeballs of mammals is an extracellular matrix with high hydration and consists of a network that contains hyaluronan polyanionic macromolecule (HA), versican, collagen IX and collagen fibrils. The macromolecule has few quantitative variations that are not significant among some mammals.[25] Vitreous fluid of cow eyeballs (VFCE) contains the highest hyaluronan compared to other sources, such as *haemolytic* zoepidemicus or velour roosters.[26] VFCE is known to contain widely distributed hyaluronan in the ECM and has been proven to influence the migration of cells, adhesion cells and angiogenesis.[27-29]

Because of the considerable role of HA on the function of the cell, it is very interesting to research the effects of VFCE against chronic wound healing in vivo. Cow eyeballs are easily obtained from animal butcheries, but until now, they have not been used as a source for the broad range of materials needed for the healing of chronic wounds. Empiric research of VFCE against chronic wound healing will be very useful, given the importance of the role of HA in wound healing.

METHODS

This is an *in vivo* experimental study using 32 adult mice obtained from the Animal Model Care Unit, Universitas Gadjah Mada (UGM). The study was conducted from March-April 2015 in the Integrated Research Laboratory, Faculty of Medicine, Public Health and Nursing UGM.

Simple random sampling was done to divide the mice into 4 groups, and each mouse was marked with a number on the leg. The number was sorted from 1 to 32. Then, numbered 1 to 32 were rolled and put inside a bottle and shaken, and the numbers were picked out one by one.

The first eight numbers were grouped as the 0.9% NaCl control group, which was treated with 0.9% NaCl. The second 8 numbers were grouped as the povidone iodine group, which was treated with povidone iodine. The third eight numbers were grouped as the Vitreous Gel group (VGG), which was treated with vitreous gel. The rest were grouped as the combination group, which was treated with 1% povidone-iodine and vitreous gel.

Isolation of cow eyeball vitreous liquid

Two cow eyeballs were taken from a 2-year-old cow after being euthanized at the animal slaughtering site. The cow eyeballs were put into a container filled with sterile 0.9% NaCl and placed into an ice box during transfer into the laboratory. In the laboratory, the cow eyeballs were washed with 10% povidone iodine and then with 0.9% NaCl three times. The vitreous liquid was aspirated using the aseptic technique and then placed inside a sterile tube and centrifuged at 200 g for 10 minutes. Supernatant liquid was isolated and stored at 4°C until use.

Wounding

Wistar mice aged 16–22 weeks with weights of 250-300 grams were used in this study. After the mice were anaesthesised with 30mg/kg BW intramuscular ketamine, hair from the back of each mouse was shaved and cleaned with 10% povidone iodine.

A full thickness wound model was made with ±2cm diameter in the back. Then 1ml topical mitomycin-C at a concentration of 0.5mg/ml was given for 5 minutes using a sterile bandage. This concentration was chosen based on related studies to avoid necrosis in the treated site. After 5 minutes, the wound was washed with 10ml 0.9% NaCl.

Treatment for each group

All mice were divided into 4 groups, each with 8 mice. After 24 hours, each wound group was given 1% povidone iodine, cow eyeball vitreous liquid at 50% concentration (vitreous liquid obtained from a couple of cow eyeballs aged 2 years old then centrifuged at 200 g for 10 minutes; the supernatant parts from those centrifugated results were used during the study, the VFCE was then added to aquades until a 50% concentration was formed), a mixture of 1% povidone iodine and 50% or 100% VFCE and 0.9% NaCl as a control topically and then closed with a sterile bandage. A previous study reported that a 1% concentration of povidone iodine had no negative effects on capillary blood flow after up to 60 minutes of exposure, while the use of 5% povidone iodine was associated with an early but rapid transient decrease in blood flow.[30]

Result measurement

The process of wound healing was observed on days 3, 7 and 14, and the developments were photographed. The wound was documented with a digital camera equipped with a

millimeter marked ruler. Then, it was processed digitally with a computer program to measure the wound area width daily by comparing the wound pixel with a 1cm^2 pixel.

The degree of wound repair (DR) was determined on days 3, 7 and 14, where it was calculated based on the initial area (measured on day 0) and stated as a percentage, according to the following formula:

$$DRi = \frac{A_0 - A_i}{A_0} \times 100\ \%$$

DR_i shows wound healing rate for i day

A_0 and A_i are wound areas wide in zero and i days, respectively.

