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Evaluate the effect of the metranidazole gel on healing of partial thickness flap in rats (serum TNF-a)

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Abstract

Background: Metronidazole is a broad-spectrum antimicrobial activity against protozoan infections and anaerobic bacteria. In the late 1950^s, Metronidazole was first introduced for the treatment of trichomoniasis. At present, the antimicrobial metronidazole is one of the most widely used as antibacterial compounds in the treatment of periodontal disease ⁹. Beside the periodontal pocket debridement (subgingival scaling and curettage), topical application of metronidazole seems to be very efficacious. Metronidazole efficiently inhibited anaerobic microorganisms in the periodontal pockets ¹⁰.

Objective: Evaluating the effect of metronidazole gel into surgically healing process of the partial thickness flap, throughout immunological study and comparing it with the chlorhexidine gel

Animals and methods: The present study was carried out in Hawler Medical University, Medical research center, College of Dentistry

Chemicals and Reagents:- (Rat TNF-a ELISA) kit for estimation of serum tumor necrosis factor (TNF-a), KOMA BIOTECH INC. USA)

Chlorhexidine 1% gel (VITIS, France), Commercial metronidazole gel 10% (Spanish). Fifty five healthy male rats were used in this study of the same species and with a body weight range of 200-250g. These (55) rats were randomly divided into three main groups:- **(group K):** This group subdivided randomly into (5) subgroups;. Each subgroup exposed to .surgical partial thickness flap, incision was carried out at the attached gingival extends between the two mandibular right and left central incisors partial thickness flap filled with Metronidazole gel (10%). after a specific time intervals of 3days(K1), 5days(K2), 7days(K3), 10days(K4), and 14days(K5), of Metronidazole gel inserted into surgical site. metronidazole gel inserted into the performed surgical site test group, **(group L):** This group (25 rats) was also subdivided randomly into (5) subgroups; Blood samples were collected from the heart of rats in the same manner as in the test groups and at the same time intervals of 3 days (L1), 5days (L2), 7days (L3), 10days (L4) and 14days (L5), after insertion of chlorhexidine gel (1%) into the surgical site. Each subgroup (5rats) exposed to surgery and filled with chlorhexidine gel which consisted of (25) rats, chlorhexidine gel inserted into the surgical site test group, which consisted of (25) rats, and normal (non surgical) group which consisted of (5) rats: **(group M):** Blood samples were also collected from (5) rats as normal group (group M), that they did not receive any surgery, to obtain a baseline data of normal rats. Results: In table 1 the current study shows the mean and standard deviation of both Metronidazole gel and Chlorhexidine gel at different time

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intervals for all the study and control groups at P value < 0.05. The statistical result show significant difference at 3 days and highly significant difference at 5days post surgical treatment, then it return back to the normal level at end of the experiments, (i.e at 14days post surgical treatment). Another table shows Mean and SD of Metronidazole gel and control group at different time intervals at P value < 0.05, the statistical result show non significant difference at 3days, and highly significant difference at 5days post surgical treatment, then it return back to its normal level at end of the experiments, (i.e at 14days post surgical treatment)Conclusion: In conclusion this experimental study has shown that application of 10% metranidazole gel to the partial thickness flap induced modulating effects leading to heal earlier. As the metranidazole gel application to the wound is non irritant, non toxic. Further studies are required to investigate that the gel effect on other wounds facing animals during their life span.

Key words: Metronidazole gel, Chlorhexidine gel, Cytokines, Tumor necrosis factor-alpha (TNF-a).

Introduction

Authors reported that older adults have slower process of wound healing and increased risk of skin breakdown with formation of ulcerated lesions, and chronic ulcers of the lower limbs, generating early retirements¹ and being the second cause of absence from work².

In most public institutions, there are scarce resources to meet the demands of low-income population, which consequently develops diseases with greater severity. To control costs, private institutions and insurance companies are setting increasingly strict criteria to allow the use of resources and sophisticated technologies³.

