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Exploring the Role of Regadenoson As an Ointment Dosage Form in Inducing Wound Healing in Mice

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Abstract

Background: Wound healing is a multi-phase and well-organized dynamic process to repair damaged tissue. Adenosine (A2A) receptor agonists were found to have a role in wound healing process by increasing collagen deposition and stimulating angiogenesis and re-epithelization.

Aim: To investigate the healing effect of regadenoson ointment in experimentally induced wounds in mice.

Methodology:

Preparation of regadenoson ointment: Regadenoson ointment (0.25% and 0.5% w/w) was prepared by dispersing regadenoson in castor oil followed by the addition of petrolatum base (Vaseline®) to obtain the final ointment preparation.

***In vivo* study:** Sixty female albino mice were enrolled in this study where they divided into five groups (N=12/group).

Group I: normal control. Group II: induced control which has received a petrolatum base only. Group III were treated topically with β-sitosterol ointment (0.25% w/w) as a positive control. Group IV and V were treated topically with regadenoson ointment (0.25% and 0.5% w/w) respectively. These products were applied once daily for 10 consecutive days.

Results: Regadenoson ointment produced a highly significant reduction in wound size in comparison with petrolatum base ($P \leq 0.001$).

In histological study, re-epithelization and angiogenesis scores of all treatment groups showed a significant increase in comparison with petrolatum base group ($P \leq 0.05$) but in collagen scores, the significant increase just occurred with β-sitosterol and the highest concentration of regadenoson 0.5% w/w in comparison with petrolatum base group ($P \leq 0.05$).

In immunological study, regadenoson groups produced a significant increase in VEGF-A.

Conclusion: According to the present findings, we can conclude that regadenoson ointment is more efficient than petrolatum base and comparable in efficacy to β-sitosterol ointment in accelerating wound healing.

Keywords: Regadenoson, adenosine A2A receptor agonist; wound assessment; histopathologic and immunologic grading.

INTRODUCTION

Wound healing is an essential homeostatic mechanism that involves a series of coordinated and multi-phases including [1]: hemostasis, inflammation, proliferation and remodeling [2, 3]. In acute inflammation, tissue damage is followed by resolution. Whereas in chronic inflammation, damage and repair continue for a long duration [4]. Inflammatory cells such as neutrophils phagocyte invading pathogens, remove waste and debris, also promote angiogenesis which in turn may enhance the recruitment of inflammatory cells and the subsequent laying down extracellular matrix to repair tissue damage [5]. Prolong inflammation may lead to tissue damage as well as aberrant or inadequate repair can lead to poorly ordered matrix deposition and fibrosis which affects normal tissue architecture [6].

Role of adenosine in wound healing

Based on previous *in vitro* studies and in experimental animal models [7], we are proposing a new strategy for promotion of impaired wound healing, is the use of adenosine receptor agonists.

Adenosine regulates cell functions by acting at specific receptors on the cell surface [8]. Generally, A1 and A3 receptors activate the Gi (inhibitory) family of G proteins, whereas A2A and A2B receptors activate the Gs (excitatory) family. Wound treatment with an adenosine receptor agonist accelerates the healing of the wound by acting on several stages involved in the wound healing process such as fibroblast migration, a rise in matrix and promotion of angiogenesis in the wound [9].

The effects of adenosine on inflammation

Instantly after the injury, the inflammatory response begins. The innate immune system is activated by evoking a local

inflammatory response that recruits various types of inflammatory cells to the site of the wound [10]. Despite A2A agonists are inhibitors of the neutrophil oxidative flow, they do not prevent the oxidative blast. Some of the oxidative blasts are resistant to inhibition by A2A agonist binding [11]. In addition to A2A receptors, neutrophils contain A1A receptors [12] and possibly A3 receptors that control neutrophil function [13].

The effects of adenosine in angiogenesis

Several findings suggest that adenosine A2A is important for angiogenesis by modifying the release of angiogenic factors from numerous cells and tissues [14].

Dermal collagen production

Matrix production is increased in wound healing by stimulation of the A2A receptors [15]. Collagen production also increased by activation of the A2A receptors [16].