The mean wound healing rate (WHR) was also measured that showed how mm^2 wound area had decreased for a certain amount of time from t1 to t2, stated in mm^2/day and determined by

$$WHR = \frac{A(t2) - A(t1)}{t2 - t1}\ (mm^2/day)$$

A(t1) and A (t2) are wound areas wide in time t1 and t2, with t2>t1

For the microscopic examination, the tissues at the site of wounds were stained with hematoxylin-eosin and van Gieson. Both semiquantitative (wound reepithelization; presence of inflammatory cells, fibroblasts, new vessels, and collagen) and quantitative methods (polymorphonuclear leucocyte/tissue macrophage ratio, percentage of re-epithelization, area of granulation tissue) were used to evaluate histological changes during wound healing.[31]

Data Analysis

The data obtained were then evaluated and analyzed using one-way ANOVA if the data were normally distributed; otherwise, Friedman's test was used, followed by a post hoc test. One-way ANOVA was used because it fulfilled the condition of more than two unpaired groups, normal distribution, and the same variation. If a p value <0.05 was obtained, there was a significant difference in the treatments of the four different groups. The post hoc test was used to understand which group had those significant differences.

RESULTS

Macroscopic

The results of wound area-wide measurements on days 3, 7, and 14 are displayed in Figure 1 (C). The macroscopic examination of tissues was evaluated in the 4 different groups progressively on days 0, 3, 7 and 14. The most significant improvement in wound healing was shown in the single administration of VFCE compared to the combination or single povidone iodine group macroscopically.

Wound healing level

The process of healing artificially clean wounds in Wistar rats in the back of the skin area (Figure 1) ranged from 7-14 days. The epithelialization process will normally complete entirely in less than ten days. The increase in fibroblast proliferation reaches its peak in 7 days as the increase in collagen is synthesized.

Table I shows an increasing trend of wound healing proportional to day, where the vitreous gel group showed a higher healing rate than the control, povidone-iodine, and combination groups starting from day 3 until day 14.

Post hoc analysis showed significance ($p<0.05$) in the DR 0-3 vitreous gel group compared to the control and combination groups, where there was an increase in healing acceleration in the vitreous gel group compared to the control and combination groups on day 3 (32% vs 8,85% vs 17.52% vs 15,8%). However, there was no significant difference with the povidone-iodine group ($p>0.05$). There was also no significant difference between DR 0-7 and DR 0-14 ($p>0.05$) (Figure 2A).

Wound healing rate

Table I shows the decrease in the wound healing rate trend in the povidone-iodine, combination and vitreous gel groups on days 3, 7 and 14. However, the NaCl control group showed an increased wound healing rate trend on days 3, 7 and 14.

Post hoc analysis showed significance ($p<0.05$) in WHR 0-3 vitreous gel compared to the control and combination groups. This result showed a higher increase in the wound healing rate in the vitreous gel group compared to the control and mixed groups on day 3 (37 vs 8.5 vs 21.68 vs 17.66 mm2/day). However, no significant difference was found in the povidone iodine group ($p>0.05$).

At WHR 0-7, the vitreous gel group showed a significant difference ($p<0.05$) compared to the control group. However, compared with the povidone iodine and mixed group, it showed no significance ($p>0.05$). At WHR 0-14, those 4 groups showed no significant difference ($p>0.05$) (Figure 2 B, C).

Microscopic

There were four aspects that were observed in the experiments: the number of fibroblast cells and polymorphonuclear cells (PMNs), epithelisation and collagen density (Figure 3).

Fibroblast cell

The measurement of fibroblast cell amount was done by calculating 3 field of views in hematoxylin eosin-stained preparation on days 3, 7 and 14 in each group.

Figure 4(A) shows an increasing trend of fibroblast cell amount in both the vitreous gel group and the control group, which peaked on day 7. Statistical analysis showed no significant difference ($p>0.05$) between each group on days 3, 7 or 14.

