Healing enhancement assessment of thermosensitive in situ gelling formulation containing metronidazole and diclofenac potassium for ligature-induced periodontitis in rats

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Received: 28 April 2021

Accepted: 20 December 2021

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DOI 10.5001/omj.2022.66

Abstract:

The aim of this study was to investigate the healing efficacy of thermosensitive in situ gelling formulation containing metronidazole and diclofenac potassium on ligature induced periodontitis (LIP) in rats.

Materials and methods: The experiment was carried out on thirty (30) adult male Sprague-Dawley rats. A total of twenty four (24) rats were subjected to LIP while the remaining 6 rats were used as control. The 24 rats with LIP were further subdivided into 4 groups of 6 rats each. Group I received 5% metronidazole loaded gel formulation; Group II received 0.15 % diclofenac potassium loaded gel formulation; Group III received both 5% metronidazole and 0.15% diclofenac potassium and Group 4 did not receive any treatment. Treatment was administered on Day 0 and the animals were monitored over 7 days. Specimens were collected for histological examination to assess healing process.

Results: A statistically significant difference ($p<0.05$) in the inflammatory and repair parameters of the healing process between different treatments was noticed. Combination of two drugs significantly ($p<0.05$) accelerated the healing compared to drugs given alone.

Conclusion: The use of a combination of diclofenac and metronidazole could potentially accelerate the healing process compared to the use of a single drug of either diclofenac or metronidazole.

Introduction

Oral cavity environment is full of normal flora and some opportunistic bacteria. Microbial colonization on the teeth is a major source of pathogens responsible for periodontal disease. (Arigbede, et al., 2012). Periodontitis is a serious gum inflammation that damages the soft tissue in response to bacterial infections. It is chronic disease that affects a major part of world population. Periodontal disease is also associated with other diseases like heart diseases and other systemic diseases (Wade et al.,2013; Könönen et al., 2019).

The main target of periodontal disease treatment is to eliminate the pathogens responsible for infection and decrease tissue inflammation. Antimicrobial agents and non-steroidal anti-inflammatory drugs have been used to treat periodontal disease (Haffajee et al., 2007; Johnson et al., 2009; Karthikeyan et al., 2012; Johnston et al., 2013).

Local delivery of moxifloxacin against bacterial periodontitis was a successful development for an effective site-specific drug delivery system for the periodontitis treatment (Beg et al., 2020). 25% metronidazole gel showed a major decrease in the probing depth when compared with the control groups local therapies (Gomes et al., 2020).

Thermo-reversible hydrogel loaded with anti-inflammatory Lipoxin A_4 and/or antibiotic doxycycline reduced bacterial load and pro-inflammatory interleukin-8 level. This finding showed that the thermo-reversible hydrogel is a safe and effective vehicle for periodontal drug delivery (Wang et al., 2020).

In order to study the disease response on animal, an experiment on periodontal disease should be established on animal gum. One of the methods to induce periodontitis is ligature model (Liu et al., 2012; Ionel et al., 2015). Induction was achieved through fixing ligature using silk or cotton around the teeth on sub marginal position of the necks of the teeth. The advantages of the ligature model is that disease can be initiated and facilitated at a known time with predictable sequence of events of inflammation of the periodontal ligaments within a few days (Graves, 2008). The removal of the ligature helped to investigate the resolution of inflammation and the healing response after applying the treatment (Assuma et al., 1998; Oz and Puleo, 2011; Tamaddon et al., 2020).

The aim of this study was to evaluate the healing efficacy of syringable, in situ gelling formulations containing metronidazole, diclofenac potassium or combination of the two drugs on ligature-induced periodontitis in rats. The inflammation parameters namely, presence of PMN (polymorphonuclear cells), ligament organization, granulation, necrosis and edema, before and after treatment were assessed as shown in Table 2.

Materials and methods

2.1 Materials

Poloxamer 188 and Poloxamer 407 were obtained from BASF Chemicals Company, Limburgerhof, Germany. Metronidazole was purchased from Ranbaxy, India. Diclofenac potassium and xanthan gum were obtained from National Pharmaceutical Industries, Muscat,

Oman. HR40 nonresorbable sterile silk thread (polyamide monofilament nonabsorbable or Barbour's linen suture thread) was obtained from Huaian Conley Properties, Wanjia, China.

2.2 Preparation of thermosensitive *in situ* gelling formulation

Thermosensitive in situ gelling formulation containing 30% Poloxamer (10% Poloxamer 188 and 20% Poloxamer 407) and 2% w/w Xanthan gum was prepared using cold method (Johnson et al., 2013). Sets of in situ gelling formulations containing 30% Poloxamer188 and 407 were prepared and xanthan gum was incorporated at concentrations of 1.5, 1.75 and 2%. It was found based on comparative in vitro release studies that one containing 2% was the optimized gel formulation and hence was used for further studies. Metronidazole (5%w/w), diclofenac potassium (0.15%w/w) or a combination of metronidazole (5%w/w) and diclofenac potassium (0.15%), were incorporated to prepare three thermosensitive *in situ* gelling formulations. The formulations were stored in polyethylene containers under refrigeration at 8°C until used.

