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Comparative Wound Healing Potential of Topical Application of Phenytoin, Epidermal Growth Factor, Dimethyl Sulfoxide and Colostrum Against Normal in Excisional Wound Model in Mice.

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ABSTRACT

Despite advances in wound healing treatments, new therapeutic agents that improve the wound healing are required. The present study was planned to evaluate the wound healing potential of topical application of Phenytoin sodium cream(1%) , Epidermal growth factor cream (30µg/gm), Dimethyl sulfoxide cream (60%) and Colostrum (lactating human mother's milk) as compared to normal wound healing process in mice. *Swiss* albino mice were procured and divided into 5 groups (n=6) .In excisional model, standard 1*1cm wound was created using ketamine anesthesia, taking aseptic precautions. The drugs were applied topically. The wound area measured on days 4, 8, 12,16 and a biopsy on days 4,10,16 for histo-pathological scoring . The hydroxyproline estimation in tissue was done. On 16th day.The measurement of wound area showed faster healing and histo-pathological analysis showed mature granulation tissue as early as day 4 in groups treated with Phenytoin and Colostrum . Also, the hydroxyproline content was higher in these groups. Thus Phenytoin and Colostrum showed promising results in wound healing potential.

Keywords: Phenytoin , Colostrum , DMSO, EGFR, Wound healing , excisional model, Mice

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INTRODUCTION

Wounds remain a challenging clinical problem in practice, with early and/or late complications presenting a frequent cause of prolonged hospital admissions [1]. In addition to this, chronic hard to heal wounds due to systemic abnormalities like diabetes, peripheral vascular disease, Raynaud's phenomenon present another challenge to the treating physician.

The present practice in wound healing is topical application of antibiotics and disinfectants. They have no role in a non-infected wound. Also, chief drawback of this practice is that it might take long time and may lead to undue scarring, hyperpigmentation, keloid formation and retractile scar tissue. Therefore, there is continuous search for an effective remedy to achieve early wound healing with minimum adverse sequel.

A particularly intriguing adverse effect of systemic administration of Phenytoin namely gingival hyperplasia; due to its stimulatory effect on collagen metabolism suggests an exciting possibility as an agent for wound healing [2-10]. Also topical applications, growth factors alter wound healing by acting on their receptors in granulation tissue and thus affect scar remodeling [11-21]. Many studies with animals demonstrated that Dimethyl Sulfoxide has several pharmacological actions such as membrane penetrant, anti-inflammatory, local analgesic, bacteriostatic, and collagen solvent. So it was tested for its effects on wound healing as a topical agent as compared to the rest of the drugs [22-26].

After topical application of Colostrum the defensins, macrophages, and more prominently the stem cells in Colostrum may provide an impetus for quick wound healing by affecting collagen metabolism. Till date, we could not find any study testing the effect of topical administration of Colostrum on wound healing.[27-35] Hence, the purpose of the present study was to find an agent for wound management which would heal the wound in the shortest time possible, with minimal scarring**.**

MATERIALS AND METHODS

This study was conducted after getting an approval from Animal Ethics Committee. Swiss albino mice of either sex and of similar age i.e.10 days old were kept in separate cages. They were provided with normal diet and water ad libitum. All the experiments were performed between 10 a.m. to 4 p.m.

Drugs

- Phenytoin cream- Pure Phenytoin powder was procured from National Chemicals Laboratory, Pune. After dissolving it in lanolin and mixing it with hot cocoa butter, a cream was prepared in the concentration of 1% w/w.
- EGFR Gel- Recombinant human Epidermal growth factor gel.(30µg/gm) Expiry date-1/2014 Batch no-55AL1, Manufacturing date: 02/2012 Marketed by: Maxter, lupin ltd.
- Dimethyl sulfoxide cream: After dissolving it in lanolin and mixing it with hot cocoa butter, a 60% w/w cream was prepared.
- Colostrum was collected from Human Milk Bank .After obtaining consent from the concerned authorities,1st day milk was collected from the bank in 2ml containers. It was refrigerated at -18◦C. On each day of the study 2 ml of milk was applied from a single container and the remainder was discarded. The pasteurized milk was obtained from mother's who were tested for absence of infections that could be transmitted through milk (HIV, Syphilis, Malaria, Toxoplasmosis).