Table I: Wound healing rate (%) and the mean of wound healing rate (mm²/days) on days 3, 7 and 14

Wound Healing Rate % (mm²/days)	Control	Povidone Iodine	Povidone Iodine & Bovine Vitreous Gel	Vitreous Gel
DR 0 – 3	8.85 ± 3.720 (8.50 ± 3.61)	17.52 ± 3.97 (21.68 ± 6.09)	15.80 ± 5.00 (17.66 ± 6.22)	32.13 ± 6.89 (37.04 ± 8.88)
DR 0 – 7	23.62 ± 7.2 (9.76 ± 3.17)	33.49 ± 6.28 (15.85 ± 2.59)	35.96 ± 7.29 (16.5 ± 4.2)	46.75 ± 9.26 (23.08 ± 4.51)
DR 0 – 14	58.18 ± 3.75 (12.54 ± 0.91)	53.57 ± 8.51 (12.75 ± 2.19)	56 ± 1.02 (12.31 ± 2.88)	74.06 ± 4.06 (17.27 ± 0.56)

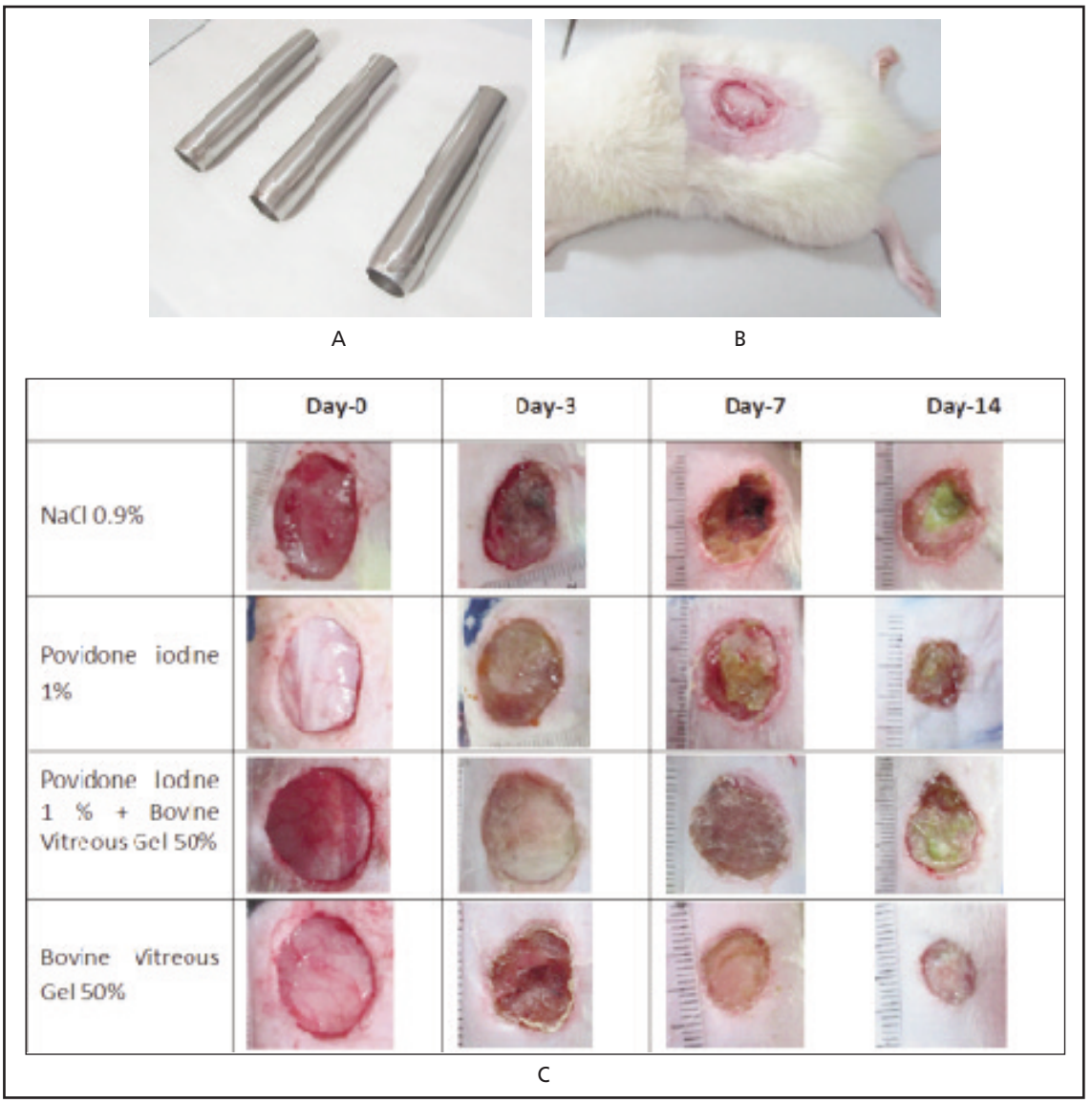

Fig. 1: (A) Wounding Equipment. (B) Mice back post-wounding. (C) Wound evaluation macroscopically in each group on days 0, 3, 7 and 14.

Polymorphonuclear cell (PMN)

The measurement of PMN amount was performed by calculating 3 fields of view in hematoxylin eosin-stained preparations on days 3, 7 and 14 in each group (Figure 5 (B)).

