Original Article

In vivo Wound Healing Potential of Raloxifene Nanoemulsion Gel for the Management of Postmenopausal Cutaneous Wounds

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ABSTRACT

Background: Depletion in estrogen level(s) especially in postmenopausal women is reported to have delayed wound healing effects; hence we have evaluated the wound healing potential of raloxifene in rat model. **Objectives:** Investigating the wound healing effects of raloxifene nanoemulsion for the management of postmenopausal cutaneous wounds. **Materials and Methods:** The optimized nanoemulsion gel contains 0.072% raloxifene hydrochloride. Female Wistar rats were used to investigate its wound healing effects. After three months of ovariectomy, wound healing effect was observed in terms of breaking strength, tensile strength, area of wound contraction, wound closure time, hydroxyproline content and histopathological changes. **Results:** The nanoemulsion gel exhibited better retention (34.31%) than its nanoemulsion. The raloxifene nanoemulsion gel has no erythema and no eschar formation recorded, and it is safe for topical use. In the incision wound model in ovariectomized rats, breaking (898 ± 25g) and tensile strengths (4.47 ± 0.12 g/mm^2) in raloxifene treated groups were found to be higher than the untreated control group. Additionally, in ovariectomized rats, wound contraction was found to be 100% in the treated group s following 20 days of post-wounding, where as in control group only 88% was contraction was observed. Also, more hydroxyproline content in raloxifene treated ovariectomized rat was observed that recommend more collagen content than the untreated ovariectomized rat but approximately similar effects to untreated non-ovariectomized rats. Histopathological studies confirmed that the raloxifene treated groups had more re-epithelialization, neo-vascularization, fibroblast proliferation, and collagen deposition than the control group. **Conclusion:** These results confirms that the raloxifene nanoemulsion gel has significant wound healing potential, as observed in ovariectomized rats, which will be helpful in postmenopausal cutaneous wound healing.

Key words: Raloxifene, Nanoemulsion gel, Ovariectomized, Postmenopausal, Breaking strength, Wound contraction, Hydroxyproline, Histopathology.

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INTRODUCTION

Wound healing, a multi-phase phenomenon consisting of homeostasis, inflammation, proliferation and remodeling.[1-5] Although these phases are arbitrary and overlap with each other, but these are affected by various systemic and local factors. Systemic factors that indirectly influence delayed healing response in individuals with diabetes, obesity, stress and old age,[6] specifically in elderly women after menopause,[7] while in local factors including infection, oxygenation and foreign bodies that directly affect the delayed wound healing locally.

Menopause is a biological process that occurs in middle-aged women around age 50 years,[8] in whom menstruation ceases which having decreased estrogen levels because of damaged ovarian follicles. Estrogen deficiency induces psychological changes, and postmenopausal women

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suffer from many health-related problems, including loss of fertility,[9] increase risk of osteoporosis,[10] atherosclerosis,[10,11] and delayed woundhealing.[4,12] In postmenopausal women, a variety of healing regimes have been reported for the acceleration of wound healing, but the most important one seems to be the hormone estrogen.[13-16] Interestingly, locally bio-available estrogen levels are reported to be altered due to lack of gonadal hormone secretion, which is particularly significant in postmenopausal women.[7] The reduction in estrogen levels at the menopausal stage has a delayed cutaneous wound healing effect. This delayed wound healing is linked to skin atrophy, decreased collagen level, decreased water content, and a loss of elasticity.[17] Estrogen accelerates healing potential through reducing inflammation, potentiating re-epithelialization, and collagen biosynthesis.[18] It also play a crucial role in maintaining dermal thickness, structural integrity and promoting extracellular matrix collagen levels. Previously, selective estrogen receptor modulators (SERMs) were tested for therapeutic potential where identifying the therapeutic effects of estrogen via the estrogen receptor (ER) was the main aim. SERMs are an innovative drug class, with both agonistic and antagonistic effects.[19] The two predominant ERs, ERα and ERβ, are differentially expressed in different tissues. The differential expression occurs in the ERs due to the involvement of different co-activators, co-repressor and promoter, which exert tissue-specific estrogenic effects.

Raloxifene hydrochloride (RLX), a class of SERMs has been found to be effective for wound healing , especially in ovariectomized rats as similar to postmenopausal women.[4,17,20] RLX strongly induces ERβ expression, while preventing ERα expression. The mechanism of action via tissue-specific estrogenic (agonist) or antiestrogenic (antagonist) is related to the structural differences between the raloxifene-ERα or -ERβ complex and the estrogen-ER complex. Furthermore, *ex vivo* and *in vivo* studies,[4,21] RLX in skin fibroblast cells has proven outcome in collagen biosynthesis, which is an important aspect of effective wound healing.

The development of nanoemulsion gel (NE-gel) has been principally focused for their thermodynamic stability, iso-tropical transparency, and dispersion of two immiscible liquids with a combination of oil and water phase, including surfactant and a co-surfactant.[22] Droplets having nanoscale size are equipped with enhanced physical properties, optical transparency, solubilization capacity, thermodynamic stability and kinetic parameters. These collectively promotes enhanced Brownian motion and reduced destabilization, including coalescence, creaming and sedimentation.[22,23] Interestingly, RLX loaded NE-gel application has been found to be more selective and target localized for the management of wound healing in postmenopausal women. Since due to the minimal metabolic drug disbursement with improved bioavailability, reduced systemic side effects, high penetration into stratum corneum responsible for the intact appearance of RLX within the skin. Moreover, topical application of NE-gel was found to be safe and good alternative for RLX delivery. Also, topical application of a NE-gel delivers localized epidermal sensitivity resulting in wound healing process to elevated thus nourishing the wounded skin more efficiently, as compared to other applications of RLX.[24]

MATERIALS AND METHODS

Materials

Reagents and chemicals of analytical grade were purchased from SD Fine Chem Ltd., Mumbai, India. Raloxifene hydrochloride was procured from Cadila Pharma (Ahmedabad, Gujarat, India), as a kind gift for the present study and with their ethical permission. Labrafil M2125CS, Cremophor RH40 and Transcutol P were received from Gattefosse, India for the present study.

Animals

In this study, female Wistar rats aged 10-weeks were used in experiments (obtained from the animal house at IFTM University, Moradabad, India) and in-housed environment with standard conditions (temperature (24 ± 2°C) and humidity, with 12 hr light and dark period cycles. In addition, they received standard food, rat chow pellets, and water *ad libitum*. Ethical approval for the present study was received from Animal Ethical Committee, IFTM University, Moradabad, India, with an approval no; 2019/837ac/Ph.D./01 following the OECD guidelines.[25,26] A brief sketch of the experiment performed in this study is depicted in Figure 1.