Groups

The animals were randomly allocated to 5 different groups, each group containing 6 animals(n=6). 1st, 2nd, 3rd and 4th groups were treated with topical applications of Phenytoin cream , EGFR cream, Dimethyl sulfide cream and Colostrum ,once daily for 20 days in excisional model. The 5th group was left untreated and served as control.

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Excisional Model

A standard 1*1 cm wound was created on the shaven back by using a stamp under ketamine anaesthesia (10 mg/kg). The wound area was charted on day 4,8,12 and 16 after wounding using a butter paper and measured using a graph paper.

Hydroxyproline (HP) Content

Hydroxyproline (HP) content per 10mg of dried granulation tissue from edges was assessed. Hydroxyproline was estimated colorimetrically by using UV spectrophotometer at 540 nm as suggested by NEUMAN and LOGAN [37].

Histopathological Assessment :

Sequential Histopathological assessment of wound edges was done on day 4, 10 and 16. All the slides were graded by 2 independent observers after blinding for the treatment.

The Histopathological scoring system used is as follows [36]:

	Criteria / Score	0	1	2
1)	Epidermal structure	Total destruction/absence	Partial necrosis/ulcer	Normal
2)	Dermo-Epidermal junction and micro blisters	<25% attachment of epidermis-dermis, blisters	25%-75%attachment of epidermis to dermis, no blisters	Normal/no micro blisters
3)	Collagen bundles and dermal organization	Amorphic dermis, destruction of bundles	Edematous/disorganized bundles, partial necrosis of dermis	Normal collagen bundles
4)	Epidermal regeneration	<25%	25-75%	>75%
5)	Leucocyte infiltration	>16/HP slide	6-15/HP slide	<5/HP slide

Statistical Analysis

The results are expressed as mean ± 2SD and analysed using SPSS software. The analysis between the groups was done initially using the 'ANOVA' test. Any significant difference was further evaluated using the 'Unpaired t' test. All qualitative data was evaluated using the 'Chi Square' test. The result was considered statistically significant if p value was less than 0.05. The power of the study to detect false negative or β error was 80%. The histopathological data was analysed by Kruskal Wallis and Mann-whitney Z test.

RESULTS

Table 1: The wound area in excisional model on day 16 as compared to control (n=6)

Group	Wound area(mm^2)		Unpaired t	p
	Mean	SD		
Phenytoin	15.92***	9.494	11.119	<0.001***
EGFR	53.00	13.842	1.856	0.093
DMSO	68.75	10.158	1.020	0.332
Colostrum	14.83***	10.265	10.671	<0.001***
Control	64.08	4.737		

Table 1 shows that Groups treated with phenytoin and Colostrum causes reduction in wound area which is highly significantly ($p \leq 0.001$) as compared to control on day 16 of the study. This shows that topical application of phenytoin and Colostrum has led to acceleration of wound healing

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Table 2: The intra-group comparison of wound area in excisional model on day 16 (n=6)

Group	Phenytoin		EGFR		DMSO	
	t	p	t	p	t	p
Phenytoin						
EGFR	5.412	<0.001***				
DMSO	9.308	<0.001***	2.247	0.048*		
Colostrum	0.190	0.853	5.425	<0.001***	9.145	<0.001***

Table 2 shows that Groups treated with Phenytoin and Colostrum differ significantly (p ≤0.001) as compared to EGFR and DMSO in wound area measurement on day 16.Group DMSO differs significantly (p = 0.048) as compared to EGFR .There is no significant difference between groups Phenytoin and Colostrum. It is evident that topical application of phenytoin and Colostrum has led to faster wound healing as compared to rest of the groups.

Table 3: The histopathology score of wound tissue in excisional model on day 16 as compared to control.

