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*RESEARCH ARTICLE*

# Evaluation of Anti-Inflammatory Activity of Metronidazole Treatment On Carrageenan Induced Paw Edema in Mice

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***Evaluation of Anti-Inflammatory Activity of Metronidazole Treatment On Carrageenan Induced Paw Edema in Mice***

***SUMMARY***

*Metronidazole is a nitroimidazole derivative antibiotic that has been used against protozoa and anaerobic organisms for a long time. Furthermore, it has been used in non-infectious inflammatory diseases such as acne, Crohn's disease, periorificial dermatitis, rosacea and seborrheic dermatitis recently. However, the studies about this issue are very few and its mechanism of action is unknown. The aim of our study is to evaluate the possible anti-inflammatory activity of metronidazole in vivo by using the mice- carrageenan-induced paw edema method. Mice were administered a single dose of 2, 20 or 200 mg/kg metronidazole via oral gavage. One hour later, 2% carrageenan was injected sub-plantar to the hind paws. The paw thickness of mice was measured just before the carrageenan injection and at 1, 2, 3, 4, 24 and 48 hours after injection by dial thickness gauge. For comparison, another group of mice received indomethacin (10 mg/kg, orally) used as a reference drug. IL-1β and TNF-α levels in the paws of mice were measured by the ELISA method. ANOVA (post-hoc Bonferroni) and Student's t tests were used for statistical analysis. Metronidazole displayed equi-potent anti-inflammatory activity with indomethacin in the carrageenan-induced mouse paw edema model. It is shown that less edema occurred at all doses (2, 20 and 200 mg/kg) compared to the control group and no differences were obtained in effect between the doses. It was observed that in metronidazole treated groups, paw thickness returned to baseline values 48 hours after carrageenan injection, unlike the control group. IL-1β and TNF-α levels, which were increased with carrageenan injection, were significantly decreased with metronidazole treatment. In our study, metronidazole was found to be anti-inflammatory due to its effects on relieving edema and reducing pro-inflammatory cytokines in the paws of carrageenan-induced mice. The effectiveness of metronidazole in treating various non-infectious diseases in recent years may be due to its anti-inflammatory activity.*

***Key Words:*** *Metronidazole, anti-inflammatory activity, mouse, paw-edema test*

***Farelerde Karragenanla İndüklenmiş Pençe Ödem Modelinde Metronidazol Tedavisinin Anti-inflamatuvar Aktivitesinin Değerlendirilmesi***

***ÖZ***

*Metronidazol uzun yıllardır protozoalara ve anaerob bakterilere karşı kullanılan nitroimidazol türevi bir antibiyotiktir. Son yıllarda bunlara ek olarak, akne, Crohn hastalığı, periorifisyal dermatit, rozase ve seboreik dermatit gibi non-enfeksiyöz inflamatuvar hastalıklarda da kullanılmaya başlanmıştır. Ancak bu konudaki çalışmalar sınırlıdır ve bu etkinin mekanizması bilinmemektedir. Çalışmamızın amacı metronidazol'ün olası anti-inflamatuvar etkinliğinin karragenan ile indüklenen pençe ödemi yöntemi kullanılarak farelerde in vivo olarak gösterilmesidir. Deneylerde farelere gavaj yoluyla 2, 20 ya da 200 mg/kg metronidazol tek doz olarak uygulanmıştır. Bir saat sonra farelerin arka pençelerine inflamasyon oluşturmak için sub-plantar %2 karragenan enjeksiyonu yapılmış ve pençe kalınlığı karragenan enjeksiyonunun hemen öncesinde ve enjeksiyondan 1, 2, 3, 4, 24 ve 48 saat sonra mikrometrik kompas ile ölçülmüştür. Metronidazol'ün etkinliği, ayrı bir grup fareye referans ilaç olarak verilen (10 mg/kg, oral) indometazin ile karşılaştırılmıştır. Farelerin pençelerinde IL-1β ve TNF-α düzeyleri ELISA yöntemiyle ölçülmüştür. İstatistiksel analiz varyans analizi (post-hoc Bonferroni) ve Student's t test ile yapılmıştır. Farelerde karragenanla indüklenen pençe ödemi modelinde metronidazol, indometazinle benzer derecede anti-inflamatuvar etkinlik göstermiştir. Metronidazol her üç dozda (2, 20 ve 200 mg/kg) da kontrol grubuna kıyasla ödem oluşmasını engellemiştir ve etkide dozlar arasında anlamlı bir farklılık bulunmamıştır. Kontrol grubunda 48. saatte hala bir miktar devam eden ödem, metronidazol gruplarında kaybolmuştur. Karragenan enjeksiyonu ile artmış olan IL-1β ve TNF-α düzeyleri metronidazol tedavisi ile anlamlı olarak azalmıştır. Çalışmamızda metronidazol farelerde karragenan ile indüklenen pençe ödemini azaltıcı ve pro-inflamatuvar sitokinleri düşürücü etkileri nedeniyle anti-inflamatuvar bulunmuştur. Metronidazol'ün son yıllarda çeşitli non-enfeksiyöz hastalıkların tedavisindeki etkinliği anti-inflamatuvar aktivitesinden ileri gelebilir.*