Ashford et al. reported, for the first time in 1984, the use of oral metronidazole to control the stench of neoplastic ulcerated lesions⁴. Currently, topical metronidazole is recommended in oncology wounds with the same purpose, because it acts against the anaerobic bacteria responsible for the production of volatile acids that cause the odor, without the side effects of its oral useage⁵⁻⁷.

Trindade Neto et al.⁸ described the use of topic 0.5% metronidazole gel in a case of granulomatous rosacea with good results and referred that metronidazole gel has been successfully used in cases of mild to moderate rosacea, maintaining patients in remission.

Metronidazole is a broad-spectrum antimicrobial activity against protozoan infections and anaerobic bacteria. In the late 1950^s, Metronidazole was first introduced for the treatment of trichomoniasis. At present, the antimicrobial metronidazole is one of the most widely used as antibacterial compounds in the treatment of periodontal disease⁹. Beside the periodontal pocket debridement (subgingival scaling and curettage), topical application of metronidazole seems to be very efficacious. Metronidazole efficiently inhibited anaerobic microorganisms in the periodontal pockets¹⁰.

There is little research on the use of topical metronidazole in benign wounds healing by secondary intention, although it is a low cost medication, simple to handle and of

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easy access to the general population, being able to give back the patient's independence in his/her self care.

This study aimed to evaluate the action of a topical 4% metronidazole solution in wounds healing by secondary intention in mice, analyzing the epithelialization, wound contraction and the correlation with myofibroblasts.

Metronidazole that controls anaerobic infections is administered orally, intravenously or topically depending on the severity of the infections in burn-wound patients¹². Whether metronidazole, topical/systemic, influences healing of burn wounds is not precisely known

Recently, a topical preparation of metronidazole namely metronidazole gel USP is being used to treat anaerobic (*B.fragilis*) burn wound infections. Topical chemotherapeutic preparations meant for burns should not inhibit the reepithelization and cause injury to viable cells¹³.

Metronidazole is the most common broad-spectrum antibiotic and is active against most of the periodontal pathogens.¹⁴ As anaerobic bacteria are believed to be the predominant causative factor in periodontitis and metronidazole, a member of nitroimidazole class of antibiotics specifically targets anaerobic microorganisms it is in use in the treatment of chronic periodontitis¹⁵. The purpose of the present study was to compare the efficacy of *A. vera* to metronidazole, as an adjunct to SRP in patients with chronic periodontitis.

Metronidazole that controls anaerobic infections is administered orally, intravenously or topically depending on the severity of the infections in burn-wound patients¹⁶. Whether metronidazole, topical/systemic, influences healing of burn wounds is not precisely known

Rice, reported¹⁷ that topical metronidazole improved wound appearance of infected pressure sores, leg ulcers etc. This could be due to either bactericidal or direct prohealing property of the drug.

Prasad and Rao observed¹⁸ that oral metronidazole increased wound contraction and epithelization in non-infected skin excision wounds suggesting that it has direct beneficial effects on healing process.

Cytokines are made by many cell populations, but the predominant producers are helper T cells (Th) and macrophages. Cytokines may be produced in and by peripheral nerve tissue during physiological and pathological processes by resident and recruited macrophages, mast cells, endothelial cells, and Schwann cells. Following a peripheral nerve injury, macrophages and Schwann cells that gather around the injured site of the nerve secrete cytokines and specific growth factors required for nerve regeneration. Localized inflammatory irritation of the dorsal root ganglion (DRG) not only increases pro-inflammatory cytokines but also decreases anti-inflammatory cytokines¹⁹