Nanoemulsion gel formulation

The raloxifene nanoemulsion gel for topical delivery was formulated,[27] after selecting the optimized constituents (Labrafil M2125CS, Cremophor RH40 and Transcutol P) with a higher solubility, where O/W nanoemulsion was formulated by using aqueous titration method. Ternary phase diagrams were constructed using obtained titration values to detect the region of nanoemulsion and axis representing phase components. Finally, raloxifene nanoemulsion was found to have 0.072% (w/v) of raloxifene, 14.29% (v/v) of Labrafil M2125CS (oil phase), 33.33% (w/w) of Cremophor

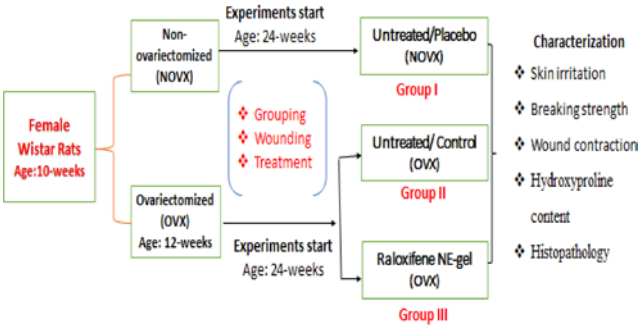

Figure 1: Scheme of the experimental protocol.

Table 1: Optimized nanoemulsion and nanoemulsion-based gel.

Excipients	Nanoemulsion	Nanoemulsion gel
Raloxifene hydrochloride (%w/v)	0.072	0.072
Labrafil M2125CS (%v/v)	14.29	14.29
Cremophor RH40: Transcutol P (Smix, 1:1) (%v/v)	33.33	33.33
Water (%v/v)	52.38	52.38
Carbopol 934 (%w/v)	-	1

RH40 and Transcutol P, as a surfactants mixture in a ration of 1:1, and 52.38% (v/v) of distilled water, optimized parameters are mentioned in Table 1. The nanoemulsion was characterized,[27] for thermodynamic stability, droplets size, zeta potential, viscosity, refractive index, transmission electron microscopy. Further nanoemulsion was changed into nanoemulsion gel by adding 1% w/w Carbopol 934 for suitability in topical application of RLX. The nanoemulsion gel was characterized,[27] for spread ability, viscosity, droplet size, zeta potential.

Permeation study

Ex vivo permeation profile of raloxifene NE-gel was assessed in rats skin, by using a Franz diffusion apparatus (Meditech Technologies, Chennai, India). Rats (200-250 g) were sacrificed as per OECD guidelines. Hairs on the skin surface were removed using deplinetry cream and complete layers of skin were excised. Subcutaneous tissue and residuals of adhering fat in the dermis were removed by surgery and wiped with isopropyl alcohol, respectively. For the histopathological investigations, rat skin in small pieces of appropriate size was mounted in between the compartments of Franz diffusion apparatus. Total studied area was 3.14 cm^2 with a receptor volume of 15 ml. The donor and receiver compartments were filled with acetonitrile: monobasic phosphate buffer (APB, 60:40, v/v), closed with aluminum foil. The assembly was put on a water bath and kept at a temperature of 37 ± 1°C for 8-10 hr with continuous automatic agitation. Raloxifene NE-gel was topically applied on the mounted skin surface and 2 ml of the samples were withdrawn from receiver compartment at fixed time intervals (0, 0.5, 1, 2, 3, 4, 6, 8, 10, and 12 hr). Additionally, similar volume of APB was added to equalize the withdrawn volume from the chambers.[25] Moreover, withdrawal samples were diluted with APB for the measurement of RLX spectrophotometrically at 289 nm.[15,21] During topical application, the drug usually act locally on skin or within the epidermis, hence minimizing the permeation of drug into the systemic circulation which in turn prevents systemic adverse effects. The cumulative permeated drug area ($\mu g/cm^2$) was plotted with respect to time and the flux (Jss, $\mu g/cm^2/hr^1$) was calculated by dividing the slope and diffusion area mounted skin, herein x-intercept representing lag time (t_L, hr). Additionally, the permeability coefficient (*K*p, cm hr^{-1}) of the drug through the mounted skin layer in the donor compartment was calculated as follow.[28-30]

$$\text{Permeability coefficient (Kp)} = \frac{\text{Flux}}{\text{(Drug conc. in donor compartment } \mu g/ml)}$$

$$\text{Cumulative amount of drug permeated} = \frac{\text{Conc. } (\mu g/ml) \times \text{Volume of diffusion cell}}{\text{Area } (cm^2)}$$

Skin retention study

The retention test was carried out for the determination of raloxifene content, which was retained by the skin. A specific amount of raloxifene must be deposited on the skin for optimal wound healing potential. Following the experiment, skin was excised from the diffusion cell and area was measured. The mounted skin (area of 3.14 cm^2) was then washed to remove any excess of the drug, after which it was thinly sliced and vortex in 10 ml of methanol. The skin was then left on a multi-tube vertex shaker (model KVS-50, Electrolab, Mumbai, India) at a temperature of 37 ± 1°C for 24 hr to ensure maximal extraction. After maximum extraction, extracted liquid mixture filtered (0.45 µM) and centrifuged (5000 rpm for 5 min) and the amount of retained raloxifene was measured spectrophotometrically (289 nm).[15,21]

Skin irritation test

Skin irritation test of optimized NE-gel was performed for the safety of the formulation through topical application. It was carried out using Wistar rats weighing 225±25 g. Before experimentation, rats were grouped into groups: Group-I Control (NE-gel) and Group-II

Raloxifene Loaded NE-gel. In a unit dose of raloxifene NE-gel contains 0.05 mg of raloxifene applied to the skin surface. The appearance of erythema and eschar formation was monitored visually for 4 days.[28]

Skin irritation test was predicted by visual inspections and scores were given as follows-

0 : No reaction

1 : Low level erythema or week spotty erythema

2 : Distinct erythema

3 : Moderate erythema- severe erythema

4 : Severe erythema-eschar formation

Ovariectomization of the rat

At 12-weeks, all the rats were subjected to non-ovariectomized (NOVX) and ovariectomized (OVX). The rats (weighing 200±25 g) were anesthetized using ketamine (0.5 ml kg^{-1}; intraperitoneal injection),[9,31,32] back hair were removed using an electric shaver, and the shaved area was cleaned by antiseptic solution containing chlorhexidine and 70% ethanol. Then the povidone-iodine solution was used to disinfect the shaved skin area. At a high degree of aseptic conditions, an incision of 2 cm was made by using surgical scalpel blade (11 no.), on the both sides (right and left) from the middle part of the abdomen.[31] After opening the peritoneal cavity, both ovaries were removed and then operated peritoneal cavity layers were stitched by an absorbable suture (Polyglactin 910, Microcryl Fast), whereas incised skin was sutured by using a non-absorbable suture (Ethicon-Mersilk NW5003). The povidone-iodine solution as a disinfectant was applied after stitching on the sutured skin surface.[31] After the surgery, rats were housed separately in a rat cage in such a way that they maintained clean and dry sheets using 100% sterilized cotton to avoid hypothermia, prevent contamination, and allow a complete recovery.[31]

Wound healing activity

After 12-weeks of ovariectomization, rats were separated based on treatment into three groups, Group I as Control-OVX (untreated), Group II as Sham-NOVX (untreated) and Group III as OVX (treated with RLX NE-gel, 0.072% w/v) with six rats in each group.[33,34] The rats were anesthetized as described above and following the anesthesia, rats were wounded according to the linear incision and circular excision wound model.[13] The NE-gel containing RLX 0.05 $mg.kg^{-1}$,[34] was applied topically twice a day until complete healing was achieved.