MATERIALS AND METHODS

Materials

Regadenoson powder was purchased from Royal pharm/China, B-Sitosterol ointment was obtained from Philadelphia/Jordan, Formalin (MW= 30.3) was provided by SOLVOCHEM/UK, castor oil was obtained from Jayant Agro-organics LTD, India and petrolatum base was provided by Regent chemicals, India.

Methods

Preparation of topical regadenoson ointment

Since regadenoson is unavailable in a topical dosage form for skin application, the first step was to prepare a topical dosage form. The selection of a suitable base for the preparation is depending on the physicochemical properties of regadenoson and its compatibility with chosen base. Two hundred fifty and five hundred milligrams of regadenoson powder was weighed separately and then, dispersed in castor oil (because regadenoson is water-insoluble compound) using spatula to obtain a smooth non-gritty dispersion. Afterwards, this dispersion was mixed well with petrolatum base by incorporation method to complete the required weight of 100 gm. In incorporation method, a small amount of petrolatum base was mixed carefully with a small volume of regadenoson dispersion for about 5 minutes until a clear homogenized ointment was obtained. The prepared regadenoson ointment was then transferred into clean and sterile plastic cups which were closed tightly and stored at normal room temperature.

Experimental animal groups

Sixty mice were involved in the experiment. Each group contained twelve mice which were selected randomly from the total mice involved in the experiment as illustrated in Figure 1.

Murine wound model

Mice were anesthetized by intraperitoneal injection of "ketamine (100mg/kg)/ xylazine (10mg/kg)". Afterwards, the back-skin hair was shaved using shaving cream and 1-cm full-thickness excisional wound was then made on the back of each mice using a sterile 10-mm biopsy punch [17] as shown in Figure 2.

Wounds size Measurement

Wounds size area for 6 mice from each group were randomly selected and measured by a ruler from edge to edge at 5th and 10th days of the experiment. The difference between wound size reduction between the experimental groups was compared using the following equation:

%Wound closure = (primary wound area - end wound area) / primary wound area * 100%

primary wound area (in day 0) was defined as 1 cm [18].

Wounds area at tenth day were carefully dissected by a sharp, sterile surgical blade. Tissues were collected without folding by forceps which were stabilized in 10% buffered formalin solution and stocked into a sterile plastic container for storage ready for embedding in paraffin wax for histopathological and immunological study.

Assessment of the histopathological changes of tissue sections. (H & E stains)

were used for the staining of paraffin wax sections [19]. The skin histopathological changes for each mouse was scored [20]. The amount of collagen, angiogenesis and re-epithelization scores were made and evaluated as: 0 = absent or a few, 1 = moderate presence, 2 = plenty.

Immunohistochemistry kit for the detection of VEGF-a

The kit was obtained from Abcam life sciences company/ UK.

Evaluation of Immunohistochemistry results

Proper and accurate application of kit instructions of Abcam company leads to the appearance of a brown precipitate in positive cells on tissue sections. The extent of reactions was measured according to the brown color intensity by using the following scale: 0= undetected, 1= low density, 2= medium density, 3=dense and 4= very dense [21].

RESULTS AND DISCUSSION

Preparation of topical regadenoson ointment

Since regadenoson is not yet available in a topical dosage form, the challenge of developing a successful semi-solid topical ointment formulation has been more promising than other topical dosage forms taking into account the properties of the active ingredient (regadenoson) which is water insoluble and the attractive properties of the ointment base (petrolatum) including its ability to cover the site of application for a long time and it will act as a barrier that prevents foreign substances and microorganisms from penetration into the tissue through the wound. In addition, the ointment base increases the hydration of the skin due to its oil nature which prevents moisture evaporation leading to an increase in drug penetration [22]. Furthermore, the prepared regadenoson ointment will be in a similar dosage form when compared to the positive control β-sitosterol ointment, since both of them have been used in an ointment dosage form. This will minimize any influence of the dosage form type on the wound healing process.