2.3 Determination of Gelation Temperature

A volume of 3 mL of the three thermosensitive *in situ* gelling formulations was measured into a 15 mL centrifuge tube and heated in a water bath. The temperature of gel formation where the gel did not fall when the tube was turned 90º was recorded as the gelation temperature. The experiment was carried out in triplicate (Johnson et al., 2013).

2.4 *Syringeability Measurement*

The work required to expel the formulation from a syringe (without needle) was determined in compression mode using Texture analyzer (TA-TX Plus, Stable Micro System, UK). The sample

was packed into a 3 mL polyvinyl chloride syringe (Terumo, Indonesia) to a height of 2.5 mL. The syringe was then placed in metallic support and immobilized with a metal clamping ring. The plunger of syringe was attached to the arm of the Texture analyzer by probe adaptor at a height of 40 mm from the syringe. The plunger descended at a constant speed of 5 mm/s and expelled the gel from syringe before returning to original position (Figure 1). The work of syringeability was calculated from the area under the resultant force-time plot (Jones et al., 1997; Borole et al., 2014; Şenyiğit et al., 2014). The experiment was performed in triplicate.

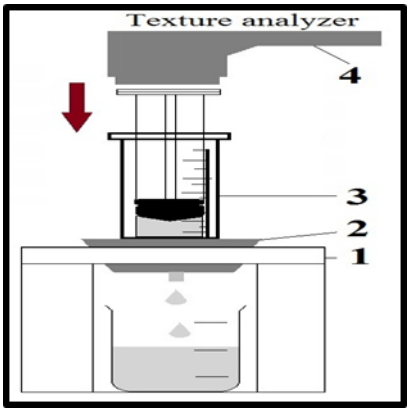


Figure 1

2.5 *In-vitro drug release studies*

The in-vitro release of metronidazole and diclofenac potassium was determined using a dissolution tester apparatus I basket method (Pharma Test, PTWS 3CE, Hainburg, Germany). One (1) mL of gel formulation was measured into muslin cloth and tied with thread before placing inside the basket. Glass tubes of 6.0 cm diameter and 17.0 cm height were used as dissolution vessel. The basket was connected to the dissolution tester and submerged in the glass tube. The study was carried out at 37.0 ± 0.5^0C with rotation speed of 100 rpm in 100 mL of 0.05M potassium phosphate buffer (pH 6.8) dissolution medium. Sample of 5 mL was collected from the dissolution vessel at preset time intervals of 1, 2, 3, 4, 5, 6, 24, 48, 72, 96, 120, 144 and

168 hours and immediately refilled with the same volume of fresh dissolution medium. The samples were analyzed using a validated HPLC method (Nida'a et al., 2013). The percentage of drug released over time was calculated and plotted.