Incision wound model

Rats from the different groups were anesthetized and the back hairs were as removed using an electric shaver and again an incision wound (2.5 cm) was made as per details mentioned above. Two para-vertebral linear incisions were made with the full thickness on both sides at a distance 1.5 cm from the vertebral column.[35] After incision, both ends of wounds were sutured by using the material detailed above at a 0.5 cm distance. All groups were treated with NE-gel according to the protocol until complete healing was achieved. All sutures and threads were detached on day 12 of post-wounding. On day 16, the breaking and tensile strength,[36-39] of wound upon healing was measured through pulling the skin strip up to of wound separation begin with a tensiometer.[40,41]

$$\text{Tensile strength} = \frac{(\text{Breaking strength (g)})}{(\text{Cross sectional area } (mm^2))}$$

Excision wound model

In excision wound model, we have estimated the area of wound contraction and wound healing closure time. Again, rats were anesthetized and a circular (diameter of 16 mm) skin layer was excised dorsally from the thoracic region with the help of 5 mm biopsy punch.[42,43] All the wounded areas of each rat were treated according to protocol using NE-gel (RLX, 0.05 $mgkg^{-1}$), and topically applied (twice in a day) until the complete healing . Post-wounding, the excised wound area was measured at different day's interval (mentioned below) by sketching the wound margin on transparent paper. The changes in the area of wound contraction was calculated using graph paper.[44-46]

$$\%\text{ of wound contraction} = \frac{\text{Wound area on day 0} - \text{Wound area on day (n)}}{\text{Wound area on day 0}} \times 100$$

Where, n are days (4^{th}, 8^{th} 12^{th}, 14^{th}, $16^{th}$and 20^{th}) post wound development.

The hydroxyproline assay

Hydroxyproline, an amino acid found in collagen and is a major collagen component. It is biosynthesized by post-translational hydroxylation of proline in the presence of prolyl hydroxylase which is among prominent biomarkers to measure the extent of wound healing. Herein, total collagen content was analyzed through estimation of hydroxyproline content in wounded tissues.[47] On day 16 of the post-wounding, hydroxyproline content was estimated through a section of healed skin area. Hot air oven was used to dried (at 60°C) the tissues up to constant tissues mass (20 mg) and then was thermo-hydrolyzed (at 120°C) in 5 ml of 6N HCl for 3 hr in covered tubes, whereas acid hydrolysate was neutralized

by adding 5 ml of 6 N NaOH. The solution was diluted 10-fold with distilled water and 2 ml from this was mixed 1 ml of chloramine-T solution. Cloramine solution was prepared by mixing these reagents viz., 0.05M chloramines T, H_2O 74 % (v/v), 2-propanol 26 % (v/v), 0.629 M NaOH, 0.140 M citric acid, 0.453 M sodium acetate and 0.112 M acetic acid), this solution was incubated at 28°C for 20 min. Onwards this, 1 ml of 0.4M perchloric acid was added to the above solution and again the mixture was further incubated for 5 min at 28°C. Finally, 1ml of Ehrlich's reagent (containing 1 M *p*-dimethylaminobenzaldehyde and prepared in 30% (v/v) HCl and 70% (v/v) 2-propanol) was added again the mixture was incubated for 20 min at 60°C. Upon cooling, the final mixture was used to measure hydroxyproline content spectrophotometrically (558 nm).[47] Standard solution: Hydroxyproline stock solution (100 µg/ml in DDW) was serially diluted by diluting 100, 200, 300,400, 500, and 600 µl of stock solution with distilled water in separate test tubes. In these tubes, chloramine T solution (1 ml) was added, mixed and incubated for 20 min at 28°C. Onwards this, 1 ml of perchloric acid (0.4M) was added to neutralized excess amount of chloramine T. After 10 min, 1 ml of Ehrlich's reagent was added to each test tube and solution was mixed by shaking the tubes. Finally, added the distilled water to each of the test tubes to make a total volume of 10 ml. The resulting mixture contained 1, 2, 3, 4, 5 and 6 µg of hydroxyproline respectively. The resulting mixture was measured spectrophotometrically at 558 nm.[13,47]

Histopathology

Rats were anesthetized as per the methods and protocols mentioned above. A circular excision wound was made dorsally in the thoracic region, with the help of a 5 mm biopsy punch.[42,43] All the wounded areas of each rat were treated according to protocol using NE-gel (RLX, 0.05 $mgkg^{-1}$; applied topically twice a day) until the complete healing of the wounds. Cross-sectional skin specimens were collected from each group on day 20 (post-wounding) and fixed in 10% buffered formalin. The collected tissue specimens were cut into thick sections (5µm) for therefore mounted on glass slides using H&E stain (hematoxylin and eosin).[48] A light microscope (Olympus CX41) was used to examine the mounted tissues, and epidermal or dermal remodeling was evaluated and graded in terms of re-epithelialization in the epidermis, fibroblast proliferation, granulation formation, neo-vascularization, and collagen synthesis in the dermis.

RESULTS AND DISCUSSION

Ex vivo permeation and retention analysis

Nanoemulsions were subjected to a penetration study through wounded skin.[25] Ideally, the drug's effective concentration would be maintained within the applied area for a long duration to achieve a good therapeutic effect. The flux and permeability coefficient of raloxifene were significantly higher in NE than its NE-gel, suggests a reduced skin permeation rate of NE-gel (Table 2). Upon investigation, retention of raloxifene in the skin was higher in NE-gel (34.31%) as compared to NE (21.56%), which may be due to gel form providing longer contact duration on skin surface (Table 3, Figure 2). Higher skin retention is preferable to permeation for topical effect in wound healing. Therefore, the NE-gel form was more suitable for topical delivery of raloxifene as it exhibited localized epidermal nourishment resulting in the induction of wound healing in particular skin area.

