Fırat Tıp Derg/Firat Med J 2015; 20(3): 133-138

Experimental Research

The Effect of Administration of Oral Clarithromycin and Tetracycline on Postoperative Adhesion Formation in Rat Uterin Horn Model

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ABSTRACT

Objective: To investigate effectiveness of oral clarythromycin and tetracycline on the prevention of postoperative adhesions.

Materials and Methods: A total of 28 Wistar-albino rats were divided into four groups. Group 1: abdomen was opened and closed, Group 2: a 2 cm linear incision was made on the right uterine horn and closed, Group 3: 15 minutes after the administration of oral clarithromycin, a 2 cm incision was made on the right uterine horn and closed, Group 4: 15 minutes after the administration oral tetracycline, a 2 cm incision was made on the right uterine horn and closed. Fifteen days later, a re-laparotomy was performed in all groups. Right uterine horn adhesions were assessed on the basis of macro-morphological characteristics and tissue cross-sections were further examined for the signs of fibrosis, angiogenesis, and VEGF and for MDA scoring. Kruskal-Wallis variance analysis and Mann-Whitney U test were used for statistical analyses.

Results: The extent and strength of adhesions were significantly lower in Group 1, Group 3 and Group 4 as compared to Group 2. Fibrosis and angiogenesis scores were significantly higher in Group 2 than in Group 1, Group 3 and Group 4.

Conclusion: Oral administration of clarithromycin and tetracycline was effective in the prevention of postoperative adhesions.

Key Words: *Adhesion, Rat, Clarithromycin, Tetracycline*

ÖZET

Klaritromisin ve Tetrasiklinin Oral Verilmesinin Rat Uterin Horn Modelinde Postoperatif Adezyon Oluşumuna Etkisi

Amaç: Oral klaritromisin ve tetrasiklinin postoperatif adezyon oluşumunu önlemedeki etkisinin incelenmesi.

Gereç ve yöntem: 28 adet Wistar-albino cinsi rat rastgele dört gruba ayrıldı. Grup 1 (G1): Batın açılıp kapatılan grup. Grup 2 (G2): Sağ uterin horna 2 cm insizyon yapılıp kapatılan grup. Grup 3 (G3): Oral klaritromisin verildikten 15 dakika sonra sağ uterin horna 2 cm lineer insizyon yapılıp kapatılan grup. Grup 4 (G4): Oral tetrasiklin verildikten 15 dakika sonra sağ uterin horna 2 cm lineer insizyon yapılıp kapatılan grup. 15 gün sonra tüm grupların batınları tekrar açıldı. Sağ uterin horndaki adezyon makromorfolojik karekteristikler ve doku kesitlerinde fibrosis, anjiogenezis, VEGF ve MDA skorlaması temel alınarak değerlendirildi. İstatistiksel değerlendirme için Kruskal-Wallis varyans analizi ve Mann-Whitney U testi kullanıldı.

Bulgular: Adezyonun şiddeti ve yaygınlığı G1, G3 ve G4' te G2 ile karşılaştırıldığında anlamlı derecede daha düşük idi. Fibrozis ve anjiogenezis skorları G2' de G1, G3 ve G4' ten anlamlı derecede daha yüksek idi.

Sonuç: Oral klaritromisin ve tetrasiklin postoperatif adezyonu önlemede etkili bulundu.

Anahtar Kelimeler: *Adezyon, Rat, Klaritromisin, Tetrasiklin*

Abdominal and pelvic adhesions represent a major source of postoperative morbidity associated with abdominal or pelvic pain, infertility, or small intestinal obstruction and are responsible for nearly 30 to 41% of all cases with intestinal obstruction (1). Furthermore, mechanic blockade of the fallopian tubes due to pelvic adhesions is an important cause of infertility (2, 3).

Human peritoneal capillaries and arteriolar endothelial cells synthetize VEGF and other angiogenic factors that regulate proteolytic enzymes and their inhibitors. VEGF is a critical cytokine for the development of peritoneal adhesions and is an endothelial cell-specific mitogen playing an essential role in the induction of angiogenesis (4-7).