***Anahtar Kelimeler:*** *Metronidazol, anti-inflamatuvar aktivite, fare, pençe-ödem testi*

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## INTRODUCTION

Metronidazole is a nitroimidazole derivate antibiotic drug that has been used against protozoa and anaerobic bacteria for many years. It has been used alone or in combination with other antibiotics in many diseases such as pelvic inflammatory diseases, endocarditis and bacterial vaginosis. Besides its conventional use, it has been started to use both topically and orally in different indications such as acne vulgaris, Crohn's disease, periorificial dermatitis, rosacea and seborrheic dermatitis (Boeck, Abeck, Werfel, & Ring, 1997; Nishimuta & Ito, 2003; Pradhan, Madke, Kabra, & Singh, 2016; Seckin, Gurbuz, & Akin, 2007; Zip, 2010). These diseases are found to be related to inflammation (Dainichi, Hanakawa, & Kabashima, 2014) and it has been suggested that metronidazole has anti-inflammatory, immunosuppressive and antipruritic properties as well as antibacterial activity (Pradhan et al., 2016). Although the usage of metronidazole has become widespread, it is not known whether its effectiveness in such a wide range of diseases is due to its antibiotic, immunosuppressive or anti-inflammatory properties (Nishimuta & Ito, 2003).

There are a few studies in the literature investigating the anti-inflammatory effect of metronidazole. In one of them, metronidazole was shown to inhibit leukocyte-endothelial cell adhesion (Arndt, Palitzsch, Grisham, & Granger, 1994). In other studies, metronidazole altered the neutrophil activity and reduced the radical oxygen derivatives (Akamatsu et al., 1990; Del Rosso & Baum, 2008; Miyachi, 2001; Miyachi, Imamura, & Niwa, 1986). However, these studies are not sufficient for discussing the in vivo anti-inflammatory effects of metronidazole. Furthermore, the functional role of metronidazole on acute inflammation is not known yet. Therefore, in our study we aimed to induce inflammation acutely in mice by using the carrageenan to investigate the acute and long-time anti-inflammatory effect of metronidazole at increasing doses. Furthermore, we also investigated the possible mechanism of anti-inflammatory action of metronidazole, measuring some important cytokine levels in inflammation.

## MATERIAL AND METHODS

### Animals

Male, 8-12 weeks old Swiss albino mice were used in the experiments. Mice were housed in a room with a 12-hour day/night cycle at constant humidity and temperature (22 °C) and fed with a standard pellet diet. All animal use was approved by the Kobay DHL A.Ş. Local Ethics Committee (Decision approval date and number: 11.09.2020 and 503). All the procedures with animals were performed according to the rules of the "Guide for the Care and Use of Laboratory Animals".

### Metronidazole Treatment

Metronidazole (Flagyl® 125 mg / 5 mL Oral Suspension, Sanofi Sağlık Ürünleri Ltd. Şti., İstanbul) was diluted to 0.3 mL of distilled water and administered to mice by gavage. The groups were administrated with 2, 20 or 200 mg/kg metronidazole as a single dose. Indomethacin (I7378, Merck, Darmstadt, Germany), known to be a well-established anti-inflammatory drug, was used as a reference drug (orally;10 mg/kg). The same volume of water was given to the control group mice by gavage. In each of the groups, eight mice were used.