Wound size reduction

The data obtained has revealed a highly significant reduction in the wound size of the treatment groups as compared with induced control group (petrolatum group) ($Pa \leq 0.001$). However, there were no significant differences in comparisons between the treatment groups (Pb, $Pc > 0.05$) as shown in Table 1 and Figure 3. This proves the efficacy of regadenoson on the wound size reduction and acceleration of wound closure. Victor-Vega *et al*. and Montesinos *et al*. have found that a selective A2AAR agonist accelerates wound healing in the wild but not knockout mice which are in agreement with the findings of the present study in that an adenosine (A2A) agonist accelerates wound healing [23, 24]. The proposed mechanism for this wound reduction is by a contraction that reduces wound size with a central gravitational motion of dermis and epidermis [25].

Histopathological scores at the tenth day

Re-epithelization score

The data has shown that there was a significant decrease in the petrolatum group in comparison to apparently healthy group (Pa≤ 0.05). While, in all treatment groups, there were no significant differences with the apparently healthy group (Pa>0.05) respectively as illustrated in Table 2.

Furthermore, all treatment groups had a significant increase in re-epithelization score as compared to the petrolatum group (pb≤0.05) respectively. Though, a nonsignificant difference has been demonstrated between the treatment groups as shown in Table 2 and Figure 4. Cronstein explained the expected mechanism that may be involved in increasing re-epithelization by adenosine agonist is that adenosine stimulates macrophage differentiation into M2-type macrophages which promotes wound healing by increasing growth factors such as VEGF that stimulates repair of tissue at sites of injury [26]. Montesinos *et al.* and Valls *et al*. also used histological examination to assess the re-epithelization of the wound and found a significant increase in the CGS-21680 (A2A adenosine agonist)–treated animals as compared to controls, this agreed with the present findings [7, 24].

Angiogenesis score

In a comparison of apparently healthy group with other groups, petrolatum group was significantly lowered in inducing angiogenesis than apparently healthy group (Pa≤0.05). For other treatment groups, it was found that there are no significant differences with apparently healthy group (Pa>0.05) respectively as shown in Table 2. A comparison of petrolatum group with each treatment groups have shown that all treatment groups were significantly higher than petrolatum group (pb≤0.05) as demonstrated in Table 2 and Figure 4. In addition, there were no significant differences in comparisons between the treatment groups (Pc and Pd>0.05) as shown in Table 2. Both regadenoson concentrations of (0.25% and 0.5% w/w) were found to significantly increase angiogenesis compared to petrolatum base group. When compared to β-sitosterol ointment group show rather a higher but statistically insignificant difference than β-sitosterol group suggesting that they have a greater effect on angiogenesis. Feoktistov *et al.* proposed the mechanism of the angiogenic effects of adenosine acting on the subtype (A2A) receptor occurs directly via enhanced endothelial cell migration and indirectly by the increasing VEGF generation by macrophages [27]. Additionally, Linden claimed that adenosine A2A receptor activation inhibits the generation of thrombospondin I, which is a powerful inhibitor of angiogenesis [28].

Collagen score

The results have shown that the C-Collagen score for petrolatum group is significantly lower than apparently healthy group (Pa≤0.05) as illustrated in Table 2. In a comparison of petrolatum group with each treatment groups, it was found that there is a significant increase in β-sitosterol and regadenoson (0.5% w/w) groups (Pb≤0.05) respectively. While there was no significant increase in the regadenoson (0.25% w/w) group (Pb>0.05) as shown in Table 2 and Figure 4. Moreover, there were no significant differences in comparison between treatment groups (Pc and Pd>0.05) as demonstrated in Table 2. Although, there were no significant difference between both regadenoson and β-sitosterol treatment groups, regadenoson has shown a strong effect on collagen synthesis and deposition. Feoktistov *et al.* highlighted that adenosine A2A receptor activation motivates fibroblasts to generate type I and III collagen at a high level comparable to that stimulated by the transforming growth factor [27]. In addition, Valls *et al.* observed that A2AR activation can immediately promote collagen and matrix production [7]. Those results are in agreement with the present findings.