Proinflammatory cytokines are important mediators of inflammation, immunity, proteolysis, cell recruitment and proliferation. Tumour necrosis factor TNF- α and TNF- β , and interleukin IL-1 α and IL-1 β have received much attention over the past decade^{20,21,22}, and both TNF and IL-1 have been demonstrated in the rheumatoid lesion by immunohistochemistry²³. TNF reportedly plays a pivotal role in the pathogenesis of RA^{21,22} especially its ability to regulate IL-1 β expression, this being important for the induction of prostanoind and matrix metalloproteinase production by synovial fibroblasts and chondrocytes

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^{20,24}. Cellular interactions mediated by TNF and IL-1, cytokines that are mainly produced by activated macrophages, have become prominent factors in the numerous reviews that have proposed a sequence of events leading to cartilage damage in RA ^{21,22,24}. The precise factors that induce monocyte/macrophages to produce TNF and IL-1 remain obscure, however, although recent studies ^{25,26,27} have suggested a role for IL-15.

Chlorhexidine (CHX) remains the gold standard of chemical antiplaque agents and remains one of the most effective topical antiseptics reported to date that has been successfully used for treating plaque-related gingivitis (Quirynen, 2001)²⁸. Chlorhexidine has been reported to have some reversible local side effects, such as staining designed to compare the efficacy of topically applied CHX, MTZ and the combination of these two gels over a period of 24 weeks in subjects with gingivitis.mucosa. Staining is largely dose-dependent, whereas desquamation of the oral mucosa and perturbation of taste is largely concentration-dependent (Brex *et al.*, 1993)²⁹.

TNF α and IFN γ , like IL - 1 β , are both pro - inflammatory cytokines and TNF α has similar effects to IL - 1 β in many cells. Bronchoalveolar lavage fluid from subjects with symptomatic asthma contains significantly elevated levels of a number of pro - inflammatory cytokines including IL - 1 β and TNF α (Broide *et al.*, 1992)³⁰ and TNF α and IFN γ , when used together with IL - 1 β cause increases in PGE₂ release, COX activity and induction of COX - 2 enzyme (Mitchell *et al.*, 1994)³¹

Aim of the study

Evaluating the effect of metronidazole gel into surgically

healing process of the partial thickness flap, throughout immunological study and comparing it with the chlorhexidine gel.

Animals and Methods

Setting of the study:-The present study was carried out in Hawler Medical University, Medical research center, College of Dentistry; the animals were kept in animal's house in college of Medicine. The experimental part was carried out during study period of January- July 2018.

Chemicals and Reagents:- Rat TNF- α ELISA kit for estimation of serum tumor necrosis factor (TNF- α), KOMA BIOTECH INC. USA) Chlorhexidine 0.1% gel (VITIS, France) Commercial metronidazole gel (Spanish)

Animals, Experimental studies and sampling:-Fifty five healthy female rats were used in this study of the same species and with a body weight range of 200-350g. All animals were conditioned at room temperature at a natural photoperiod for 2 weeks before experiment execution. These animals were supervised by the staff of animal house. A commercial balanced diet and tap water ad libitum were provided. The duration of experiment was 8weeks. Each (2) rats were kept in a special breeding cage.

Immunological studies:-Fifty five rats were used for these studies. These (55) rats were randomly divided into three main groups:- metronidazole gel inserted into the performed surgical site test group, which consisted of (25) rats,

chlorhexidine gel inserted into the surgical site test group, which consisted of (25) rats, and normal (non surgical) group which consisted of (5) rats:-

A-Metronidazole gel inserted into the surgical site (group K): This group subdivided randomly into (5) subgroups; K1, K2, K3, K4, and K5. Each subgroup (5 rats) exposed to surgical partial thickness flap filled with Metronidazole gel (10%). The surgical partial thickness flap was carried out at the mandibular right and left central incisors. Before the surgery, the rats were weighted and anesthetized with subcutaneous injection of a combination of Ketamine-HCL and Xylazine 2% in a dose of (35mg/kg) and (0.5mg/kg) respectively. Blood samples for immunological studies were collected by cardiac puncture, and the serum prepared through centrifuging at 2500 ×g for 15 minutes at 30°C, after a specific time intervals of 3days(K1), 5days(K2), 7days(K3), 10days(K4), and 14days(K5), of Metronidazole gel inserted into surgical site.