There was an increasing trend of PMN cell amount in the vitreous gel group and reached a peak on day 7. Statistical analysis showed no significant difference ($p>0.05$) between each group on days 3, 7 and 14 in each group (Figure 4 (B)).

Epithelization

For epithelization, the 4 groups had a positive trend but showed no significant difference statistically ($p>0.05$)

Collagen density

For collagen density, the 4 groups had a positive trend but showed no significant difference statistically ($p>0.05$).

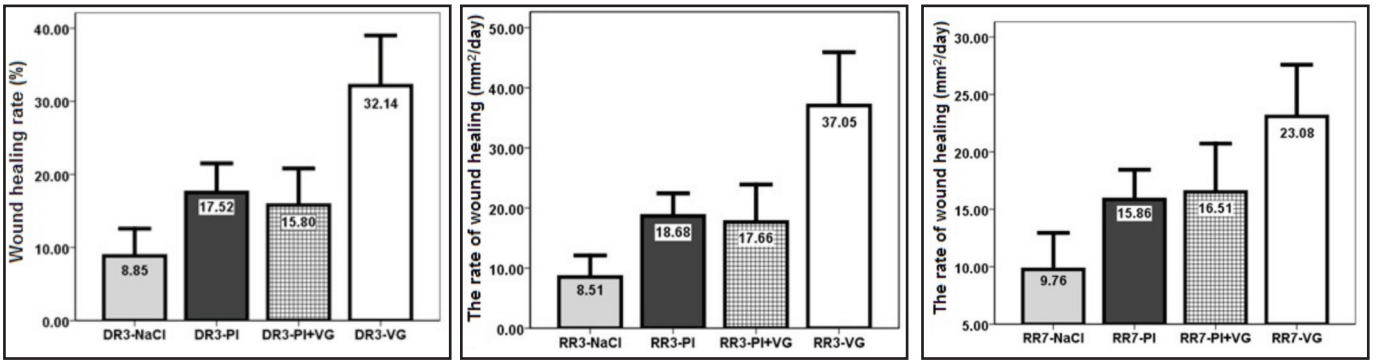

Fig. 2: (A) Degree of wound repair in day 3 showed significance difference ($p<0.05$) of vitreous gel with control and mixed groups; (B) Rate of wound healing in day 3 showed significant difference ($p<0.05$) of vitreous gel with control and mixed groups: (C) Rate of wound healing in day 7 showed significant difference ($p<0.05$) of vitreous gel with control group (DR: degree of wound repair, RR: wound healing rate, PVI: povidone-iodine, PVI+VG: povidone iodine + vitreous gel, VG: vitreous gel).