Skin irritation test

In this study, raloxifene nanoemulsion gel did not show visible signs of skin irritation (erythema and eschar). Additionally, only rubefaction was visible on skin area in some rats only on day 1st and 2nd, which also disappeared before the last day of experiment. The mean irritation score was found to be 0.29 and determined that the non-irritant potential of the raloxifene NE-gel. The results

Table 2: *Ex vivo* permeability parameters of optimized formulation across the wistar rat skin after 12 hr.

Optimized formulation	Flux (*Jss*, µg cm^{-2} hr^{-1}) ± SD	Permeability co-efficient (*Kp*, cm hr^{-1}) ± SD
NE	16.28 ± 0.7	0.023 ± 0.003
NE-gel	11.96 ± 0.4	0.017 ± 0.001

Values represented as mean ± standard deviation (SD), n = 3, *Jss* = Steady-state transcutaneous flux of raloxifene was obtained by dividing the slope with the permeated area, *Kp* =Permeability coefficient: was obtained by dividing flux with the initial concentration of the drug in the donor compartment.

Table 3: Skin retention and permeation studies of developed raloxifene nanoemulsion gel.

Formulation	Conc. in µg/ml	Amount of drug retained in skin (µg) (mean ± SD) (n=3)	% of Raloxifene retained in skin	% of Raloxifene permeated through skin
NE	205.80	770.81 ± 28.84	21.56	26.68
NE-gel	245.29	1226.46 ± 39.23	34.31	22.38

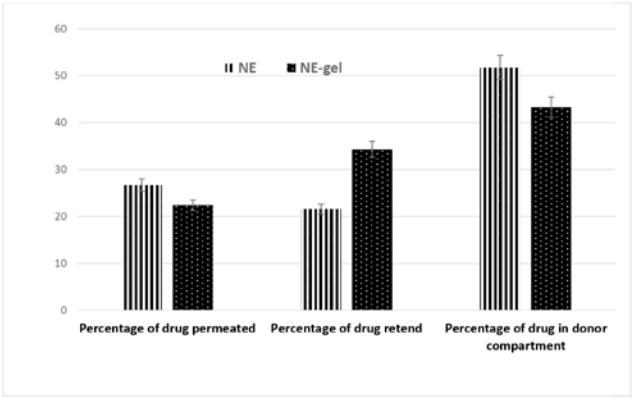

Figure 2: *Ex vivo* permeation and retention studies of different raloxifene formulations.

Table 4: Skin Irritation study of optimized nanoemulsion gel.

Group	Average irritation score (Erythema and Eschar) formation on visible inspection each days*				Mean irritation Score
	Day 1	Day 2	Day 3	Day 4	
Control-OVX (Placebo NE-gel)	0.50	0.33	0.17	0	0.25
OVX (Raloxifene NE-gel)	0.67	0.33	0.17	0	0.29

*n = 6 Wistar rats in each group
Skin irritation score denoted as- No erythema 0, Low level erythema or week spotty erythema 1, Distinct erythema 2, Moderate to severe erythema 3 and Severe erythema to eschar formation 4.

Table 5: Formulations' effect on the breaking and tensile strength of the skin at day16 of post-wounding.

Treatment	Group	Breaking strength Mean ± SD. (g)	Tensile strength (g/mm²)
Placebo NE-gel	NOVX	783 ± 20	3.90 ± 0.10
	Control-OVX	552 ± 15	2.75 ± 0.07
Raloxifene NE-gel	OVX	898 ± 25	4.47 ± 0.12
Normal Skin	Unwounded	1300 ± 40	6.47 ± 0.20

Determining the breaking and tensile strength of the wounded skin of different groups with respect to normal (unwounded) skin viz. group-I NOVX (placebo NE-gel), group-II control-OVX (placebo NE-gel) and raloxifene NE-gel treated group OVX.
n = 6 rats in each group

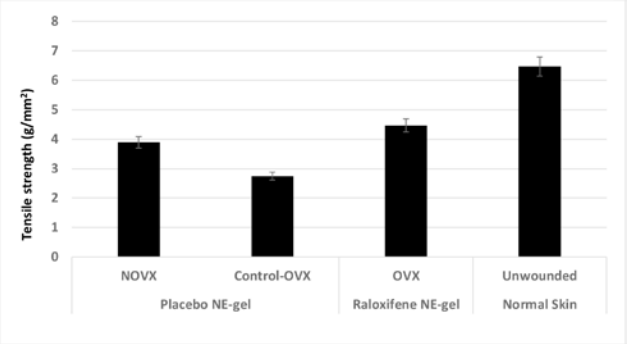

Figure 3: Effect of a topically applied raloxifene nanoemulsion gel on skin, the tensile strength of female (NOVX and OVX) Wistar rats on day 16 of post-wounding. The effect was compared to untreated (NOVX and Control-OVX) and Raloxifene treated OVX with respect to unwounded skin.

suggest that raloxifene nanoemulsion gel was safe for topical delivery (Table 4).

Tensile strength

On day 16 of post wounding, the breaking and tensile strength were determined by specific treatment of different groups of rats, Group I untreated (NOVX), Group II untreated(control-OVX) and Group III OVX rats treated with 0.05 mg of raloxifene loaded NE-gel,[34] were applied topically throughout the period twice daily. The breaking strength and tensile strength parameters are depicted in Table 5 and Figure 3, where untreated NOVX and raloxifene NE-gel treated OVX groups breaking strength were found to be 783 ± 20 g and 898 ± 25 g respectively, compared to control OVX group 552 ± 15 g. The breaking strength of raloxifene treated OVX rat had significantly higher breaking strength than control-OVX while untreated NOVX rat had slightly less or nearly equal to the raloxifene treated OVX rat. That's proved that raloxifene has wound healing potential in OVX rats. Also, none of the constituents of gel (i.e., oil, surfactant and co-surfactant) have shown the ability of wound healing except raloxifene. It clears that wound healing ability more in NOVX equivalent with premenopausal female than OVX equivalent with the postmenopausal female. According to the above test data, we conclude that the higher the breaking strength more is the collagen fiber deposition to the wounded tissue consequently faster the wound healing ability.

Percentage of wound contraction

The extent of raloxifene's effect on wounded skin in Wistar female (NOVX and OVX) rats was determined by topical application of raloxifene-loaded NE-gel. The area of wound contraction was measured post-wounding (on day 4, 8, 12, 16, and 20) in all the groups, shown in Table 6, Figure 4 (A, B). In untreated NOVX and raloxifene NE-gel treated OVX female rats; the extent of wound contraction was nearly equal from days 4 to 20 of post-wounding, as shown in Figure 4. In excision wound model, the contraction percentage

Table 6: Effect of the formulations on the area of wound contraction in the circular excision wound model.