B-Chlorhexidine gel inserted into the surgical site (group L): This group (25 rats) was also subdivided randomly into (5) subgroups; L1, L2, L3, L4 and L5. Each subgroup (5rats) exposed to surgery and filled with chlorhexidine gel (1%) (after weighting and anesthetizing) into the same area as in the test groups. Blood samples were collected from the heart of rats in the same manner as in the test groups and at the same time intervals of 3 days (L1), 5days (L2), 7days (L3), 10days (L4) and 14days (L5), after insertion of

chlorhexidine gel into the surgical site .

C-Non-surgical group (group M): Blood samples were also collected from (5) rats as normal group (group M), that they did not receive any surgery (the blood samples were taken in the same way as in the group K and group L, to obtain a baseline data of normal rats.

Surgical procedure:-

Preparation of the animals for surgical procedure:-The animals were anesthetized using subcutaneous injection of a combination of Ketamine-HCL and Xylazine 2% in a dose of (40mg/kg) and (4mg/kg) respectively. The labial surface of the mandibular right and left central incisors was the site of the surgical procedure.

***Surgical procedure:-** A partial thickness flap incision was carried out at the attached gingival extends between the two mandibular right and left central incisors. This was carried out by using a scalpel blade No.11. The flap was reflected by periosteal elevator. The surgical site was filled with Metronidazole gel of 10% and considered as an experimental group. The surgical incision was filled by chlorhexidine gel 1% as a control group, and as a normal group, no any surgical procedure was performed. The incision was sutured by one stitch with (4/0) black silk in order to close the wound and replaced into the same previous position. The area of surgical site was pressed with sterilized gauze to reduce bleeding that might happen after the operation.

Statistical analysis:

Statistical analysis was performed using Graph- pad prism program (version 7). Values are expressed as Mean, \pm standard deviation, differences among days with controls were compressed with the analysis of variance (ANOVA) and Turkey s multiple comparison test was used. Differences among days were compared with repeated ANOVA and dunnett s test was also used. P value < 0.05 (two tailed significance) were considered statistically significant.

Immunological results:

Table 1: shows the mean and standard deviation of both Metronidazole gel and Chlorhexidine gel at different time intervals for all the study and control groups at P value < 0.05 . The statistical result show significant difference at 3 days, and highly significant difference at 5days post surgical treatment, then it return back to the normal level at end of the experiments, (i.e. at 14 days post surgical treatment).

Discussion

The results of the present study show that metronidazole gel accelerates healing while the chlorhexidine gel is neutral to healing.

The pro-healing effect of metronidazole gel in partial thickness flap corroborates with the findings of Prasad and Rao who reported that metronidazole would promote contraction and epithelization of excision wounds¹⁸

However, this is contrary to what was observed by Borden in fascial wounds³². While the systemic metronidazole did not materially alter contraction of skin wounds, it did depress the breaking strength of fascial wounds.

The elimination of infection by metronidazole eventually leading to promotion of healing.

Systemic metronidazole could still be assumed to have scavenged the free radicals and prevented the generation of lipid peroxides that are known to be present during burn stress. A reduction in lipid peroxides of burns may reduce the further loss of tissue in burned area and may thus promote healing. If this were to be true, one would expect similar findings with topical metronidazole. We opine that the direct cytotoxicity of topical metronidazole, in such a high concentration, might have a more pronounced retarding effect on the healing as compared to its indirect effect as an antioxidant. However, this needs a detailed appraisal.