Immunohistochemistry at the tenth day

Vascular endothelial growth factor-alpha

Apparently healthy skin group when compared with other groups, there were significantly higher values in all groups (Pa≤0.05) respectively as shown in Table 3 and Figure 5. A comparison of each treatment group with petrolatum group, there were a significant increase in those groups (Pb≤ 0.05) respectively. The comparison of β-sitosterol group with both regadenoson groups, has revealed that there is a significant increase in both regadenoson groups (Pc≤0.05) respectively as illustrated in Table 3. Valls *et al.* noticed that adenosine A2A receptors activation promotes VEGF production by a macrophage [7]. Additionally, Montesinos *et al*. found that topical usage of adenosine A2A receptor agonists has increased angiogenesis in wounds by stimulating systemic and local VEGF generations [24] which are in agreement with the present findings.

CONCLUSION

Topical application of A2A adenosine receptor agonist (regadenoson ointment) once daily for 10 days on induced wound seems to be more effective in accelerating wound healing in comparison to petrolatum base and comparable in efficacy to β-sitosterol ointment in all parameters scores that have been measured.

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CONFLICT OF INTEREST

None

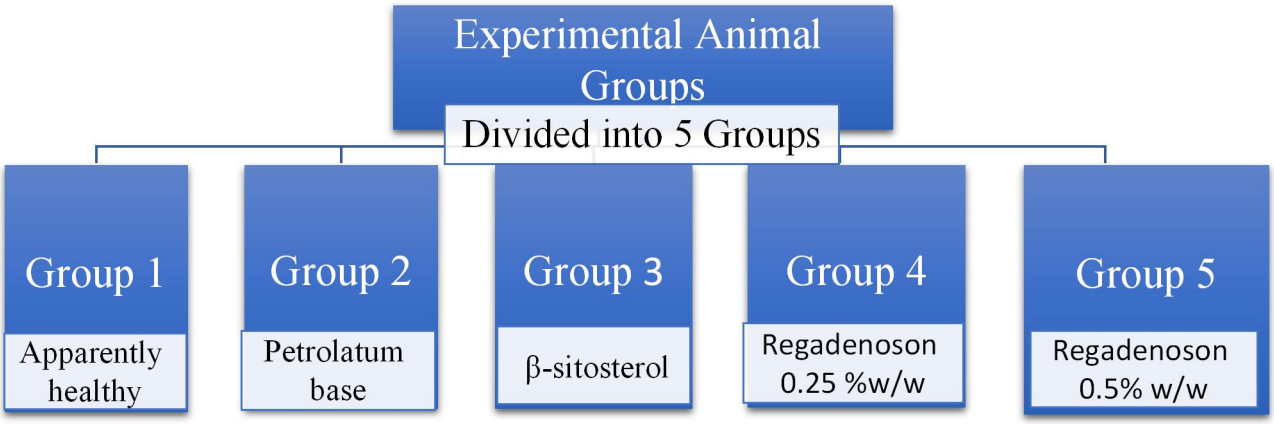

Figure 1. Experimental Animal Groups

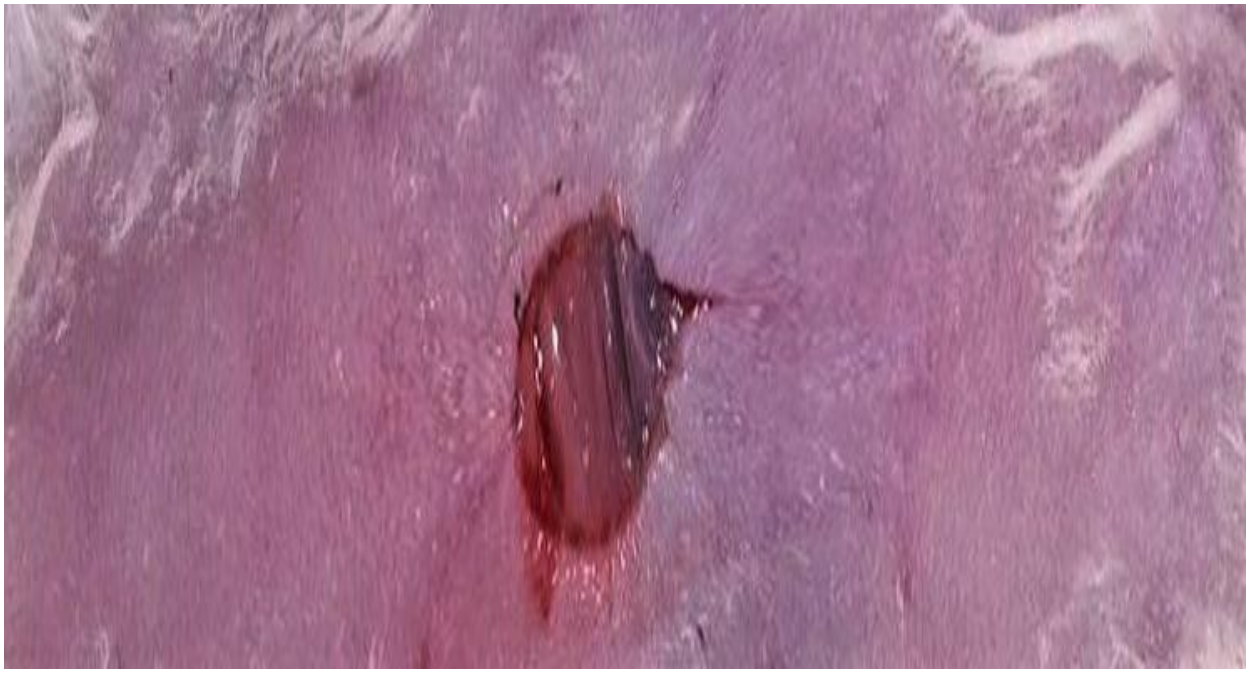

Figure 2. Experimental Wound induction by punch biopsy

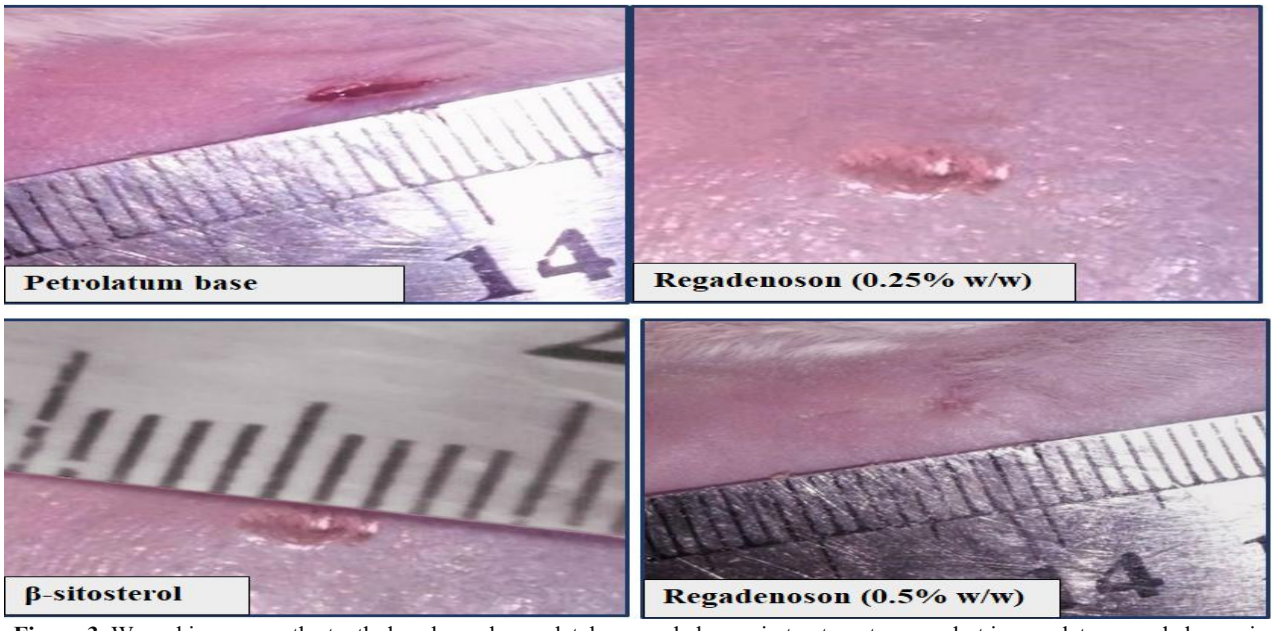