Tetracyclines and their derivatives have an impact on inflammation, immune modulation, cell proliferation, and angiogenesis (8). These agents are utilized in a wide variety of disorders for therapeutic purposes including arthritis, acne, periodontitis, and non-infectious diseases (9). On the other hand,

clarithromycin is known to reduce mucus secretion, suppress the production of pro-inflammatory molecules such as IL-8, IL-1, IL-6, and TNF-alpha, and inhibit the synthesis of VEGF and VEGF mRNA (10-12).

Objectives: To examine the effect of oral tetracycline and clarithromycin on the development of postoperative intra-abdominal adhesions in a rat uterine horn adhesion model.

MATERIALS AND METHOD

Protocol: This experimental study was undertaken at the Experimental Animal Laboratory, Fırat University following approval from the Ethics Committee of Fırat University. All experimental manipulations were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animals: A total of 28 regularly menstruating female Wistar-Albino rats weighing between 200 and 220 g were housed in cages of 7 with 12-h cycles of light and darkness and were fed with standard pellet food and tap water. Twelve hours prior to experimentation, food intake was discontinued and only water was allowed.

Experimental Groups: Rats were randomly allocated into the following four groups:

Group 1(n=7): The abdominal cavity was opened and closed 15 minutes after oral placebo.

Group 2(n=7): A 2-cm linear incision was made in the right uterine horn and the abdominal cavity was then closed using 4/0 vicryl sutures 15 minutes after oral placebo.

Group 3(n=7): A 2-cm linear incision was made in the right uterine horn and the abdominal cavity was then closed using 4/0 vicryl sutures 15 minutes after oral administration of clarithromycin at a dose of 10 mg/kg (Klacid oral suspension 125 mg/5 ml, Abbott, Italy).

Group 4 (n=7): A 2-cm linear incision was made in the right uterine horn and the abdominal cavity was closed using 4/0 vicryl sutures 15 minutes after orally administered tetracycline (Monodoks 100 mg kapsül, Deva Ilaç, Tekirdağ) at a dose of 10 mg/kg.

Experimental Design: Anesthesia in rats was induced by Ketamine (Ketalar, Eczacıbaşı Warner-Lambert, İstanbul, Turkey) 60 mg/kg and Xylazine (Rompun, Bayer, İstanbul, Turkey) 7 mg/kg administered intramuscularly in the left hind extremity. The rats were placed on the surgical table in the supine position. The surgical area was cleaned by 10% povidone and a midline abdominal incision was made. A 2 cm longitudinal midline incision was made in the right uterine horn in all rats at the anti-mesenteric side. Then the incision was closed using 4/0 vicryl and abdominal layers were closed using 3/0 continuous silk sutures. Fifteen days after the initial procedure, a transverse subcostal incision was made again in all rats under general anesthesia and the incisions in the abdominal cavity and uterine horns were examined.

Adhesion Assessment and the Adhesion Severity Score: Adhesions in the uterine horn, intra-abdominal organs, and abdominal wall were evaluated by independent surgeons outside the study team who used the method proposed by Linsky et al. (13) to score the extent and severity of the adhesions as follows:

Adhesion size:

No adhesions = 0 points,

Adhesions in 25% of the traumatized area = 1 point,

Adhesions in 25% to 50 of the traumatized area = 2 points

Adhesions in 50% to 100% of the traumatized area = 3 points.

Adhesion severity:

No resistance to surgical separation = 0 point

Moderate resistance to surgical separation= 0.5 points

Significant resistance requiring sharp dissection = 1 point

Histological examination: The traumatized uterine horn including adhesions was promptly removed, fixed in 10% formaldehyde. Paraffin blocks were prepared for histologic and histochemical assays. The 5 to 6 μm thick cross-sections obtained from the paraffin blocks were stained with Masson's trichrome staining. Histopathologic assessment of fibrosis was based on the semi-quantitative scoring system proposed by Hooker et al. (14). Accordingly, histopathologic adhesion was graded between 0 and 3 based on the presence and extent of fibrosis as follows: Grade 0, no fibrosis; Grade I, mild fibrosis; Grade II, moderate fibrosis; and Grade III, severe fibrosis.