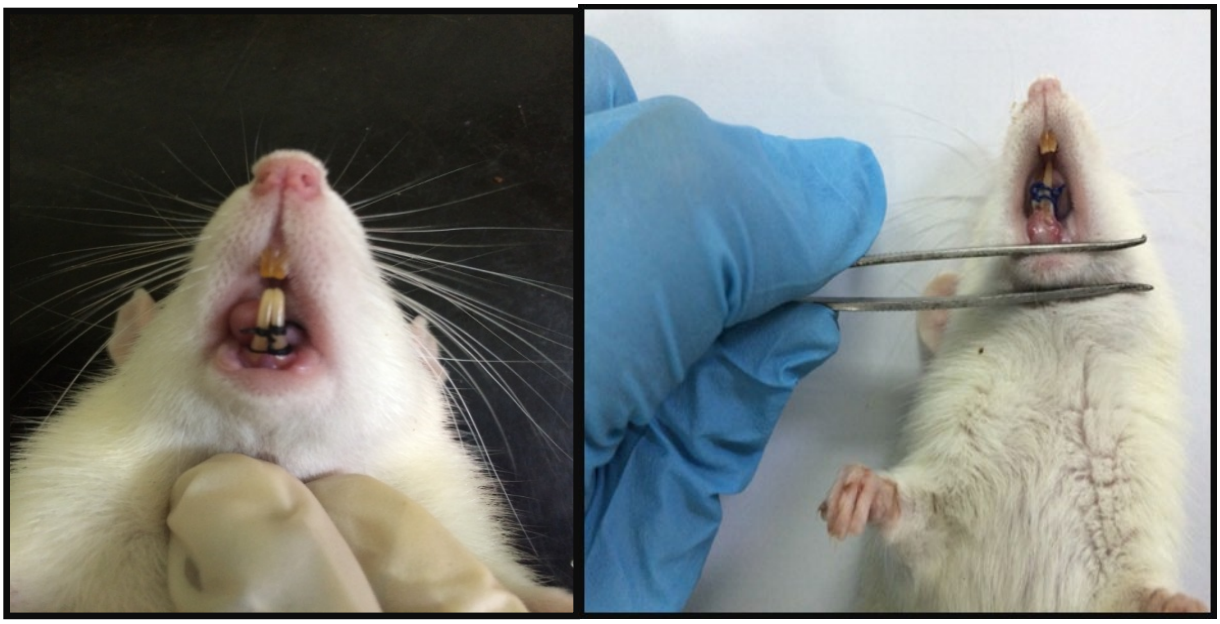
2.6 Animals

The animals were supplied by Animal Research and Service Center (ARASC), USM. The animals showed healthy intact teeth and no previous problem of periodontal disease. Five animals were housed in one plastic cage under standard conditions (12 h light/dark cycle and 22±2 °C), with free access to commercial standard rodent chow. Tap water was given ad *libitum*. The animals were allowed to adapt to the laboratory environment for about 5 days before the experiment. The study protocol was approved by the Animal Ethics Committee, School of Pharmaceutical Sciences, USM, Penang, Malaysia (Clearance No(103)(805), 2016).

2.7 Ligature-induced periodontitis in rats

Thirty (30) adult male Sprague-Dawley rats (220 ± 20g body weight) were used. A total of twenty- four (24) rats were subjected to ligature-induced periodontitis, while the remaining 6 rats were used as a control group. General anesthesia was achieved through intramuscular injection of 0.2 mL/100g body weight containing 10% ketamine & 2% xylazine (100mg/ kg and 10mg/kg, respectively) in a ratio of 2:1 (Fernandes et al., 2010). Ligatures in "8" with HR40 nonresorbable sterile silk thread was placed around the cervix of the lower incisor frontal tooth and knotted on the buccal side (Ionel et al., 2015, Huet et al., 2021**)**.

The clinical manifestation of periodontitis was observed on Day 5, which included edema and redness of gingiva and bleeding upon probing (Figure 2).



On Day 0, after confirmation of the existence of periodontitis, the 24 rats were further divided into two groups, 6 rats in the untreated group and another 18 rats were further subdivided into 3 groups of six animals in each group.(Since ligature was left for five days for induction of periodontitis, some animals could not survive due to the infection and hence could not be used

in final results). Group 1 received 5% metronidazole loaded gel formulation, Group 2 received 0.15 % diclofenac potassium loaded gel formulation and Group 3 received 5% metronidazole and 0.15% diclofenac potassium loaded gel formulation. The gel of 0.2 mL was administered using 1 mL syringe with needle of 20G (Terumo, Japan, Tokyo) to the infected periodontium (Figure 3).

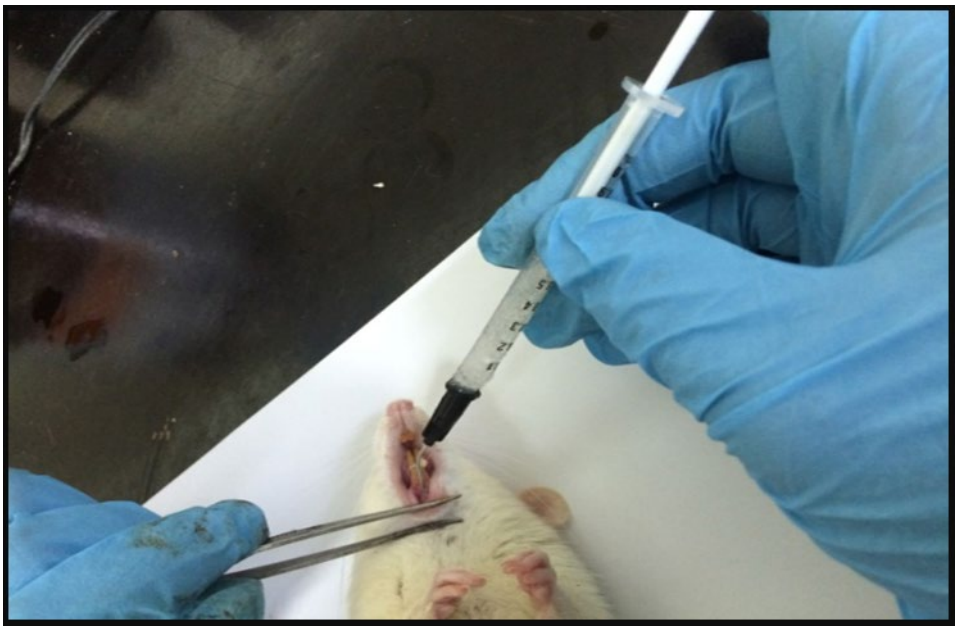


Figure 3

After administration of gel, cyanoacrylate n-butyl-2-cyanoacrylate was applied in thin layer over the pocket to prevent leakage of gel from the pocket (Eskandari et al., 2006; Sagar et al., 2015). On Day 7, all the animals were euthanized by exposing to ether and their maxilla excised and examined in Histology Laboratory, Lam Wah Ee hospital (Penang, Malaysia). The samples were decalcified by 1/1 mixture of 8 % formic acid and 8 % chlorhydric acid for two weeks. The tissue was trimmed and dehydrated using isopropyl alcohol at different concentrations of 70 %, 90 %, and 100 % clarified in xylene. The tissue was fixed on paraffin block and sectioned before staining with hematoxylin and eosin. It was examined histologically and score evaluations were applied under a microscope (Olympus, Germany). The indicators used for evaluation were