Days of post-wounding	Wound contraction area (%)		
	NOVX (Placebo NE-gel)	Control-OVX (Placebo NE-gel)	OVX (Raloxifene NE-gel)
4	20.89	16.25	22.56
8	56.54	30.33	64.49
12	71.02	54.74	88.15
16	93.10	73	96.52
20	100.00	88	100.00

Contraction of excision wound area of different rats grouped- Group-I NOVX (treated with placebo NE-gel), Group-II control-OVX (placebo NE-gel) and Group-III OVX (treated with raloxifene NE-gel).

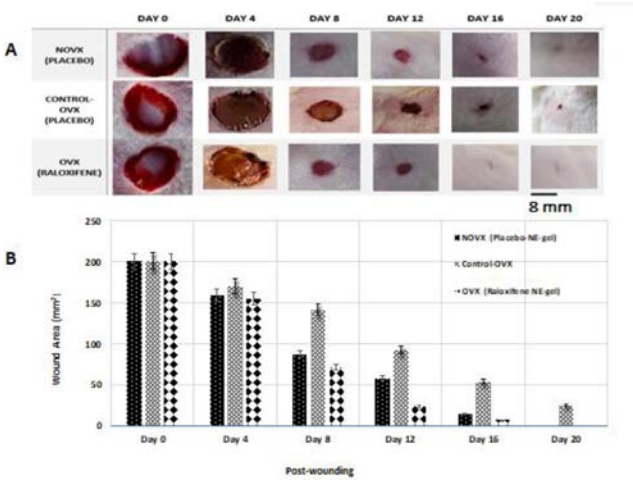

Figure 4: Influence of raloxifene in wound contraction effect: (A) Circular wounds of nearly 16 mm in diameter were excised dorsally from the thoracic region of rats. All the wounded area of rats were treated topically with 0.072% w/w of raloxifene as nanoemulsion gel. Bar, 8 mm. (B) Wound area (mm^2) of contraction in untreated NOVX (placebo NE-gel), control-OVX (placebo NE-gel) and 0.05 mg. Kg^{-1} raloxifene treated OVX rats on days 0, 4, 8,12,16, and 20 of post-wounding. Values are mean ± S.E. for six different observations.

in ovariectomized rats increased in the all groups, but more rapidly in raloxifene-treated group, compared to control (Figure 4). The wound closure rate significantly decreased from days 0 to 4. However, treated groups shown faster closure of wounds as compared to the untreated groups from days 4 to 8. A 100% wound closure was achieved on day 20 of post-wounding in the NOVX and raloxifene-treated OVX rats, whereas only 88% was observed in the control group. This is because the non-ovariectomized rats maintain estrogen content in the skin. This result also demonstrated that except raloxifene, none of the components of nanoemulsion gel have wound healing properties. The nanoemulsion gel with raloxifene applied topically to wounded skin displays significant acceleration of wound contraction. The above result proved that raloxifene had significant properties for wound healing in OVX (postmenopausal) female rats which might be due to contracting the wound area, accelerating wound healing and accelerating re-epithelialization.

Hydroxyproline content

The hydroxyproline content was assessed in the excisional wound model of OVX female rats at days 4, 12, and 20 of post-wounding, given in Table 7. There was a consistent increase in hydroxyproline concentration from day 4 to 20 of post-wounding if raloxifene was applied topically in the OVX female rat. But, on day 20, the raloxifene-treated OVX rats exhibited a higher content of hydroxyproline of 43.63 µg/gram tissue weight, whereas in control on day 20 is 29.90µg/gram tissue weight. Throughout healing, hydroxyproline content was found to be elevated in raloxifene treated group, comparing to the control group.

Histopathology

The histopathological examination of the excised wound was evaluated and scored of healing processes (fibroblasts proliferation, neo-vascularization, re-epithelialization, collagen deposition) and healing phase (inflammation, proliferation, and remodeling). The results were summarized and compared between the raloxifene treated OVX and the untreated (NOVX and OVX) groups in Table 7. Histopathological examination of normal skin tissue showed epidermis layer and dermis layer with blood vessels, hair follicles, and collagen (Figure 5). In untreated NOVX female rats wound tissues, there were slow regular healing processes indicates re-epithelialization, fibroblast proliferation, collagen fiber synthesis, regeneration of hair follicle, neo-vascularization, formation of the mononuclear cell (Figure 6) as graded in Table 8. In comparison to control-OVX wound, tissues showed persistent inflammation, little proliferation and slow re-epithelialization occur that indicate poor healing is shown in Figure 7, while in

Table 7: Effect of topical application of raloxifene nanomeulsion gel for estimation of hydroxyproline content in the wounded skin.

Treatment	Group	Hydroxyproline content (µg/gram tissue)		
		d(4)	d(12)	d(20)
Placebo NE-gel	NOVX	16.17	26.32	41.84
	Control-OVX	9.00	19.75	29.90
Raloxifene NE-gel	OVX	14.97	29.30	43.63

raloxifene treated OVX rats exhibited increased collagen deposition, increased re-epithelialization, increased neo-vascularization, indicative of high wound healing rate Figure 8. In respect to the above result, the untreated OVX group recorded more inflammation, progression and remodeling than the untreated OVX group, while the untreated NOVX group give a nearly similar result. This is because ovariectomized rats (similar to the menopausal stage) unable to maintain effective estrogen levels. This result also proved that raloxifene has wound healing properties in the estrogen deficient group.

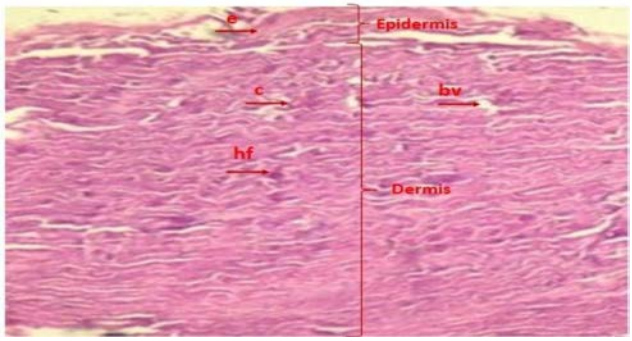

Figure 5: Histopathological examination of normal skin tissue. Photograph of histological section of skin layer show (a) Epidermis- re-epithelial cell (b) Dermis- bv-blood vessels, hf-hair follicles, c-collagen fibre. (Hematoxylin and Eosin stain).