Group	Histopathology score		Mann-Whitney Z	p
	Mean	SD		
Phenytoin	7.08**	0.664	2.918	0.004
EGFR	4.58	.585	1.185	0.236
DMSO	3.67	1.366	0.494	0.622
Colostrum	8.03**	0.664	2.913	0.002
Control	3.92	1.02		

Table 3 shows that the histopathology score of Groups treated with Colostrum (P =0.002) and Phenytoin (p=0.004) were significantly more as compared to control. It is seen that topical application of phenytoin and Colostrum have led to improvement in wound healing by affecting vascularization and collagen formation.

Table 4: The histopathology score of wound tissue in excisional model on different days

Group	Day4	Day10	Day16
Phenytoin	3.416±0.861***	4.58±0.376***	7.08±0.664***
EGFR	0.67±0.516	2.67±0.606	4.58±0.585
DMSO	0.50±0.548	2.33±1.033	3.67±1.366
Colostrum	3.50±0.632***	5.66±0.605***	8.03±0.664***
Control	0.67±0.516	2.17±0.753	3.92±1.02

Table 4 shows sequential analysis of histopathological scoring in excisional model on days 4,10 and 16 as compared by Mann Whitney Z-test. The groups treated with Phenytoin and Colostrum differed significantly as compared to control. The wound healing appears to be faster in groups with topical application of Phenytoin and Colostrum.

Table 5: The intra-group comparison of histopathological score in excisional model on day 16 (n=6)

Group	Phenytoin		EGFR		DMSO	
	Z	p	Z	p	Z	p
Phenytoin						
EGFR	2.918	0.004*				
DMSO	2.918	0.004*	1.636	0.102		
Colostrum	2.221	0.026*	2.913	0.004*	2.913	0.004*

Table 5 shows Groups Colostrum and Phenytoin differ significantly with respect to both DMSO as well as EGFR (p =0.004). However group treated with EGFR did not differ significantly from DMSO group.

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Table 6: The hydroxyproline content (µg/10 mg of dried tissue) in excisional model on day 16 as compared to control

Group	Hydroxyproline(µg)		Unpaired t	p
	Mean	SD		
Phenytoin	63.028205***	14.0656362	7.278	<0.001
EGFR	28.036667**	4.9519558	3.264	0.009
DMSO	36.907692**	2.9788684	7.477	0.003
Colostrum	58.387179***	24.7485655	3.872	<0.001
Control	18.383333	5.2869071		

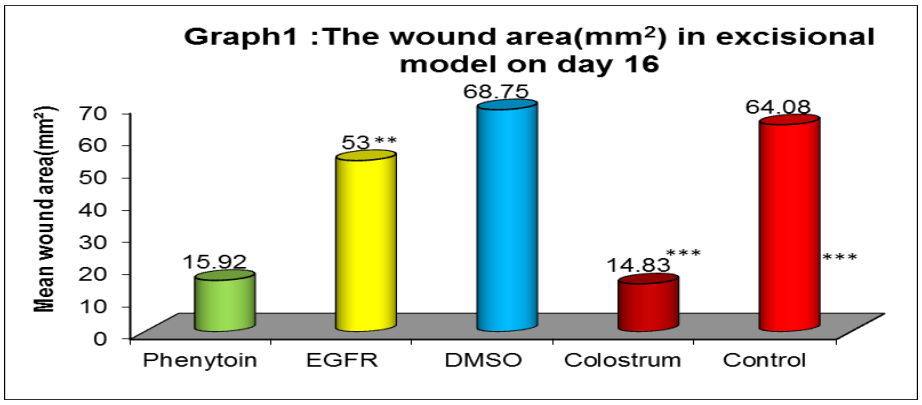
Table 6 shows that Group EGFR (p =0.009) and DMSO (p=0.003) differs significantly and Groups Colostrum and Phenytoin differ highly significantly (p<0.001) as compared to control.

The hydroxyproline content of wound in Excisional Model is significantly increased by all but more with phenytoin and Colostrum.