Immunohistochemical examination: The 5–6 μm thick cross-sections prepared from paraffin blocks were placed on polylysine coated microscopic slides. Routine immunohistochemical staining was performed according to Kuloğlu et al.'s (15) method, and VEGF (vascular endothelial growth factor, E2611, Spring Bioscience, USA) and MDA (Rabbit polyclonal Anti-Malondialdehyde antibody, ab6463, Abcam, Cambridge, UK) immunoreactivity were studied with avidin-biotin-peroxidase methodology. Light microscopy and photography were done using an Olympus BX 50 light microscopy. The cytoplasmic immune staining was graded in a 5-point scale in the following manner: 0, no staining; 1, suspicious staining; 2, mild; 3, moderate; 4, strongly positive.

Inflammatory reaction was also graded similarly: 0, none; 1, trivial; 2, mild; 3, moderate; and 4, strong.

Statistical analyses: Statistical analyses were performed using SPSS 17.0 for Windows (SPSS Inc., Chicago, Il, USA). A Kruskal-Wallis analysis of variance was performed. For pairwise comparisons between groups for parameters with a p value of less than 0.05 Mann-Whitney U test was used. Again, for p values of less than 0.05 a MWU test was done with Bonferroni correction (0.05/6 = 0.008). A p value of less than 0.008 was considered significant.

RESULTS

Adhesion assessment: The experiment was accomplished successfully in all rats. Assessment of the adhesions revealed the following results:

Adhesion size: The adhesion size in G2 (Image 2) was significantly greater as compared to G1 (Image 1), G3 (Image 3) and G4 (Image 4) (p<0.008, MWU test). G1, G3 and G4 were similar in terms of the adhesion size (p>0.008, MWU test), (Table1).