2, 20 and 200 mg/kg doses of metronidazole were chosen in accordance with the dose previously applied to rats and took into consideration of the therapeutic dose for patients (Ganrot-Norlin, Stalhandske, & Karlstrom, 1981; Reagan-Shaw, Nihal, & Ahmad, 2008). Metronidazole, indomethacin, and distilled water were administered to mice 1 hour before carrageenan injection according to the study showed that the metronidazole reaches the maximum concentration in the blood one hour after oral administration (Ralph, 1983).

### Carrageenan-induced Paw Edema Test

*In vivo* anti-inflammatory activity was evaluated

by a carrageenan-induced paw edema test (Posadas et al., 2004). Inflammation was induced by sub-plantar administration of 0.01 mL of 2% carrageenan (Sigma-Aldrich, Germany) into the hind paws of mice. The increase in paw thickness indicates inflammation-related edema and the reduction in edema reflects the anti-inflammatory activity of the administered drug. A dial thickness gauge was used to measure the paw thickness of mice (Dial Thickness Gauge, Ozaki Co., Japan; 0.01-1.0 mm). The paw measurements were performed just before ($x_0$) and 1, 2, 3, 4, 24 and 48 hours after the carrageenan injection (x) (Murat Ulu, 2019). The calculations were performed according to the formulas given below.

Change in paw thickness ($\Delta$)=x- $x_0$

Edema inhibition (%) = [(Control $\Delta$ – Treatment $\Delta$) / Control $\Delta$] x 100

Potency was calculated according to the formula below. Because edema occurred mostly 2 hours after the carrageenan injection, the edema inhibition % of the 2$^{nd}$ hour was selected for the potency calculations.

Potency = [% edema inhibition of metronidazole of each animal (at 2$^{nd}$ hour) / the mean of % edema inhibition of indomethacin (at 2$^{nd}$ hour)] x 100

**ELISA Assays**

One series of mice were sacrificed at the second hour of injection. Their paws were isolated and froze immediately with liquid nitrogen. Tissues were kept at -80 °C until experiment day. Interleukin-1β (IL-1β) and Tumor Necrosis Factor alpha (TNF-α) levels were measured using ELISA kits (Invitrogen IL-1 beta Mouse ELISA Kit #BMS6002 and TNF alpha Mouse ELISA Kit #BMS607HS) according to the manufacturer's instructions.

**Statistical analysis**

Two-way repeated or one-way ANOVA and *post hoc* Bonferroni test was performed for statistical analysis. GraphPad Prism 5 Software (San Diego, USA) was used for analysis. Results are given as mean ± standard error of mean and a *p* value less than 0.05 is considered statistically significant.

**RESULTS**

Carrageenan-induced paw edema in the groups administered with 2, 20, and 200 mg/kg metronidazole was reduced at all measurement time points compared to the control group (Figure 1.).

Edema reached its highest level in the second hour in the control group and remained high until the fourth hour.