inflammatory cells count, necrosis, edema, presence of granulation, complete organization of periodontal ligament without tearing and re-epithelization as the repair parameters of the healing process. Each parameter was scored from 0 to 3, where 0(0%) = normal, 1(16.5%) = mild increase, 2(33.3%) = moderate increase and 3(50%) = severe increase. The histological analysis was performed by a blind examiner in all groups (Botelho et al., 2010a; Ramadan et al., 2010; Liu et al., 2012).

2.8 Statistical analysis

The results of healing parameters were evaluated using Kruskal-Wallis test (GraphPad PRISM, Version 5.01, USA). When there was a statistically significant difference, post-hoc Dunn test was performed. A statistically significant difference was considered at $p < 0.05$.

3. Results and discussion

3.1 *Determination of gelation temperature*

In general, the physical properties of polymeric solution depend primarily on concentration. However, the physical properties of Poloxamer solution are influenced by both concentration and temperature. Poloxamer solution exhibits thermoreversible gelation (Yong et al., 2003). The temperature dependent gelation of Poloxamer solutions is attributed to the changes in their micellar properties. Table 1 presents the gelation temperature values of various Poloxamer content comprising of different compositions of Poloxamer 407 and 188. Incorporation of both Poloxamer 407 and 188 could modify the PEO/PPO ratio, leading to changes in gelation temperature. The gelation temperature of 30.0 % and 37.5 % Poloxamer contents were within the temperature of 33-37 °C in the oral cavity, which was the desired gelation temperature range

and suitable for periodontal application (Choi et al., 1998; Baloglu et al., 2011). Poloxamer content of 30% was selected for subsequent experiment as lower polymer content was employed and the temperature was close to the physiological temperature of the oral cavity.

Table 1: Poloxamer mixtures with corresponding gelation temperature values. Mean ± S.D, N=3.

Poloxamer content (%w/w) (Poloxamer 407 : Poloxamer 188)	Gelation temperature (°C)
7.5 (5.0 : 2.5)	No gelation up to 45
15.0 (10.0 : 5.0)	
22.5 (15.0 : 7.5)	
30.0 (20.0 : 10.0)	35.7 ± 0.6
37.5 (25.0 : 12.5)	33.2 ± 0.8
45.0 (30.0 : 15.0)	30.2 ± 0.8
52.5 (35.0 : 17.5)	28.0 ± 1.0
60.0 (40.0 : 20.0)	27.5 ± 0.58

3.2 *Syringeability measurement*

It was reported that the work of injection/syringeability was more accurate than simple measurement of force to characterize the extrusion of syringe content (Zhang et al., 2018). The work needed to expel the gel from the syringe was 4.300 ±0.900 (N Sec). It was noted the content of Xanthan gum 2.0 % increased the work of syringeability (Karavana, 2012; Singh et al., 2014). The present study used a blend of poloxamer 407 and 188 with xanthan gum, which could affect the work of syringeability as well as the gelation temperature.

3.3 *In vitro* drug release studies

. The mean release profiles of metronidazole and diclofenac potassium of *in-situ* gelling formulation are shown in Figure 4. Metronidazole release was found to be relatively faster than that of diclofenac potassium. It could be observed that the in-situ gelling formulation could prolong the release of metronidazole and diclofenac potassium to 7 days. This could reduce the dosing frequency and improve patient compliance by administering the formulation once a week.