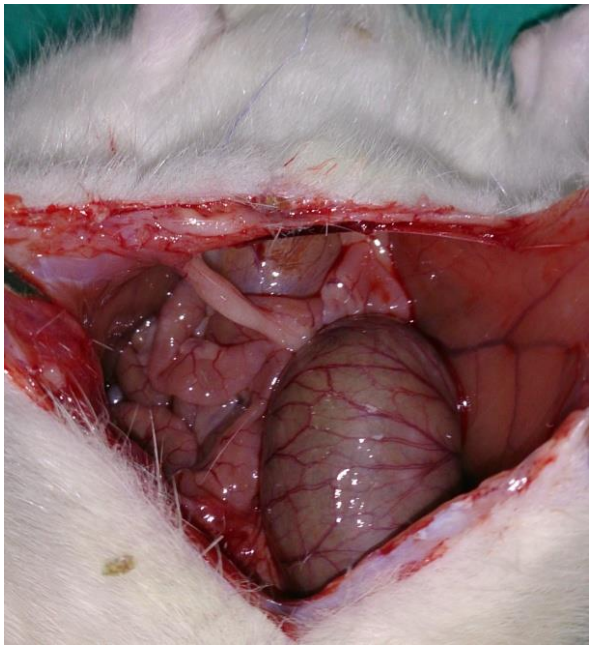

Image 1. *Adhesions in 25% of the traumatized area = 1 point,*

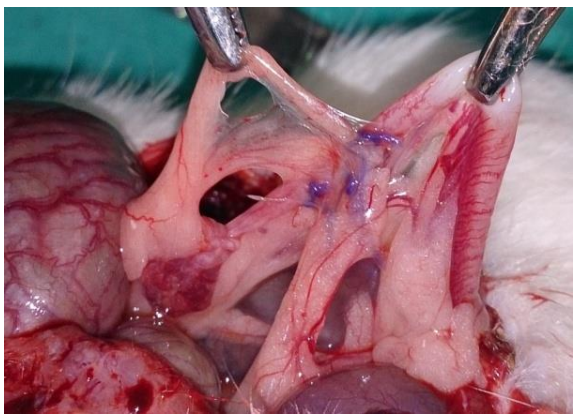

Image 2. *Adhesions in 50% to 100% of the traumatized area = 3 points.*

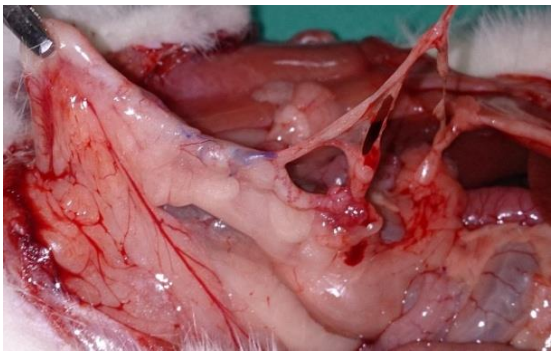

Image 3. *Adhesions in 25% of the traumatized area = 1 point,*

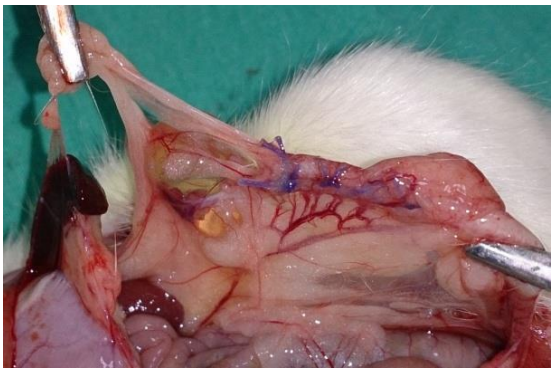

Image 4. *Adhesions in 25% to 50 of the traumatized area = 2 points*

Table 1. *Adhesion surface area and severity in study groups*

Groups	Adhesion surface area	Adhesion severity
G1	0.4±0.5¹	0.1±0.2¹
G2	2.4±0.5²	0.8±0.3²
G3	0.7±0.5¹	0.2±0.3¹
G4	0.8±0.7¹	0.2±0.3[1]

Since the 4 independent groups reflect an ordinal scale, a Kruskal-Wallis analysis of variance was performed. A MWU with Bonferroni-correction was performed when a p value of less than 0.05 was detected (0.05/6= 0.008). A P value of <0.008 was considered significant. Different values above the mean represent significant difference between means (p < 0.008). Note: The mean values have been listed in an ascending order.

Adhesion severity: Severity of adhesion in G2 was significantly higher than in the other three groups (p<0.008, MWU test). G1, G3 and G4 were similar with regard to the severity of adhesion (p>0.008, MWU test), (Table 1).

Histologic and immunohistologic results

Fibrosis: As compared to G1, G3 and G4, there was a significant increase in fibrosis in G2 (p<0.008, MWU test). G1, G3 and G4 were similar in this regard (p>0.008, MWU test), (Figure 1a, 1b, 1c, 1d), (Table 2).

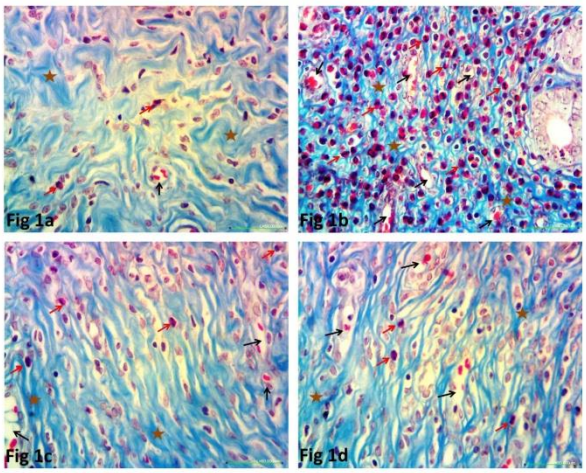

Figure 1. *Fibrosis (Brown star), angiogenesis (blackarrow) and inflammatorycells (redarrow)*

Table 2. *Histologic and immunohistochemical assessment in study groups*

Groups	Fibrosis	Angiogenesis	VEGF	MDA	İnflammatory cell score
G1	0.5±0.5¹	0.5±0.5¹	0.3±0.5¹	0.4±0.5¹	1.1±0.4¹
G2	3±0²	3±0²	2.6±0.5³	2.4±0.5²	3.7±0.5²
G3	1.4±0.1¹	1.4±0.5¹	1±0.4²	2.3±0.5²	1.3±0.5¹
G4	1.3±0.5¹	1.4±0.5¹	1.3±0.5²	2.3±0.5²	1.4±0.5¹