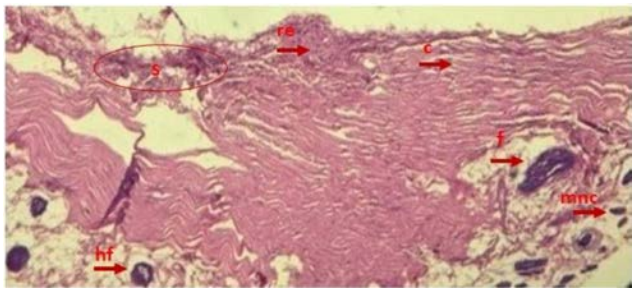

Figure 7: Histopathological examination of excise skin on day 20 of post-wounding tissue in untreated control-OVX rat. Histological section shown re-epithelialization but slower migration over the granulation tissue. Hair follicle can also seen, granulation tissue contains less collagen, and fibroblasts, presence of mononuclear cells. Circular area show vide scar between the two edge of wound closure and presence of inflammatory cell near scar. Arrow of tip show: re-re-epithelialisation, c-collagen fibre, hf-hair follicle, f- fibroblast, mnc- mononuclear cells and s- scar.(H&E stain).

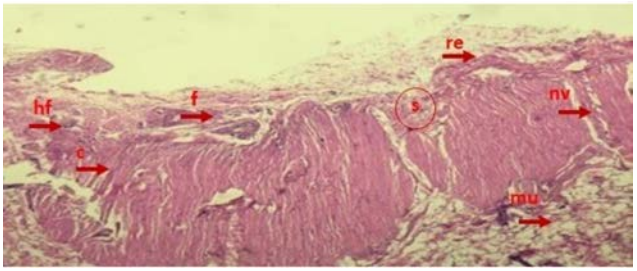

Figure 6: Histopathological examination of excise skin on day 20 of post-wounding tissue in untreated NOVX rats. Histological section shows re-epithelialization that fills the wound bed after skin wounding. The granulation tissue contains more collagen, fibroblasts and neo-vascularization, small scar and absence of inflammatory cell near scar. Skin appendage hair follicle can also be seen. Arrow of tip show: re- re-epithelialisation, hf- hair follicle, f- fibroblasts, c- collagen fibre, mu- muscle layer below dermis, nv-neo-vascularization ands- scar tissue. (H&E stain).

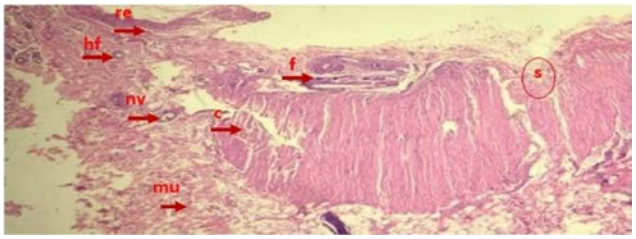

Figure 8: Histopathological examination of excise skin on day 20 of post-wounding tissue in raloxifene NE-gel treated-OVX rats. Histological section show more re-epithelialization, well developed hair follicle, granulation tissue contains more collagen, fibroblasts and neo-vascularization, while circular area show very small scar at the wound closure edge and absence of inflammatory cell near scar. Arrow of tip show: re-epithelialisation, c- collagen fibre, nv- neo-vascularisation, h- hair follicle, f- fibroblast, mu- muscle layer and s- scar. (H&E stain).

Table 8: Wound healing processes and healing phases s in different groups of treatment at day 20 of post-wounding.

Groups	Wound healing processes							Healing phases		
	S	RE	F	C	MNC	NV	HF	I	P	R
NOVX (Placebo)	+	++	+	++	-/+	++	+	-	+	-/+
Control-OVX (Placebo)	+++	+	++	-/+	++	+/-	+	++	++	+
OVX (Raloxifene)	+	++	++	++	-/+	+++	++	-	+	-/+

H-E (Haematoxylin and eosin) stained segments were marked as absent (-), mild (+), Moderate (++) and Severe (+++) for epidermal and dermal remodelling, wound healing processes denoted as: s- scar tissue, re- re-epithelialization, hf- hair follicle, f- fibroblasts, c- collagen fibre, mnc- mononuclear cell, nv- neo-vascularization and Healing phases denoted as: i- inflammation phase, p- proliferation phase and r- remodelling phase.

CONCLUSION

In this study, by applying the raloxifene hydrochloride nanoemulsion gel topically, the delayed cutaneous wound healing response in the OVX rats was speed up. *In vivo* studies of the optimized raloxifene nanoemulsion gel formulation also revealed significant increase in various strengths of skin (breaking and tensile strength), a reduction of the wound area, and an increase of the hydroxyproline concentration, which consequently increased the collagen content in OVX rats' wound tissue. The aim of this study was to evaluate whether raloxifene applied topically has potential in the management of wound healing in estrogen-deficient OVX female rats. The finding of the present study clearly suggests that *ex vivo* and *in vivo* use of nanoemulsion gel of raloxifene significantly influence the collagen deposition, wound contraction and re-epithelization of excised wounds. Histopathological examination also confirms that the raloxifene treated OVX rats have greater accelerated wound healing potential in re-epithelialization, neo-vascularization, fibroblast proliferation, and collagen deposition than the control group. These findings have clear implications for our study, which shows that a topical application of raloxifene hydrochloride nanoemulsion gel has a potential effect in the management of postmenopausal cutaneous wound healing.

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CONFLICT OF INTEREST

This study and its results have none conflict of interest.

ABBREVIATIONS

RLX: Raloxifene hydrochloride; **NOVX:** non-ovariectomized; **OVX:** ovariectomized; **µg:** micro gram; **µm:** micro meter; **ml:** milli liter; **nm:** nanometer; **hr:** hour **rpm:** revolution per minute; **g:** gram; **mg:** milligram; **ºC:** degree centigrade; **cm:** centimeter.