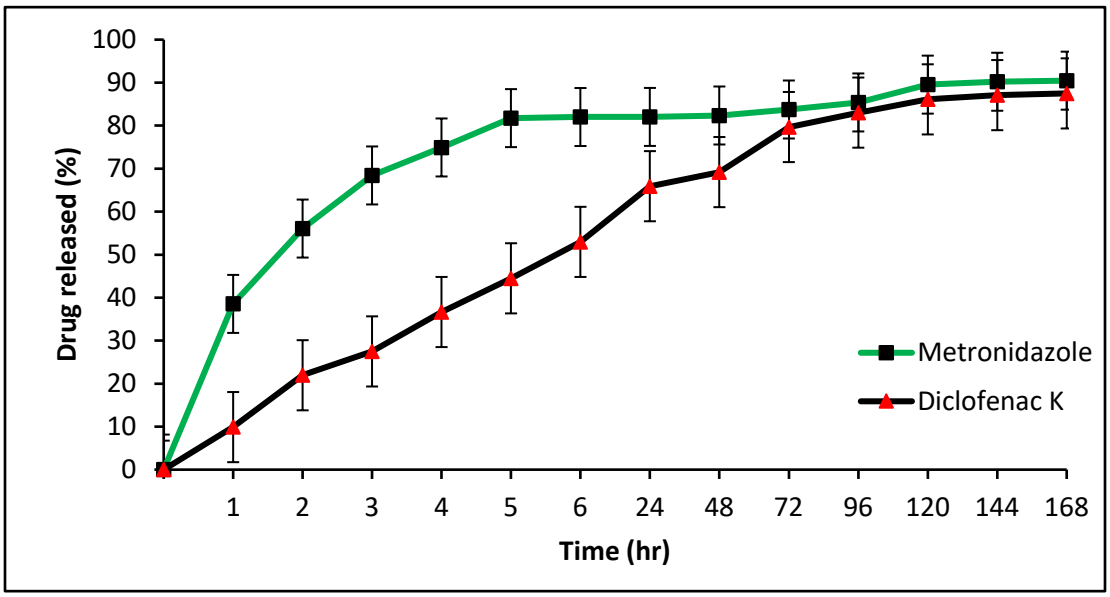
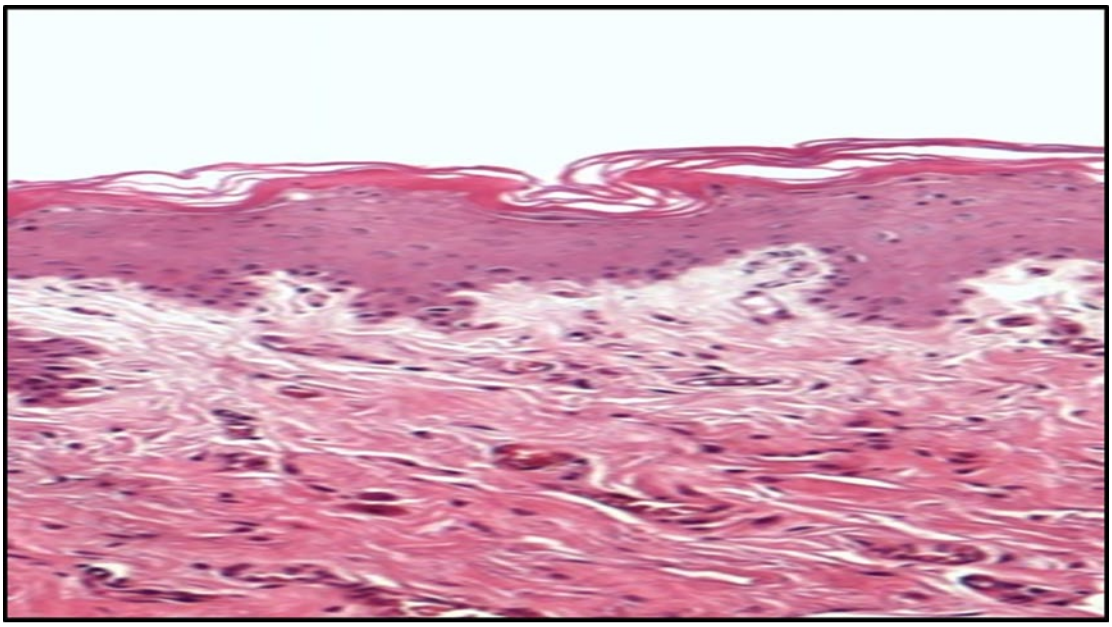