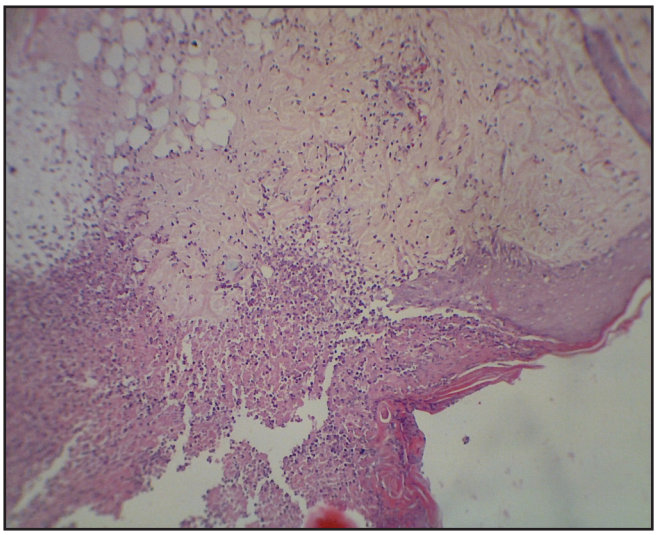

Fig. 3: Photomicrographs of fibroblasts, polymorphonuclear cells, and epithelial cells.

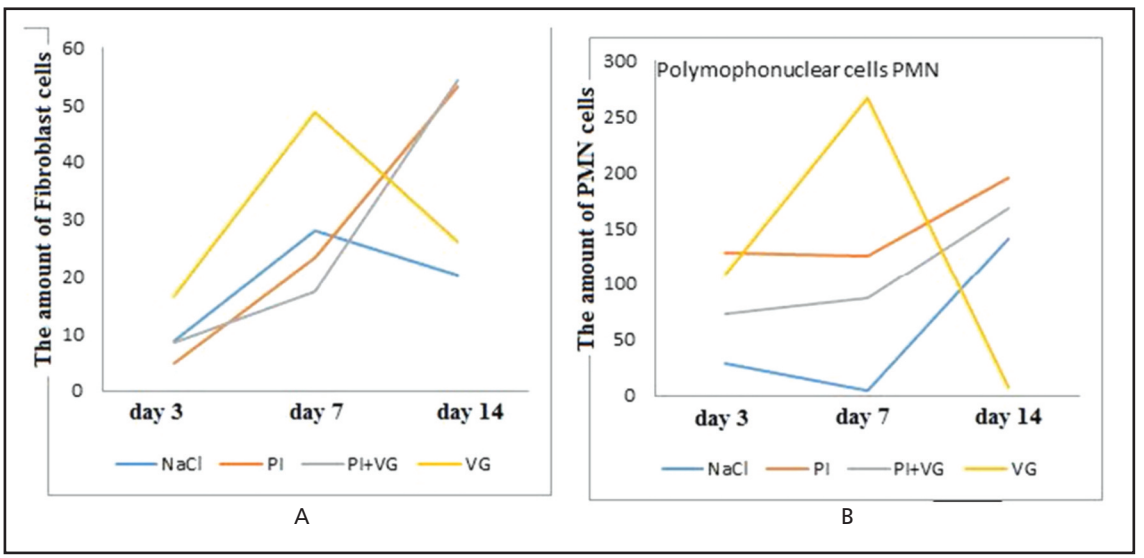

Fig. 4: (A) The number of fibroblast cells in each group on days 3, 7 and 14; (B) The number of PMN cells in each group on days 3, 7 and 14 (PMN: polymorphonuclear cells, PVI: povidone-iodine, PVI+VG: povidone iodine + vitreous gel, VG: vitreous gel).

DISCUSSION

Chronic wound or disrupted wound healing histologically showed fibroblast aging. The chronic wound model in this research was created using 0.5mg/ml mitomycin-C.[20] Continuing chronic inflammation factors and bacterial infections hold an important role in chronic wound physiology changes.[4,5,9] Therefore, to control the bacterial infection factor, 1% povidone-iodine, which is often used as wound treatment daily, was used.

From the evaluation of wound healing level and rate, the vitreous gel group showed excellent results on day 3 compared to the control, povidone iodine only or the combination of povidone iodine and VFCE and decelerated on days 7 and 14. This finding was probably connected with the inflammation process that happened during the early phase of the wound healing process, where inflammation will be prolonged in chronic wounds.