REFERENCES

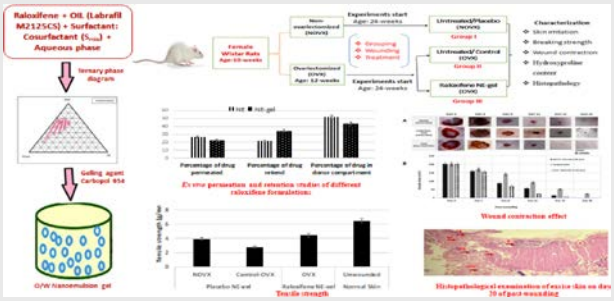
1. Janis JE, Harrison B. Wound healing: Part I. Basic science. Plast Reconstr Surg. 2016;138(3);Suppl:9S-17S. doi: 10.1097/PRS.0000000000002773, PMID 27556781.
2. Horsley V, Kulesz-Martin M, Wang XJ. Montagna Symposium 2015: Harnessing Stem Cells to Reveal Novel Skin Biology and Disease Treatments. J Invest Dermatol. 2016;136(5):893-6. doi: 10.1016/j.jid.2016.01.022. PMID 27107373.
3. Tsai HW, Wang PH, Tsui KH. Mesenchymal stem cell in wound healing and regeneration. J Chin Med Assoc. 2018;81(3):223-4. doi: 10.1016/j.jcma.2017.06.011, PMID 28716605.
4. Hardman MJ, Emmerson E, Campbell L, Ashcroft GS. Selective estrogen receptor modulators accelerate cutaneous wound healing in ovariectomized female mice. Endocrinology. 2008;149(2):551-7. doi: 10.1210/en.2007-1042, PMID 17974625.
5. Horsley V, Watt F. Repeal and replace: Adipocyte regeneration in wound repair. Cell Stem Cell. 2017;20(4):424-6. doi: 10.1016/j.stem.2017.03.015, PMID 28388424.
6. Marroni A, Cronje FJ, Meintjes J, Cali-Corleo R. Dysbaric illness. Vol. 9781402044, Handbook on Hyperbaric Medicine; 2006. p. 173-216.
7. Archer DF. Postmenopausal skin and estrogen. Gynecol Endocrinol. 2012;28;Suppl 2:2-6. doi: 10.3109/09513590.2012.705392, PMID 22849791.
8. Stanford JL, Hartge P, Brinton LA, Hoover RN, Brookmeyer R. Factors influencing the age at natural menopause. J Chronic Dis. 1987;40(11):995-1002. doi: 10.1016/0021-9681(87)90113-5, PMID 3654908.
9. Parhizkar S, Ibrahim R, Latiff LA. Incision choice in laparatomy: A comparison of two incision techniques in ovariectomy of rats. World Appl Sci. 2008;4(4):537-40.
10. Lasota A, Danowska-Klonowska D. Experimental osteoporosis-different methods of ovariectomy in female white rats. Rocz Akad Med Bialymst. 2004;49;Suppl 1:129-31. PMID 15638397.
11. Sato M, Rippy MK, Bryant HU. Raloxifene, tamoxifen, nafoxidine, or estrogen effects on reproductive and nonreproductive tissues in ovariectomized rats. FASEB J. 1996;10(8):905-12. doi: 10.1096/fasebj.10.8.8666168, PMID 8666168.
12. Gilliver SC, Ruckshanthi JPD, Hardman MJ, Nakayama T, Ashcroft GS. Sex dimorphism in wound healing: The roles of sex steroids and macrophage migration inhibitory factor. Endocrinology. 2008;149(11):5747-57. doi: 10.1210/en.2008-0355, PMID 18653719.
13. Ashcroft GS, Greenwell-Wild T, Horan MA, Wahl SM, Ferguson MWJ. Topical estrogen accelerates cutaneous wound healing in aged humans associated with an altered inflammatory response. Am J Pathol. 1999;155(4):1137-46. doi: 10.1016/S0002-9440(10)65217-0, PMID 10514397.
14. Hardman MJ, Ashcroft GS. Estrogen, not intrinsic aging, is the major regulator of delayed human wound healing in the elderly. Genome Biol. 2008;9(5). doi: 10.1186/gb-2008-9-5-r80.
15. Ashcroft GS, Dodsworth J, Van Boxtel E, Tarnuzzer RW, Horan MA, Schultz GS, *et al*. Estrogen accelerates cutaneous wound healing associated with an increase in TGF-β1 levels. Nat Med. 1997;3(11):1209-15. doi: 10.1038/nm1197-1209, PMID 9359694.
16. Ashcroft GS, Mills SJ, Lei K, Gibbons L, Jeong MJ, Taniguchi M, *et al*. Estrogen modulates cutaneous wound healing by downregulating macrophage migration inhibitory factor. J Clin Invest. 2003;111(9):1309-18. doi: 10.1172/JCI16288, PMID 12727922.
17. Mukai K, Urai T, Asano K, Nakajima Y, Nakatani T. Evaluation of effects of topical estradiol benzoate application on cutaneous wound healing in ovariectomized female mice. PLOS ONE. 2016;11(9):e0163560. doi: 10.1371/journal.pone.0163560, PMID 27658263.
18. Stevenson S, Sharpe DT, Thornton MJ. Effects of oestrogen agonists on human dermal fibroblasts in an *in vitro* wounding assay. Exp Dermatol. 2009;18(11):988-90. doi: 10.1111/j.1600-0625.2009.00864.x, PMID 19469894.
19. Jordan VC. SERMs: Meeting the promise of multifunctional medicines. J Natl Cancer Inst. 2007;99(5):350-6. doi: 10.1093/jnci/djk062, PMID 17341726.
20. Mahdavian Delavary BM, Van der Veer WM, Van Egmond M, Niessen FB, Beelen RHJ. Macrophages in skin injury and repair. Immunobiology. 2011;216(7):753-62. doi: 10.1016/j.imbio.2011.01.001, PMID 21281986.
21. Surazynski A, Jarzabek K, Haczynski J, Laudanski P, Palka J, Wolczynski S. Differential effects of estradiol and raloxifene on collagen biosynthesis in cultured human skin fibroblasts. Int J Mol Med. 2003;12(5):803-9. doi: 10.3892/ijmm.12.5.803, PMID 14533013.
22. Chen H, Mou D, Du D, Chang X, Zhu D, Liu J, *et al*. Hydrogel-thickened microemulsion for topical administration of drug molecule at an extremely