Figure 4



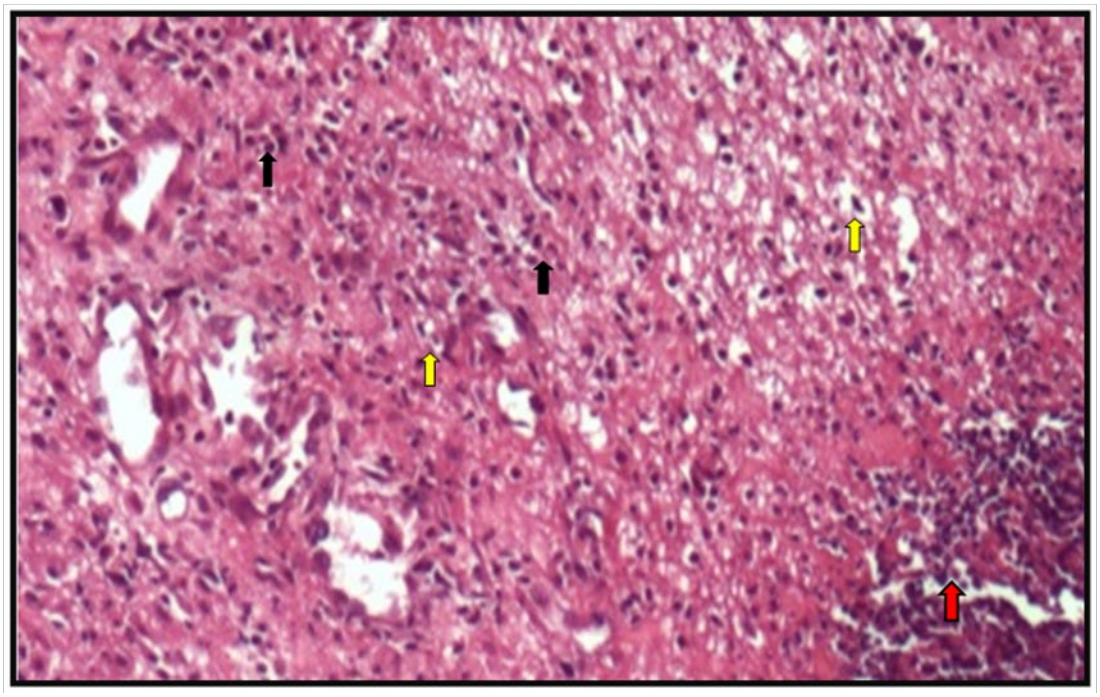


Figure 5

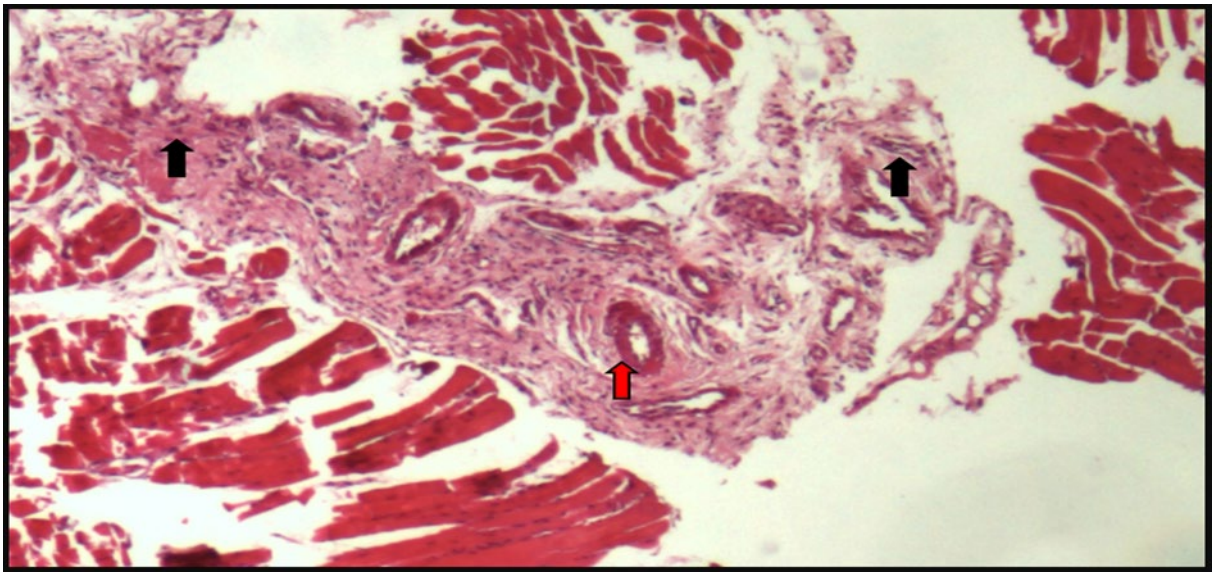
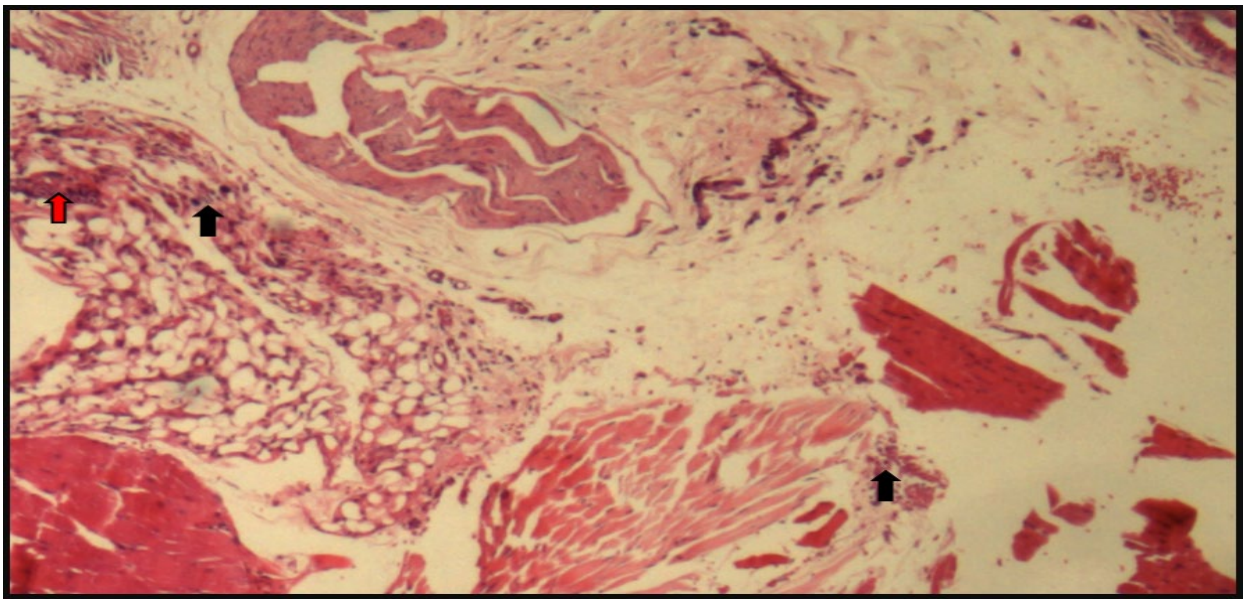