Figure 3. Wound images on the tenth day showed completely wound closure in treatment groups but incomplete wound closure in petrolatum base group.

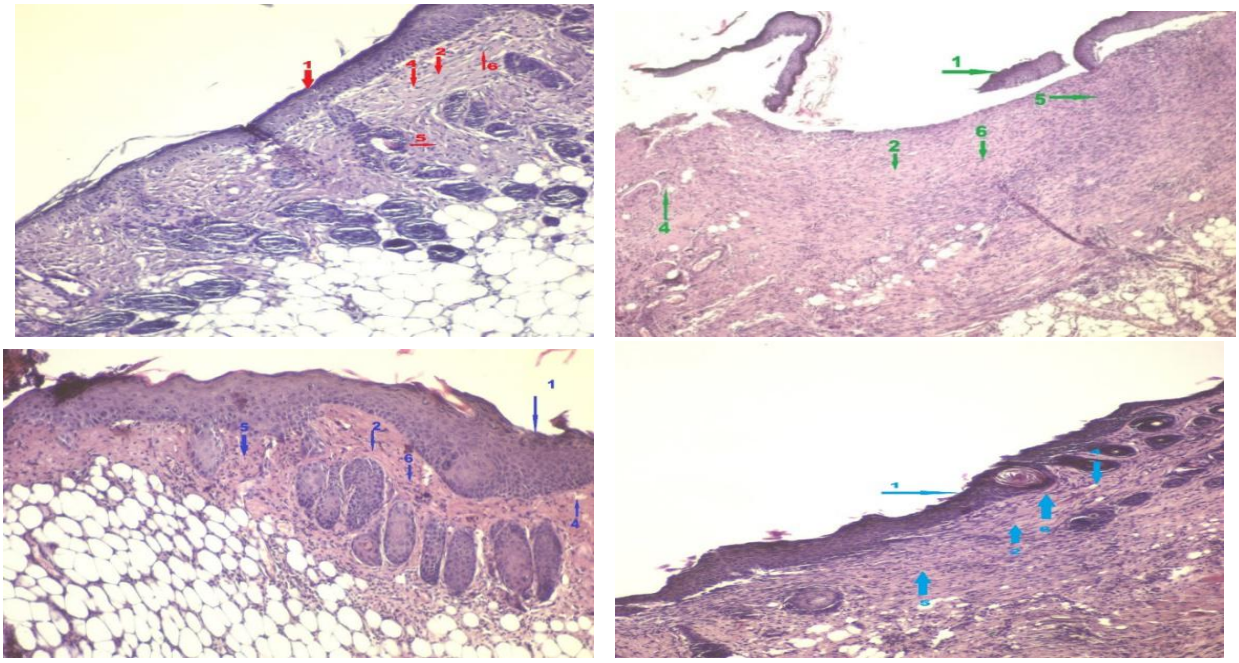

Figure 4. Cross-section of wound tissue at tenth day for petrolatum base (green arrow) showed high presence of inflammatory cells and fibroblasts but mild collagen, angiogenesis and re-epithelization, β-sitosterol (red arrow), regadenoson (0.25%) (bright blue arrow) and regadenoson (0.5%) (blue arrow) groups showed the presence of few inflammatory cells and fibroblasts, high angiogenesis, collagen and re-epithelization on microscopical examination (H&E staining) (10x). 1. Re-epithelialization. 2. Collagen. 3.Hair follicle. 4. Blood vessels. 5. Inflammatory cell. 6. Fibroblast.