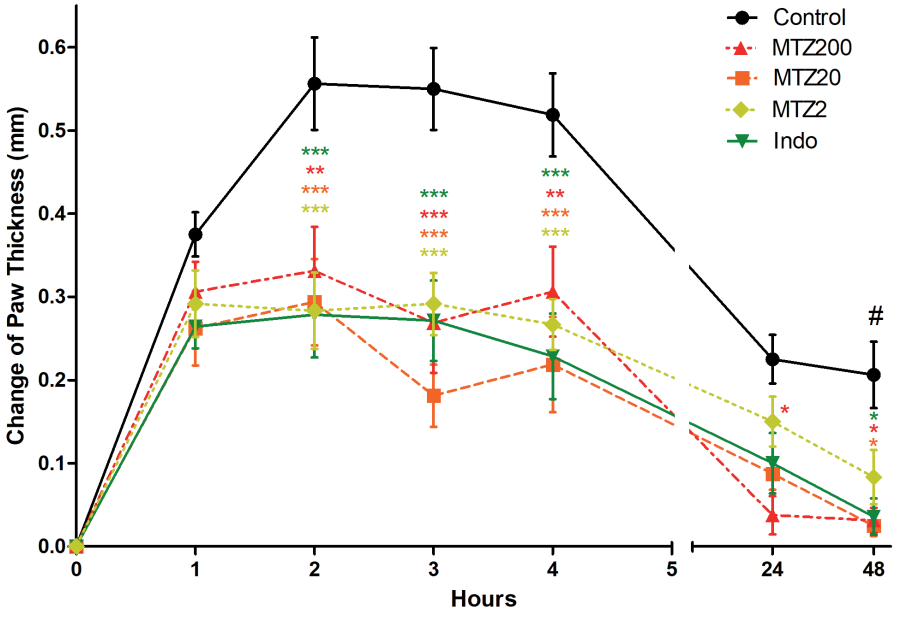


**Figure 1.** Changes of paw thickness after carrageenan injection

**Figure 1.** Time-dependent changes of paw thickness after carrageenan injection in mice treated with metronidazole (MTZ) (2, 20 and 200 mg/kg, orally). Data are expressed as mean ± standard error of mean. Eight mice were used for each group. Statistical analysis was performed with GraphPad Prism 5 Software. Two-way ANOVA on repeated measures and post-hoc Bonferroni test was used (* $p<0.05$; ** $p<0.01$; *** $p<0.001$ compared to time matched control group; #, $p< 0.001$ compared to initial paw thickness of control group).

One hour after carrageenan injection, the decrease of paw edema in metronidazole-treated mice did not show a statistically significant difference compared to the control group (Figure 1.). However, in the following hours, metronidazole significantly reduced carrageenan-induced paw edema at all doses compared to the control group (Figure 1.). There is no significant difference in edema relieving effect of metronidazole between different doses.

The developed edema did not dissipate even after 48 hours (#, $p=0.0019$) in the control group, whereas the paw thickness returned to the baseline values after 48 hours in all metronidazole groups (Figure 1.). Indomethacin significantly reduced carrageenan-induced paw edema compared to the control group (Figure 1.). There was no statistically significant difference between the metronidazole groups and the indomethacin group (Figure 1.). Metronidazole groups were found to be as potent as the indomethacin group, comparing the edema inhibition % of the metronidazole groups to the indomethacin group (Table 1.).

**Table 1.** Edema inhibition % and potency of metronidazole (MTZ) groups

| | Indomethacin | MTZ 2 mg/kg | MTZ 20 mg/kg | MTZ 200 mg/kg |
|---|---|---|---|---|
| **% Edema inhibition[a]** | 49,92 ± 9,23 | 49,06 ± 11,54 | 47,19 ± 9,32 | 40,45 ± 9,53 |
| **Potency[a]** | 100 ± 18,50 | 98,28 ± 23,12 | 94,53 ± 18,68 | 81,02 ± 19,08 |

[a]Comparison of edema inhibition % and potency of metronidazole (MTZ) groups with indomethacin group 2 hours after carrageenan injection. Data are given as mean ± standard error of mean.

The carrageenan injection in the paws of mice induced an inflammatory reaction in mice as shown by significantly increased levels of IL-1β and TNF-α (Figure 2.). Indomethacin decreased IL-1β and TNF-α levels compared to the control group. Metronidazole treatments at all doses also significantly reduced the levels of IL-1β and TNF-α compared to the control group (Figure 2.).