The VFCE contains hyaluronan (HA) with the highest concentration of 430-555µg/ml and a molecular weight of 500.000-800.000 Da. It provides high hydration for the tissue, which is very important in the process of wound healing.[32] In other animal experiments, topically applied HA has also been shown to accelerate skin wound healing and showed that HA prevents free radical damage to granulation tissue in rats.[33] In chronic wounds, such as venous leg ulcers, HA application has been shown to promote healing.[34] Ialenti and Di Rossa[35] have also directly demonstrated the inflammation-moderating effect of HA in standard models of acute and chronic inflammation, including in rats. A recent systematic review also concluded that HA is safe and efficacious for use in skin repair.[30]

The fibroblast cell and polymorphonuclear cell numbers in the vitreous gel group were the highest on day 7 compared to those in the control, povidone-iodine, and combination groups. Quan et al., reported that HA could increase local mechanical strength, thereby improving the morphology and function of old fibroblasts. This improvement can restore the response of old fibroblasts to the stimulation of TGF-β in the proliferation and synthesis of collagen.[18]

HA has a crucial role in healing both acute and chronic wounds. HA activated and moderated the inflammatory process and facilitated the proliferation, mitosis and migration of fibroblast cells and angiogenesis of granulated tissue formation. Moreover, HA is also an integral part and extracellular matrix of basal keratinocytes, proliferation, and migration of the re-ephitelization process of normal epidermal formation.[36] In chronic wounds, the inflammation phase will be accelerated and can be slowed down with the administration of vitreous gel, demonstrated by the high amount of PMN and fibroblast cells on day 7 and then decreased on day 14. The core factor in poor healing of chronic wounds is replicative senescence of fibroblasts that are unresponsive to TGF-β1 stimulation. Hyaluronic acid in the vitreous gel was able to improve TGF-β1 signaling in senescent cells, and a recent study showed that VFCE can be used to stimulate replicative senescence of human dermal fibroblasts (HDFs) with a higher proliferation index, migration rate and collagen deposition, thereby stimulating healing of chronic wounds.[32] Meanwhile, for epithelization and collagen density, all treatment groups showed positive healing trends. However, these findings could not be thoroughly evaluated because of the unfinished wound healing process.

HA also has an essential function of maintaining extracellular space and providing tissue hydration for nutrient exchange in the epidermis. The function is an integral part of the ECM, as an antidote to free radicals, and in the proliferation and migration of keratinocytes, which are very influential in the process of reepithelialisation.[36]

Furthermore, VFCE also contains approximately 60 µg/ml collagen. Fibroblasts synthesize collagen, fibronectin, and another basic substance to heal wounds influenced by interferon-γ and TGF-β. The resulting matrix will connect and unite the two ends of the wound. Eventually, collagen synthesis increases and fibroblast proliferation decreases, creating a balance between the synthesis and degradation of extracellular matrices.[37]

In this study, we compared the efficacy of VFCE and povidone iodine in the wound healing process and found that VFCE is superior in terms of the wound healing process. Although previous studies have found that povidone iodine can promote wound healing due to its antimicrobial spectrum, lack of resistance, efficacy against biofilms, good tolerability and its effect on excessive inflammation, several studies also found that povidone iodine could delay epithelialization and dermal healing and prolong inflammation.[38,39]

There are several limitations in this study that need to be addressed and may benefit future research on the effect of VFCE in wound healing. First, using different concentrations of the VFCE can explain whether the wound healing capability differs between different concentrations. Second, longer duration of observation of the wound ideally until the wound completely heals can be more appropriate, especially in examining the efficacy on epithelization and collagen formation.

CONCLUSION

VFCE proved to have an accelerating effect on the wound healing process. VFCE increased the amount of fibroblast and PMN cells upon mitomycin-C exposure in the white mice experiment, which occurred during the inflammation phase. Further research is needed to determine the possible role of VFCE as an astringent and aid in acute and chronic wound healing.

LIST OF ABBREVIATIONS

ECM: extracellular matrix; DR: degree of wound repair; WHR: wound healing rate; bFGF: basic fibroblast growth factor; EGF: epidermal growth factor, HA: hyaluronic acid; PDGF: platelet-derived growth factor; TGF-β: transforming growth factor β; CTGF: connective tissue growth factor; MMC: mitomycin; DNA: deoxyribonucleic acid; PVP-1: povidone iodine 10%; PMN: polymorphonuclear cell.

DECLARATIONS

Ethics approval and consent to participate

The ethical clearance of this study was by the ethical committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada (KE/FK/594/EC/2015).

COMPETING INTERESTS

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The work had no funding sources.

ACKNOWLEDGMENT

We thank all those who provided excellent technical support and assistance during the study.

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