low concentration. Int J Pharm. 2007;341(1-2):78-84. doi: 10.1016/j.ijpharm.2007.03.052, PMID 17570625.
23. Shafiq S, Shakeel F, Talegaonkar S, Ahmad FJ, Khar RK, Ali M. Development and bioavailability assessment of ramipril nanoemulsion formulation. Eur J Pharm Biopharm. 2007;66(2):227-43. doi: 10.1016/j.ejpb.2006.10.014, PMID 17127045.
24. Puro D, Athawale R, Pandya A. Design, optimization and characterization of nanostructured lipid carriers of raloxifene hydrochloride for transdermal delivery. NANOASIA. 2018;10(1):57-67. doi: 10.2174/2210681208666181106124337.
25. Hardman MJ, Waite A, Zeef L, Burow M, Nakayama T, Ashcroft GS. Macrophage migration inhibitory factor: A central regulator of wound healing. Am J Pathol. 2005;167(6):1561-74. doi: 10.1016/S0002-9440(10)61241-2, PMID 16314470.
26. Guideline O. The FOR, of T. Uterotrophic Bioassay Rodents October. 2007 (October).
27. Singh TP, Ahmad FJ, Jain GK, Verma N. Formulation development and characterization of nanoemulsion-based gel for topical application of raloxifene hydrochloride. Indian J Pharm Educ Res. 2021;55(4):996-1007. doi: 10.5530/ijper.55.4.200.
28. Baboota S, Shakeel F, Ahuja A, Ali J, Shafiq S. Design, development and evaluation of novel nanoemulsion formulations for transdermal potential of celecoxib. Acta Pharm. 2007;57(3):315-32. doi: 10.2478/v10007-007-0025-5, PMID 17878111.
29. Azeem A, Rizwan M, Ahmad FJ, Iqbal Z, Khar RK, Aqil M, *et al*. Nanoemulsion components screening and selection: A technical note. AAPS PharmSciTech. 2009;10(1):69-76. doi: 10.1208/s12249-008-9178-x, PMID 19148761.
30. Shakeel F, Baboota S, Ahuja A, Ali J, Aqil M, Shafiq S. Nanoemulsions as vehicles for transdermal delivery of aceclofenac. AAPS PharmSciTech. 2007;8(4):E104. doi: 10.1208/pt0804104, PMID 18181525.
31. Khajuria DK, Razdan R, Mahapatra DR. Description of a new method of ovariectomy in female rats. Rev Bras Reumatol. 2012;52(3):462-70. PMID 22641600.
32. Caetano GF, Fronza M, Leite MN, Gomes A, Frade MAC. Comparison of collagen content in skin wounds evaluated by biochemical assay and by computer-aided histomorphometric analysis. Pharm Biol. 2016;54(11):2555-9. doi: 10.3109/13880209.2016.1170861, PMID 27180834.
33. Burks RI. Povidone-iodine solution in wound treatment. Phys Ther. 1998;78(2):212-8. doi: 10.1093/ptj/78.2.212, PMID 9474112.
34. Marini H, Polito F, Altavilla D, Irrera N, Minutoli L, Calò M, *et al*. Genistein aglycone improves skin repair in an incisional model of wound healing: A comparison with raloxifene and oestradiol in ovariectomized rats. Br J Pharmacol. 2010;160(5):1185-94. doi: 10.1111/j.1476-5381.2010.00758.x, PMID 20590611.
35. Ehrlich HP, Hunt TK. The effects of cortisone and anabolic steroids on the tensile strength of healing wounds. Ann Surg. 1969;170(2):203-6. doi: 10.1097/00000658-196908000-00007, PMID 5796705.
36. Kokane DD, More RY, Kale MB, Nehete MN, Mehendale PC, Gadgoli CH. Evaluation of wound healing activity of root of Mimosa pudica. J Ethnopharmacol. 2009;124(2):311-5. doi: 10.1016/j.jep.2009.04.038, PMID 19397984.
37. Suguna L, Singh S, Sivakumar P, Sampath P, Chandrakasan G. Influence of *Terminalia chebula* on dermal wound healing in rats. Phytother Res. 2002;16(3):227-31. doi: 10.1002/ptr.827, PMID 12164266.
38. Lodhi S, Pawar RS, Jain AP, Singhai AK. Wound healing potential of *Tephrosia purpurea* (Linn.) Pers. in rats. J Ethnopharmacol. 2006;108(2):204-10. doi: 10.1016/j.jep.2006.05.011, PMID 16806763.
39. Bellare A, Epperly MW, Greenberger JS, Fisher R, Glowacki J. Development of tensile strength methodology for murine skin wound healing. MethodsX. 2018;5(April):337-44. doi: 10.1016/j.mex.2018.04.002, PMID 30050753.
40. Galeano M, Bitto A, Altavilla D, Minutoli L, Polito F, Calò M, *et al*. Polydeoxyribonucleotide stimulates angiogenesis and wound healing in the genetically diabetic mouse. Wound Repair Regen. 2008;16(2):208-17. doi: 10.1111/j.1524-475X.2008.00361.x, PMID 18318806.
41. Mukai K, Komatsu E, Nakajima Y, Urai T, Nasruddin SJ, Sugama J, *et al*. The effect of 17β-estradiol on cutaneous wound healing in protein-malnourished ovariectomized female mouse model. PLOS ONE. 2014;9(12):e115564. doi: 10.1371/journal.pone.0115564, PMID 25518000.
42. Morton JJ, Malone MH. Evaluation of vulneray activity by an open wound procedure in rats. Arch Int Pharmacodyn Ther. 1972;196(1):117-26. PMID 5059357.
43. Tramontina VA, Machado MA, Nogueira Filho Gda R, Kim SH, Vizzioli MR, Toledo Sd. Effect of bismuth subgallate (local hemostatic agent) on wound healing in rats. Histological and histometric findings. Braz Dent J. 2002;13(1):11-6. PMID 11870955.
44. Nagappa AN, Cheriyan B. Wound healing activity of the aqueous extract of *Thespesia populnea* fruit. Fitoterapia. 2001;72(5):503-6. doi: 10.1016/s0367-326x(01)00275-1, PMID 11429243.
45. Rashed AN, Afifi FU, Disi AM. Simple evaluation of the wound healing activity of a crude extract of *Portulaca oleracea* L. (growing in Jordan) in Mus musculus JVI-1. J Ethnopharmacol. 2003;88(2-3):131-6. doi: 10.1016/s0378-8741(03)00194-6, PMID 12963132.
46. Sadaf F, Saleem R, Ahmed M, Ahmad SI, Navaid-ul-Zafar. Healing potential of cream containing extract of *Sphaeranthus indicus* on dermal wounds in Guinea pigs. J Ethnopharmacol. 2006;107(2):161-3. doi: 10.1016/j.jep.2006.02.022, PMID 16765545.
47. Woessner JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys. 1961;93(2):440-7. doi: 10.1016/0003-9861(61)90291-0, PMID 13786180.
48. Srinivas Reddy B, Kiran Kumar Reddy R, Naidu VGM, Madhusudhana K, Agwane SB, Ramakrishna S, *et al*. Evaluation of antimicrobial, antioxidant and wound-healing potentials of Holoptelea integrifolia. J Ethnopharmacol. 2008;115(2):249–56.

SUMMARY

With an aim to study the wound healing effects of the raloxifene nanoemulsion gel using topical application the incision and excision wound models in ovariectomized and non-ovariectomized female Wistar rats were used for the management of postmenopausal cutaneous wound healing. Female Wistar rats were used to investigate raloxifene nanoemulsion gel potential for its wound healing effects. The rats were ovariectomized and, after three months of ovariectomy, they were divided into three different groups: untreated-NOVX, controls-OVX, and raloxifene (0.05 $mg.kg^{-1}$) treated-OVX group. Wound healing effects were characterized in terms of breaking strength, tensile strength, area of wound contraction, wound closure time, hydroxyproline content and histopathological characteristics. The results show that raloxifene exhibits a good wound healing effect in ovariectomized rats. The outcomes of our investigation, give a clear suggestion that raloxifene nanoemulsion gel has wound healing properties in ovariectomized rats and will be effective for postmenopausal cutaneous wound healing.

PICTORIAL ABSTRACT



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