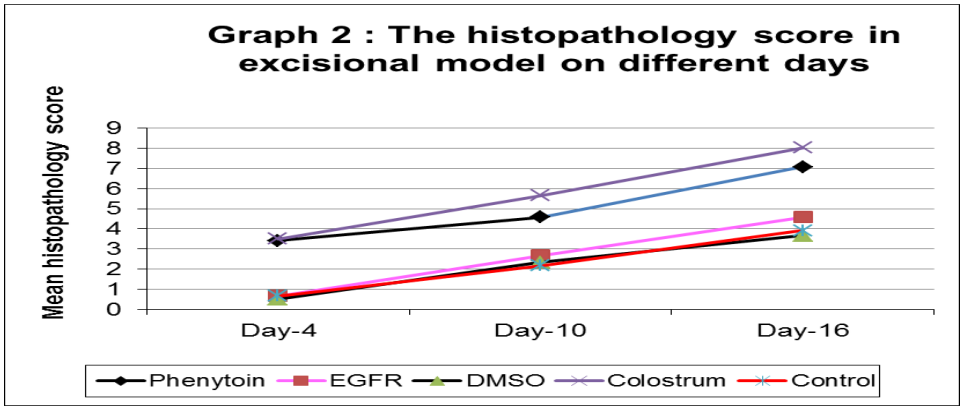
Table 7: The intra-group comparison of hydroxyproline content (µg/10 mg of dried tissue) in excisional model on day 16

Group	Phenytoin		EGFR		DMSO	
	t	p	t	p	t	p
Phenytoin						
EGFR	5.748	<0.001***				
DMSO	4.450	0.001***	3.760	0.004**		
Colostrum	0.399	0.698	2.946	0.015*	2.111	0.061

Table 7 shows that there is no significant difference between group Colostrum and Phenytoin. The hydroxyproline content of the wound appears to be in this order **Phenytoin > Colostrum>DMSO> EGFR.**



Graph 1: Shows that the wound area on day 16 is minimum in the groups treated with Phenytoin and Colostrum .



Graph 2: Shows that the histopathology score is more and improved faster with Phenytoin and Colostrum than DMSO or EGFR.

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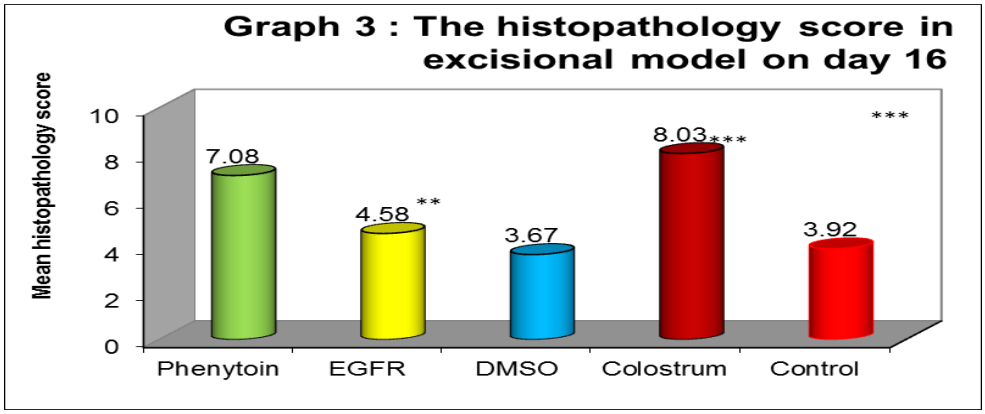
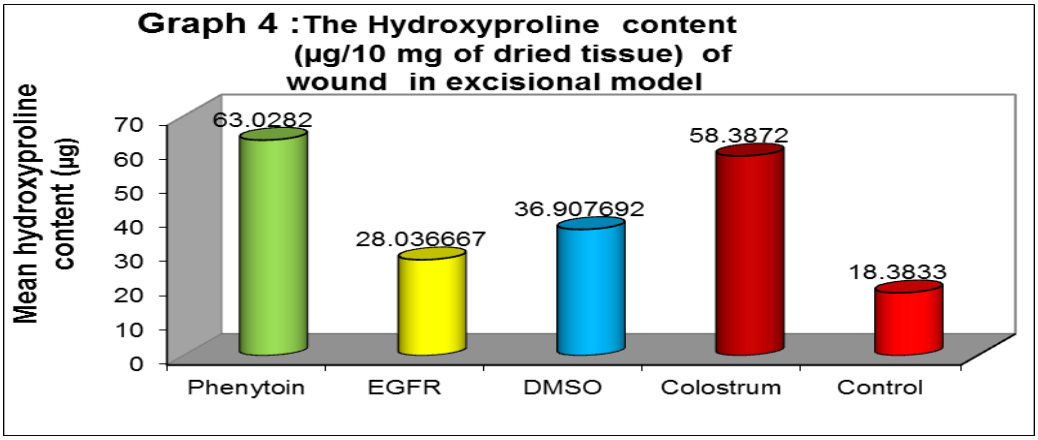