Figure 6



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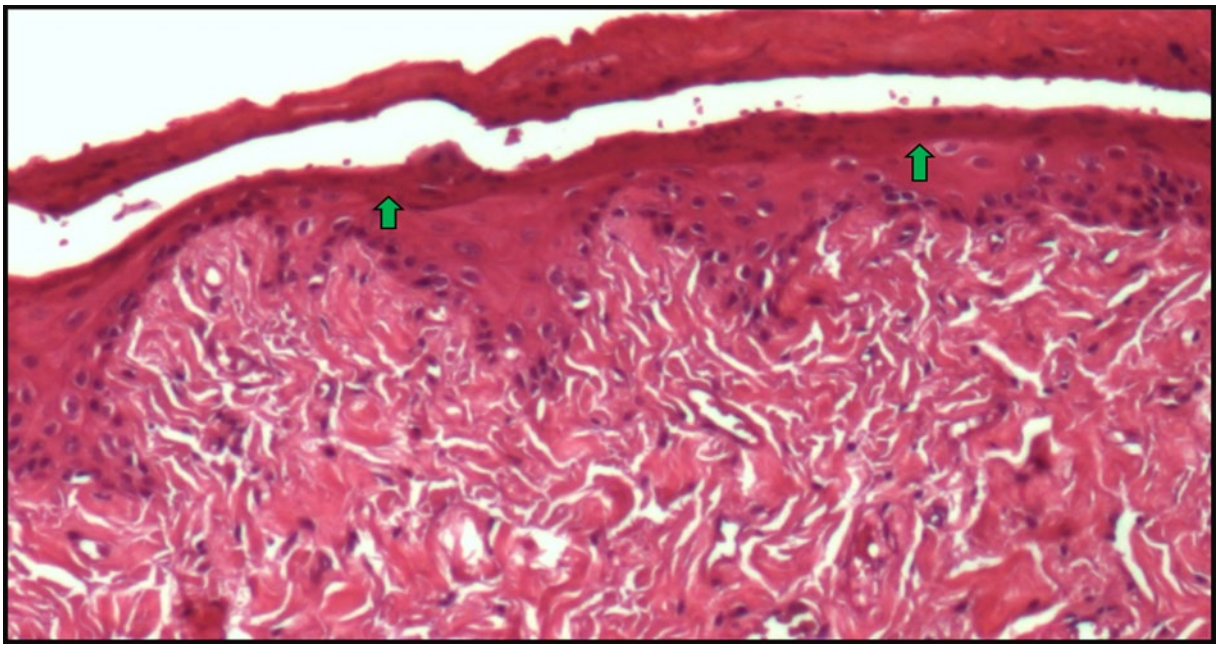

Figure 8

3.4 *Histopathological study*

The scores of inflammatory parameters of untreated and treated rats after induction of periodontitis are presented in Table 2. From histopathology examination, it was found that the untreated group revealed intense inflammatory response to irritation caused by the ligature. There was abundance of infiltration of polymorph nuclear (PMN) inflammatory cells and monocytes when compared with normal tissues without exposure to ligature-induced periodontitis (Figure 5). Edema, necrosis and granulation could be observed in the tissues. Periodontal ligaments were disorganized. No re-epithelization occurred. The findings were consistent with the results reported in previous studies (Botelho et al., 2010a; Ionel et al., 2015).

For the group treated with metronidazole loaded gel formulation, there was presence of inflammatory cells diffused among the gingival epithelial and granulation tissue. Necrosis was less than that observed in the untreated group (Figure 6). This observation was consistent with

the antibacterial activity of metronidazole against both Gram positive and negative microorganisms (Sander et al., 1994; Ramadan et al., 2010; Liu et al., 2012).