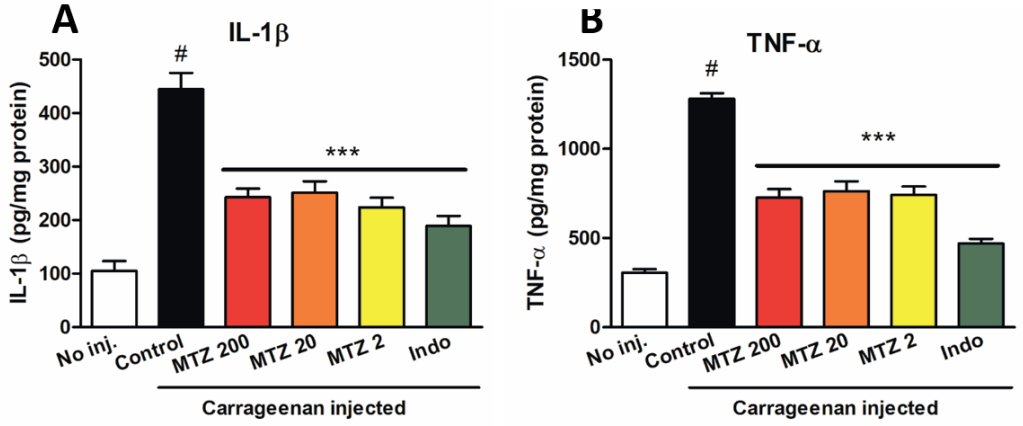


**Figure 2.** The effects of carrageenan injection on pro-inflammatory cytokines

**Figure 2.** The changes of pro-inflammatory cytokines after carrageenan injection in the paws of mice treated with metronidazole (MTZ) (2, 20 and 200 mg/kg, orally) or indomethacin (Indo) (10 mg/kg, orally). Data are expressed as mean ± standard error of mean. Four mice were used for each group. Statistical analysis was performed with GraphPad Prism 5 Software. One-way ANOVA and post-hoc Bonferroni test were used (# $p<0.001$ compared to the non-injected group; *** $p<0.001$ compared to control group).

**DISCUSSION**

Metronidazole is an antibiotic that has been prescribed for many years for its antibacterial and antiprotozoal properties. The action mechanism of metronidazole against anaerobic bacteria and protozoa is microbial DNA damage and nucleic acid synthesis inhibition (Edwards, 1993; Pradhan et al., 2016; Seidler Stangova et al., 2019). It has been used alone or in combination with other antibiotics in many diseases such as amebiasis, trichomoniasis, giardiasis, vaginitis, endocarditis, surgical chemoprophylaxis or *Helicobacter pylori* eradication (Chey, Leontiadis, Howden, & Moss, 2017; Petrin, Delgaty, Bhatt, & Garber, 1998). Since metronidazole is included in the first-line treatment known as triple therapy with proton pump inhibitor and clarithromycin for *Helicobacter pylori* infection, it is prescribed frequently (Chey et al., 2017).

Metronidazole has also been used in various non-infectious diseases in recent years (Khodaeiani et al., 2012; Pradhan et al., 2016; Seidler Stangova et al., 2019; Zip, 2010). Topical gels or creams are effective in rosacea, acne vulgaris, periorificial dermatitis and seborrheic dermatitis (Boeck et al., 1997; Del Rosso & Baum, 2008; Khodaeiani et al., 2012; Nishimuta & Ito, 2003; Seckin et al., 2007; Zip, 2010). It has been suggested that the therapeutic effect of metronidazole in rosacea and acne vulgaris may be due to its anti-inflammatory effect rather than its antibacterial effect (Nishimuta & Ito, 2003). However, the action mechanism of metronidazole in the treatment of various non-infectious diseases is not fully understood. In one of the previous studies about its anti-inflammatory activity, metronidazole prevented leukocyte endothelial adhesion (Arndt et

al., 1994). In other studies, it has been suggested that metronidazole may have an anti-inflammatory effect by changing neutrophil activity and reducing radical oxygen derivatives (Akamatsu et al., 1990; Del Rosso & Baum, 2008; Miyachi, 2001; Miyachi et al., 1986).