Topical anti-infective formulations are routinely employed in burn wounds. The bases are assumed inert. However, in the light of the present study bases cannot be assumed innocuous. They could either facilitate or retard healing. Therefore, it is imperative that a thorough evaluation of bases used in topical formulation be done prior to the incorporation of medicament could not be ruled out in this case.³³

Authors who did experimental work in rats with wound healing by secondary intention, Prasad et al.³⁴ and Rao et al.³⁵ with the use of oral metronidazole, and Rao et al.³⁶ using it topically, reported a significant increase of epithelialization in the wounds of the experimental groups, but this parameter was evaluated in all three studies as the number of days required for complete wound epithelialization. In the present study, healing proved to be earlier in the experimental group. However, the later evaluations found no difference between the wounds of both groups (metronidazole gel and chlorhexidine

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gel). Metronidazole gel probably facilitates the initial healing when used topically at a concentration of 10%

Regarding the contraction of the wound in the back of the animal, as measured by planimetry, Prasad et al.³⁴ and Rao et al.³⁶ They reported an increase in contraction with the use of metronidazole oral dose of 160 mg/kg/day and 180 mg/kg/day, respectively. Borden et al.³⁷, who used metronidazole intraperitoneally at a dose of 20 mg/kg/day, they found no significant difference in wound contraction. In this study, it was observed that the wounds of both groups significantly decreased their area as time passed. But when the groups were compared, there was no difference in any of the moments, demonstrating that metronidazole at a dose of 50mg/kg/day as topical use did not interfere with the contraction of the wound that is healing by secondary intention, the same result was obtained before by Borden et al., despite different routes of administration of the medication.

Proinflammatory cytokines are produced predominantly by activated macrophages and are involved in the up-regulation of inflammatory reactions. There is abundant evidence that certain pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α are involved in the process of pathological pain.³⁸

TNF- α , also known as cachectin, is another inflammatory cytokine that plays a well-established, key role in some pain models. TNF acts on several different signaling pathways through two cell surface receptors, TNFR1 and TNFR2 to regulate apoptotic pathways, NF- κ B activation of inflammation, and activate stress-activated protein kinases (SAPKs). TNF- α receptors are present in both neurons and glia³⁹. TNF- α has been shown to play important roles in both inflammatory and neuropathic

hyperalgesia. Intraplantar injection of complete Freund's adjuvant in adult rats resulted in significant elevation in the levels of TNF- α , IL-1 β , and nerve growth factor (NGF) in the inflamed paw. A single injection of anti-TNF- α antiserum before the CFA significantly delayed the onset of the resultant inflammatory hyperalgesia and reduced IL-1 β but not NGF levels⁴⁰. Intraplantar injection of TNF- α also produces mechanical⁴¹ and thermal hyperalgesia⁴². It has been found that TNF- α injected into nerves induces Wallerian degeneration^{40, 43} and generates the transient display of behaviors and endoneurial pathologies found in experimentally painful nerve injury⁴⁴. TNF binding protein (TNF-BP), an inhibitor of TNF, is a soluble form of a transmembrane TNF-receptor. When TNF-BP is administered systemically, the hyperalgesia normally observed after lipopolysaccharide (LPS) administration is completely eliminated³⁸. Intrathecal administration of a combination of TNF-BP and IL-1 antagonist attenuated mechanical allodynia in rats with L₅ spinal nerve transection⁴⁵.

Conclusion

The results presented here therefore indicated a relevant role for this new formulation of metronidazole gel in wound healing, which led to improvement of therapeutic resources in the treatment of surgical wounds. From an immunological standpoint, the significant differences of the inflammatory and repair stage of healing treated with this gel in comparison with control group, indicated a positive potential therapeutic effects on acceleration of surgical wound healing particularly improvement of anti-inflammatory cytokines (TNF- α) consequences after

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surgery. In wound healing, it could be useful in modulating the inflammatory response and better repair.

In conclusion, this experimental study has shown that application of 10% metronidazole gel to the partial thickness flap induced modulating effects leading to heal earlier. As the metronidazole gel application to the wound is non irritant, non toxic. Further studies are required to investigate that the gel effect on other wounds facing animals during their life span.