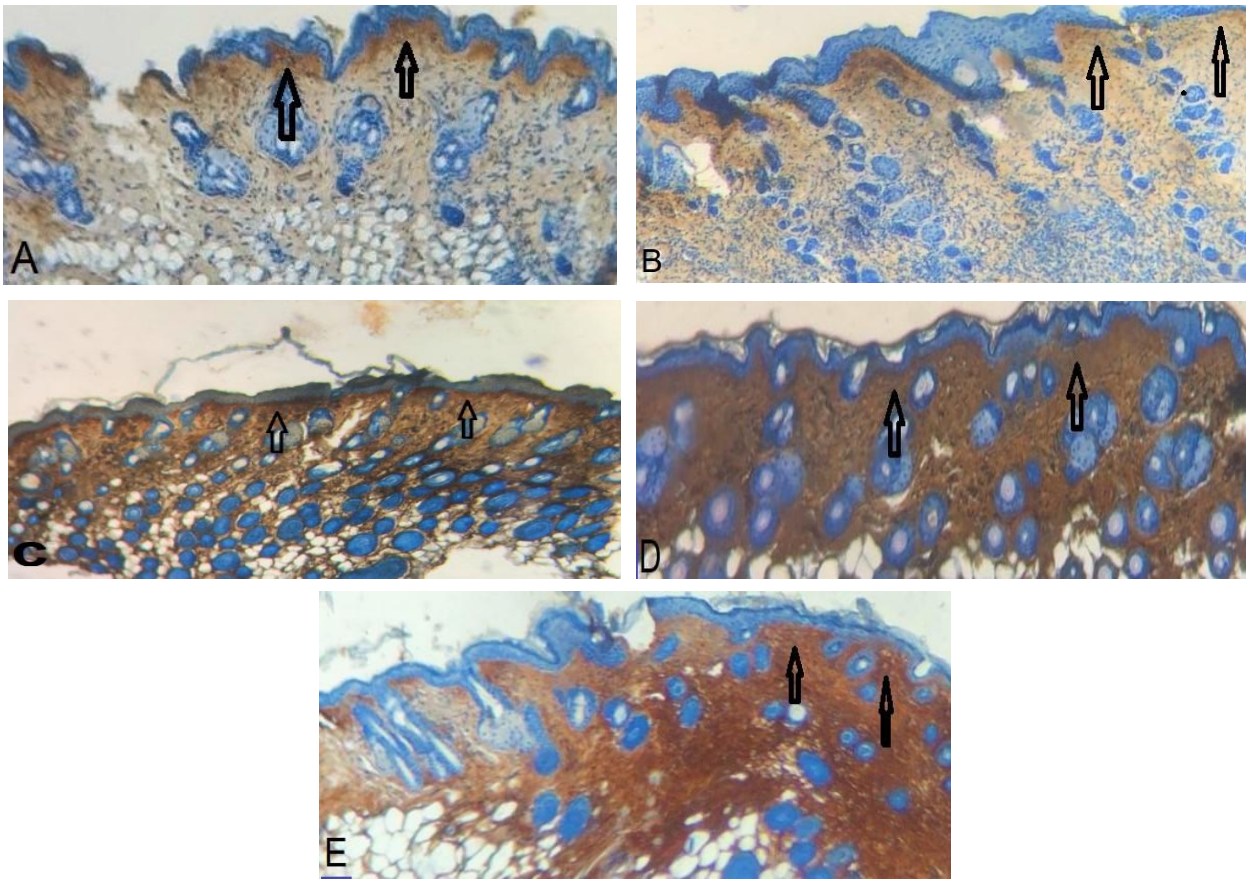

Figure 5. Extracellular immunohistochemical expression of VEGF-A within the dermis at tenth day (10x). A. Normal tissue has shown low intensity (black arrow); B. Induce wound group has shown medium intensity (black arrow); C. β-sitosterol treatment group has shown high intensity (black arrow); D and E. Both regadenoson treatment groups (0.25% and 0.5% w/w respectively) have shown high intensity (black arrow).