In our study, the anti-inflammatory activity of metronidazole was investigated in a carrageenan-induced paw edema model in mice. Metronidazole showed significant anti-inflammatory activity at all three doses via reducing carrageenan-induced edema and two important pro-inflammatory cytokines. We selected three doses of metronidazole for scanning a large dose-effect range and assessing the dose-response relation of metronidazole and its anti-inflammatory activity of it. The 20 mg/kg dose was chosen in accordance with the dose of metronidazole used in various infectious diseases in the clinic. When the body surface area normalization method was used instead of a simple conversion based on body weight for dose translation (Reagan-Shaw et al., 2008), the dose of metronidazole was calculated as 200 mg/kg for mice. Thus, a group of mice administered 200 mg/kg was also created. Finally, the low (2 mg/kg) dose was administered to test the subclinical effect. We found no difference in preventing edema between the groups that received increasing doses of metronidazole, suggesting that the anti-inflammatory effect was not dose-dependent in this dose range. According to our findings, it can be suggested that metronidazole may have an anti-inflammatory effect besides its antibacterial effect at the dose used in the treatment of various bacterial diseases in the clinic (e.g. 500 mg or 3x250 mg).

Acute inflammation is characterized by edema that develops as a result of increased vascular permeability, extravasation of fluids and proteins, cellular infiltration and accumulation of leukocytes in the inflammation area (Necas & Bartosikova, 2013; Posadas et al., 2004). The carrageenan-induced paw edema model is a well-known and widely used acute inflammation model (Necas & Bartosikova, 2013; Xue, Wu, Wu, Li, & Wang, 2019). Carrageenan injection develops edema, one of the five cardinal signs of inflammation, without causing any injury or damage to the inflamed paw (Necas & Bartosikova, 2013; Telli, Kazkayasi, & Uma, 2021). Indomethacin is a non-steroidal anti-inflammatory drug with strong anti-inflammatory activity. It has been used as a reference drug in the carrageenan-induced paw edema model (Akindele & Adeyemi, 2007). Therefore, indomethacin was used as a reference drug in our study and metronidazole groups were found to be as potent as it is.

In the carrageenan-induced paw edema model, it was observed that the control group developed severe edema starting from the second hour of injection, which is consistent with previous studies (Necas & Bartosikova, 2013; Posadas et al., 2004). In our study, measurements were continued until the 48$^{th}$ hour. The paw thickness of control group did not return to the initial values even 48 hours after injection. On the other hand, the paw thickness of metronidazole groups at 48th hour was not different from the initial value, indicating that the anti-inflammatory effect persisted.

IL-1β, which stimulates the local and systemic inflammation responses of the body is a prototypical cytokine. It stimulates the accumulation of inflammatory cells by inducing the release of adhesion molecules on endothelial cells. It also triggers the synthesis of various enzymes, which contribute to the release of inflammatory mediators (Gabay, Lamacchia, & Palmer, 2010). In our study, carrageenan injection increased IL-1β level which was decreased by metronidazole treatments. Another pro-inflammatory cytokine TNF-α, regulates vascular endothelium and endothelial leukocyte interactions. This interaction in combination with the release of chemokines may lead to the recruitment of different populations of leukocytes. In addition, TNF-α contributes to the inflammation process by inducing fever, vasodilatation and expression of pro-coagulant

proteins (Bradley, 2008). Similarly, to IL-1β, we found that carrageenan injection increased TNF-α level which was decreased by metronidazole treatments. In accordance with our results, it has been shown that metronidazole incubation was shown to reduce IL-1β and TNF-α levels in lipopolysaccharide-induced human periodontal ligament cells (Rizzo et al., 2010). Thus, the mechanism underlying the anti-inflammatory effect of metronidazole may be due to its IL-1β and TNF-α reducing effect.

## CONCLUSION

In conclusion, our study showed for the first time that metronidazole has anti-inflammatory activity in acute inflammation *in vivo* by using a carrageenan-induced paw edema model in mice. Furthermore, it has been shown that pro-inflammatory cytokines, IL-1β and TNF-α, which were increased with carrageenan injection, decreased with metronidazole treatment. If its anti-inflammatory activity can be demonstrated in different inflammation models with further studies, the rationale for using metronidazole in various non-infectious diseases may be attributed to its anti-inflammatory activity and metronidazole can be used in the treatment of many other inflammatory diseases.

## CONFLICT OF INTEREST

The authors of this article declared no conflict of interest.

## AUTHOR CONTRIBUTIONS

IK developed the hypothesis, did the literature research, experiments and data analysis and wrote the paper. GT did the experiments, reviewed and edited the paper.

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