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Table (1):- The mean and standard deviation of serum tumor necrosis factor-alpha(TNF-a) concentration in 2 surgical groups with 10% Metranidazol gel and 1% Chlorhexidine gel and control group at different time interval at P value < 0.05, ANOVA and dunnett s test

Time/Days	Serum TNF-a mean&SD (pg/ml) Metronidazole gel	Serum TNF-a mean&SD (pg/ml) Chlorhexidine gel	Serum TNF-a mean &SD (pg/ml) Control	Sig*
3	20.8±0.476	5.01±2.893	18.71 ±4.283	S
5	6.415±2.614	0.00±0.00	18.71±4.283	HS
7	8.25±8.96	8.308±4.134	18.71±4.283	S
10	10.26±5.926	12.53±9.815	18.71±4.283	NS
14	11.54±3.635	14.8±13.16	18.71±4.283	NS

Table 2: shows Mean and SD of Metronidazole gel and control group at different time intervals at P value < 0.05, the statistical result show non significant difference at 3days, and highly significant difference at 5days post surgical treatment, then it return back to its normal level at end of the experiments, (i.e at 14days post surgical treatment)

Table (2):- The mean and standard deviation of serum tumor necrosis factor-alpha(TNF-a) concentration in 10% Metronidazole gel surgical group with control group at different time interval at P value < 0.05, ANOVA and dunnett s test

Time/Days	Serum TNF-a mean&SD (pg/ml) Metronidazole gel	Serum TNF-a mean&SD (pg/ml) Control	Sig*
3	20.8±0.476	18.71 ±4.283	NS
5	6.415±2.614	18.71±4.283	HS
7	8.25±8.96	18.71±4.283	S
10	10.26±5.926	18.71±4.283	S
14	11.54±3.635	18.71±4.283	NS

Table 3: shows Mean and SD of Chlorhexidine gel and control group at different time intervals at P value < 0.05. The statistical result show highly significant difference at 3days, and at 5days post surgical treatment, then it return back to its normal level at end of the experiments, (i.e at 10,14days post surgical treatment)

Table (3):- The mean and standard deviation of serum tumor necrosis factor-alpha(TNF-a) concentration in 1% chlorhexidine gel surgical group with control group at different time interval at P value < 0.05, ANOVA and dunnett s test

Time/Days	Serum TNF-a mean&SD (pg/ml) Chlorhexidine gel	Serum TNF-a mean&SD (pg/ml) Control	Sig*
3	5.01±2.893	18.71 ±4.283	HS
5	0.00±0.00	18.71±4.283	HS
7	8.308±4.134	18.71±4.283	S
10	12.53±9.815	18.71±4.283	NS
14	14.8±13.16	18.71±4.283	NS

Table 4: shows Mean and SD of Metronidazole gel and Chlorhexidine gel at different time intervals at P value < 0.05 (two tailed significance). The statistical result show highly significant difference at 3, days, and at 5 days post surgical treatment, and it reach the same level at 7 day post surgical treatment then it return back to its normal level at end of the experiments, (i.e at 10, 14 days post surgical treatment)

Table (4):- The mean and standard deviation of serum tumor necrosis factor-alpha(TNF-a) concentration in 2 surgical groups with 10% Metronidazole gel and 1% Chlorhexidine gel at different time interval at P value < 0.05, ANOVA and dunnett s test

Time/Days	Serum TNF-a mean&SD (pg/ml) Metronidazole gel	Serum TNF-a mean&SD (pg/ml) Chlorhexidine gel	Sig*
3	20.8±0.476	5.01±2.893	HS
5	6.415±2.614	0.00±0.00	HS
7	8.25±8.96	8.308±4.134	NS
10	10.26±5.926	12.53±9.815	NS
14	11.54±3.635	14.8±13.16	NS