Table 1. Comparison of wound size reduction between induced control and each other treatment groups and among other treatment

groups by unpaired t-test.

Day	Wound reduction %	Petrolatum base N=6	β-sitosterol N=6	Regadenoson (0.25%) N=6	Regadenoson (0.5%) N=6
Day 5	Mean±SE	28.33+1.67	48.33+1.67	49.67+1.86	47.67+0.92
	P-value *a*		<0.001	<0.001	<0.001
	P-value *b*			0.605	0.733
	P-value c				0.357
Day 10	Mean±SE	67.5+1.12	98.0+1.0	95.0+1.83	97.67+1.23
	P-value *a*		<0.001	<0.001	<0.001
	P-value *b*			0.180	0.838
	P-value c				0.254

- Data represented as Mean± Standard error (SE).
- N= number of animals from each group tested per day.

(a) Comparison between petrolatum base and each treatment group, (b) Comparison between β-sitosterol and both regadenoson 0.25% and 0.5%, (c) Comparison between regadenoson 0.25% and regadenoson 0.5%.

Table 2. Comparisons of histopathology at tenth day between healthy, induced control, and each other treatment groups and among each treatment group by Mann Whitney test.

Histopath-ology		Apparent-ly healthy N=6	Petrolaum base N=6	β-sitosterol N=6	Regadeno-son (0.25%) N=6	Regadeno-son (0.5%) N=6
Re-epitheliza-tion	Mean±SE	2.0+0.0	1.0+0.0	2.0+0.0	2.0+0.0	2.0+0.0
	Median	2.0	1.0	2.0	2.0	2.0
	P-value *a*		0.002	1.000	1.000	1.000
	P-value *b*			0.002	0.002	0.002
	P-value *c*				1.000	1.000
	P-value *d*					1.000
Angioge-nesis	Mean±SE	2.0+0.0	1.0+0.0	1.83+0.17	2.0+0.0	2.0+0.0
	Median	2.0	1.0	2.0	2.0	2.0
	P-value *a*		0.002	0.699	1.000	1.000
	P-value *b*			0.015	0.002	0.002
	P-value *c*				0.699	0.699
	P-value *d*					1.000
Collagen	Mean±SE	2.0+0.0	1.17+0.17	2.0+0.0	1.67+0.21	2.0+0.0
	Median	2.0	1.0	2.0	2.00	2.0
	P-value *a*		0.015	1.000	0.394	1.000
	P-value *b*			0.015	0.180	0.015
	P-value *c*				0.394	1.000
	P-value *d*					0.394

- Data represented as Mean± Standard error (SE).
- N= number of animals from each group tested per day.

(a) Comparison between healthy and all other groups, (b) Comparison between petrolatum base and β-sitosterol, regadenoson 0.25% and 0.5%, (c) Comparison between β-sitosterol and both regadenoson 0.25% and 0.5%, (d) Comparison between regadenoson 0.25% and regadenoson 0.5%.

Table 3. Comparison of immunological parameter Vascular endothelial growth factor-A (VEGF-A) at tenth day between healthy, induced control, and each other treatment groups and among each treatment group by Mann Whitney test.

Parameter		Healthy N=6	Petrolaum base N=6	β-Sitosterol N=6	Regadenos-on 0.25% N=6	Regadenos-on 0.5% N=6
VEGF-A	Mean±SE	1.0+0.0	2.0+0.0	2.83+0.41	3.67+0.52	3.83+0.41
	Median	1.0	2.0	3.0	4.0	4.0
	P-value *a*		0.002	0.002	0.002	0.002
	P-value *b*			0.005	0.002	0.002
	P-value *c*				0.041	0.009
	P-value *d*					0.699

- Data represented as Mean± Standard error (SE).
- N= number of animals from each group tested per day.

(a) Comparison between healthy group and all other groups, (b) Comparison between petrolatum base and β-sitosterol, regadenoson 0.25% and 0.5%, (c) Comparison between β-sitosterol and both regadenoson 0.25% and 0.5%, (d) Comparison between regadenoson 0.25% and regadenoson 0.5%.

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