Graph 3: Shows that the histopathology score is maximum on day 16 with groups treated with Phenytoin and Colostrum than DMSO or EGFR.



Graph 4: Shows that the hydroxyproline content of wound is maximum in groups treated with Phenytoin and Colostrum than DMSO or EGFR.

DISCUSSION

Wound healing involves a complex series of interactions between different cell types, cytokine mediators, and the extracellular matrix. Restoration in the continuity of damaged area is brought about by housekeeping mechanism of the body viz., the repair process.

The present study was planned to assess wound healing potential of 4 drugs for topical application. Colostrum which contains lactoferrin and also stem cells was proposed to help in wound healing and the same was tested in present study.

It was observed that Colostrum led to increase in collagen content of wound, acceleration of epithelialization as well as changes in histo-pathological scoring. There was appearance of mature granulation tissue as early as on day 4. On Day 16 there was predominance of fibroblasts and new blood vessels and a more systematic arrangement of collagen fibers. In conclusion, Colostrum due to presence of lactoferrin or stem cells may be effectively used in wounds of different etiologies. It was Cost effective with Rs.0.575/mm² reduction in wound area.In one study it was seen that Milk fat globule/EGF factor 8/lactadherin played an important role intestinal epithelial homeostasis and mucosal healing suggesting a role of recombinant lactadherin in bowel injuries [28].Research has shown that colostrum contains growth factors that help to prevent and heal injurious effects of NSAIDs on bowel [29].

Phenytoin cream has led to increase in collagen content. There was a decrease in final wound area and time taken. There was appearance of mature granulation tissue as early as on day 4. On Day 16 there was predominance of fibroblasts and new blood vessels and a more systematic arrangement of collagen fibers. In conclusion, topical Phenytoin had better therapeutic outcome as compared to the rest . Also it was cost

effective (Rs 0.634/mm² reduction in wound area). It can be used as a cheap and affective alternative for wound healing.In one study phenytoin in 0.5% cream form accelerated skin wound healing with excellent cutaneous tolerability but dose in humans needs to be determined [8].

Topical application of phenytoin significantly accelerated wound healing and improved the quality and vascularity of granulation tissue, possibly by .increasing fibroblast proliferation, maturation of collagen content on one hand and decreasing collagenase activity on the other [10].

EGFR has led to increase in collagen content but no others parameters appear to have been significantly affected. In conclusion, it can be stated that delivery of growth factors at appropriate time and in proper delivery system was important. But it is costly with Rs 41/mm² reduction in wound area.Also it can be concluded that clinical application for EGF treatment could extend to deeper cutaneous injuries such as chronic wounds and burns [21].

Also, DMSO did not lead to any significant changes in parameters tested. So it can be concluded that topical DMSO did not seem to have any significant role in wound healing as compared to the rest.The cost of this therapy is Rs0.89/mm^2 reduction in wound area.

In conclusion, as Colostrum and Phenytoin cream affected all the parameters of wound healing positively in excisional wound model, they appear to be cheap and safe alternatives to costly therapies like growth factors**.**

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