Since the 4 independent groups reflect an ordinal scale, a Kruskal-Wallis analysis of variance was performed. A MWU with Bonferroni-correction was performed when a p value of less than 0.05 was detected (0.05/6= 0.008). A P value of <0.008 was considered significant. Different values above the mean represent significant difference between means (p < 0.008). Note: The mean values have been listed in an ascending order.

MDA: A significantly higher MDA score was found in G2, G3 and G4 than in G1 (p<0.008, MWU test), while G2, G3, and G4 did not differ significantly (p>0.008, MWU test), (Figure 2a, 2b, 2c, 2d), (Table 2).

VEGF: There was a significant increase in G2, G3 and G4 in comparison with G1 (p<0.008, MWU test). When compared G2 with G3 and G4, VEGF value was significantly higher in G2 (p<0.008, MWU test). G3 and G4 were similar (p>0.008, MWU test), (Figure 3a, b, c, d), (Table 2).

Angiogenesis: A significantly higher level of angiogenetic activity was found in G2 than in G1, G3 and G4 (p<0.008, MWU test). G1, G3 and G4 were similar in terms of angiogenesis score (p>0.008, MWU test), (Figure 1a, 1b, 1c, 1d), (Table 2).

Inflammatory cells: A significantly higher number of inflammatory cells was found in G2 as compared to G1 (p<0.008, MWU test). As compared to G2, significantly lower level of inflammation was found in G3 and G4 (p<0.008, MWU test). G1, G3, and G4 were similar in this respect (p>0.008, MWU test), (Figure 1a, 1b, 1c, 1d), (Table 2).

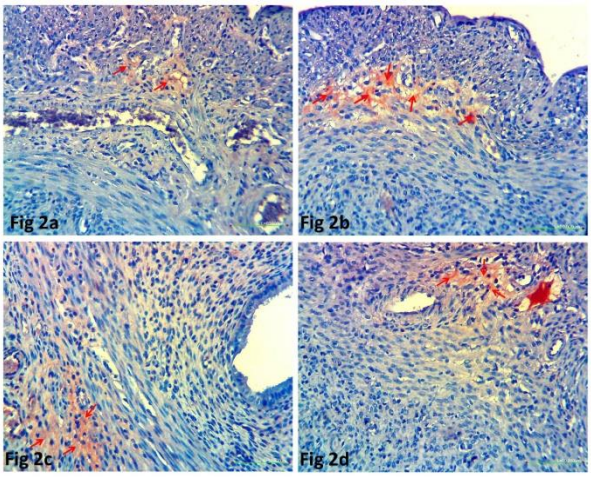

Figure 2. *Red arrow; MDA immünoreactive cells*

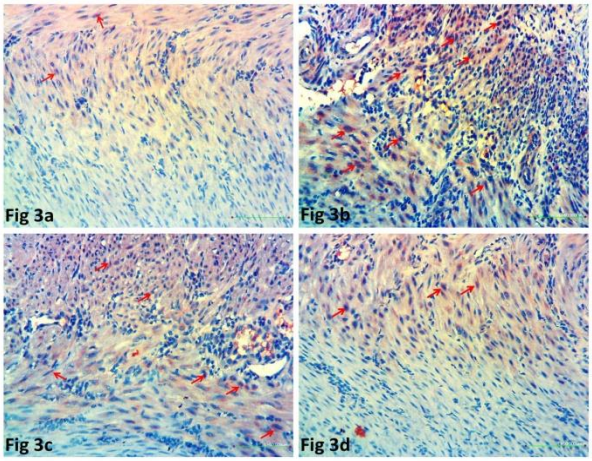

Figure 3. *Red arrow; VEGF immünoreactivecells*

DISCUSSION

Manual or surgical manipulation performed during laparotomy represents the most important cause of postoperative adhesions (16,17). In this model of rat uterine horn, both mechanical and instrumental manipulation was performed. In order to avoid the induction of chemical peritonitis in the peritoneum, tetracycline and clarithromycin were administered orally.