Table 2

Treatments	No	Score of Healing Parameters					
		Presence of PMN	Ligament organization	Granulation	Re-epithelization	Necrosis	Edema
Control group	1	0	organized	0	0	0	0
	2	0	organized	0	0	0	0
	3	0	organized	0	0	0	0
	4	0	organized	0	0	0	0
	5	0	organized	0	0	0	0
	6	0	organized	0	0	0	0
	Mean	0		0	0	0	0
	SD	0		0	0	0	0
Untreated Rat	1	3.00	disorganized	2.00	0.00	2.00	2.00
	2	3.00	disorganized	2.00	0.00	3.00	1.00
	3	3.00	disorganized	1.00	0.00	2.00	2.00
	4	3.00	disorganized	2.00	0.00	3.00	2.00
	Mean	3.00		1.75	0.00	2.50	1.75
	SD	0.00		0.50	0.00	0.58	0.50
Metronidazole loaded gel formulation	1	2.00	disorganized	1.00	1.00	1.00	0.00
	2	1.00	disorganized	0.00	2.00	1.00	0.00
	3	2.00	disorganized	1.00	2.00	1.00	0.00
	4	1.00	disorganized	1.00	2.00	1.00	0.00
	Mean	1.50		0.75	1.75	1.00	0.00

	SD	0.58		0.50	0.50	0.00	0.00
Diclofenac potassium loaded gel formulation	1	1.00	disorganized	1.00	0.00	1.00	0.00
	2	2.00	disorganized	1.00	1.00	0.00	0.00
	3	2.00	disorganized	1.00	0.00	0.00	0.00
	4	1.00	disorganized	1.00	1.00	1.00	0.00
	Mean	1.50		1.00	0.50	0.50	0.00
	SD	0.58		0.00	0.58	0.58	0.00
Metronidazole and diclofenac potassium loaded gel formulation	1	1.00	organized	1.00	3.00	0.00	0.00
	2	0.00	organized	1.00	3.00	1.00	0.00
	3	0.00	organized	0.00	3.00	0.00	0.00
	4	1.00	organized	1.00	3.00	1.00	0.00
	Mean	0.50		0.75	3.00	0.50	0.00
	SD	0.58		0.50	0.00	0.58	0.00

Similarly, the group treated with diclofenac potassium loaded gel formulation showed relatively lower scores for all the inflammatory parameters in comparison with the untreated group (Figure 7). This observation indicated that topical therapy of NSAID could reduce periodontitis disease progression via modulation of arachidonic acid metabolism. These results were consistent with the findings of other reported studies (Bräger et al., 1997; Paquette, 1998; Botelho et al., 2010b). Some studies showed that diclofenac potassium can combat multidrug-resistant bacteria and problematic antibiotic-resistant bacterial infections (Dutta et al., 2007; Worthington and Melander, 2013; Salem-Milani et al., 2013)

The healing of the group treated with metronidazole and diclofenac potassium loaded gel formulation was more obvious in terms of improvement and continual organization of

periodontal ligament, formation of new epithelial or re-epithelization and absence of edema (Figure 8). There was a statistically significant difference in the scores of the inflammatory parameters namely, presence of PMN, granulation, re-epithelization, necrosis and edema, between the untreated rats and rats treated with three different formulations, gel formulation loaded with metronidazole, diclofenac potassium or both metronidazole and diclofenac potassium.

A statistically significant improvement was observed solely for edema between the untreated and the group treated with metronidazole loaded gel formulation. There was a statistically significant improvement in edema and necrosis when treated with diclofenac potassium loaded gel formulation. When treated with gel loaded with both metronidazole and diclofenac potassium, there was significant improvement in the presence of PMN, re-epithelization, necrosis and edema. When gel loaded with single drug and two drugs were compared, a statistically significant difference was only obtained in re-epithelization and organization of the ligament between gel formulation loaded with diclofenac potassium or metronidazole and loaded with two drugs.

There was no statistically significant difference in any inflammatory parameter between the gel loaded with diclofenac potassium and the gel loaded with metronidazole. Diclofenac potassium showed a beneficial anti-inflammatory effect, which could give a more pronounced healing (Cavagni et al., 2016). Diclofenac potassium might compliment the antibacterial action of metronidazole in inhibition of DNA synthesis and eradication of the pathogens (Hersh et al., 1991; Dastidar et al., 2000).

The results revealed that the treatment of periodontal disease could be achieved successfully using a thermosensitive *in situ* gel delivery system. The use of a combination of non-steroidal

anti-inflammatory drug and antibiotic could accelerate the healing process compared to the use of antibiotic or anti-inflammatory drug alone in the treatment of periodontal disease (Mohiuddin et al., 2011). This can be seen from the significant improvement in more inflammatory parameters when both drugs were used.

Conclusion

A thermosensitive, *in situ* gelling formulation containing metronidazole and diclofenac potassium for treatment of periodontal disease was successfully developed. The gel demonstrated prolonged drug release over an extended period. This local delivery system is anticipated to improve patient compliance and clinical outcome as the gel formulation could be applied directly to the site of action. The effectiveness of the co-therapy of antibacterial and anti-inflammatory agents such as metronidazole and diclofenac potassium can play a potentially advantageous role in the clinical therapy of the periodontal disease, which is more effective than using either antibacterial or anti-inflammatory drug. The co-therapy could add benefits towards the eradication of chronic periodontal disease. This rationalizes the prescribing treatment of the combination of antibacterial and anti-inflammatory agents.

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