In fact, peritoneal adhesions are a result of the normal wound healing process. The key step in the induction of peritoneal adhesions is the injury inflicted upon the mesothelial cells on serosal surfaces. Following the exposure of subserosal connective tissue, serosanguineous exudate is produced, forming the soft fibrin gel matrix within 72 hours. Under normal conditions this fibrin gel matrix is degraded and removed through the effect of the fibrinolytic activity of the mesothelial cells. When fibronolytic activity is reduced or when the fibrinolytic activity is overwhelmed by the amount of fibrin gel formation, adhesions with significant angiogenetic activity develop within 15 days (18), consistent with our timing of re-laparotomy in our study.

Intraperitoneal adhesion formation is a complex process involving blood cell proliferation, matrix components, and angiogenesis. Theoretically, angiogenesis plays an important role in the development of intra-abdominal adhesions. Mesothelial and vascular endothelial cells giving rise to peritoneal blood vessels are known to secrete both VEGF and fibroblast growth factor-2 (FGF-2), which play a role in the formation of adhesions (19). VEGF is a powerful angiogenic cytokine that plays a role in the adhesion formation and is likely to induce new blood vessel formation in tissue injury/trauma occurring after surgery (20). Since adhesions are a result of tissue repair, angiogenesis is an integral element in adhesion formation (21), leading to the proposal that prevention of angiogenesis may halt adhesions (22,23). In our study, tetracycline and clarithromycin groups were defined on the basis of a theoretical inhibition of VEGF and angiogenesis by these two agents, both of which significantly reduced tissue angiogenesis, VEGF and fibrosis scores.

Tetracyclines inhibit metallo-proteas enzymes which play a role in a number of different processes including inflammatory diseases, wound healing, embryogenesis, tumor invasion, and angiogenesis. The non-antibiotic effects of tetracyclines include lipase production, secretion of chemotactic factors, leukocyte migration, protein kinase C inhibition, inhibition of granuloma formation, protease inhibition, anti-angiogenic activity and anti-metastatic activity (24). Lee et al. (25) showed in a murine model that doxycycline was able to provide in vivo suppression of VEGF-induced MMP (matrix metalloproteinase-9). Also, doxycycline was found to inhibit VEGF-related vascular permeability and VEGF secretion in breast cancer cells and in murine models (26,27). Again, these non-antibiotic characteristics of tetracyclines were our primary consideration in choosing these agents for the prevention of postoperative adhesions in our study. Our results show that there was a significant reduction in angiogenesis and VEGF scores in G2 than in G4. In contrast with our findings, in a study by Rappaport et al. (28), irrigation with cefazolin or tetracycline solutions resulted in increased adhesion formation in rat models. This apparent difference between the two studies may be explained by the use of intraperitoneal administration of diluted tetracycline and cephalosporins, which might have increased adhesions via peritoneal irritation (29).

Conversely, in our experiment oral administration of these agents rather than the intraperitoneal route could have helped prevent irritation, probably reflected by the significant lower number of inflammatory cells in G4 as opposed to G2. This finding may be related to the anti-inflammatory properties of tetracyclines contributing to anti-adhesive effects, in addition to their anti-bacterial effects.

Recently, an anti-inflammatory activity independent of an anti-bacterial effect has been reported for macrolide antibiotics (30). Similarly in experimental studies clarithromycin has been reported to reduce mucus secretion, suppress the production of pro-inflammatory agents such as IL-1, IL-6, IL-8 and TNF-alpha, and inhibit the synthesis of VEGF and VEGF mRNA (10-12). Furthermore clarithromycin has been shown to prevent the migration of neutrophils to the inflamed areas, and to increase apoptosis in activated lymphocytes in vitro (31,32). Similarly, a significant reduction in the number of inflammatory cells in G3 was found, as compared to a marked inflammation in G2. Absence of a significant difference between G1, G3 and G4 in this regard suggests that in addition to their anti-bacterial effects, the anti-inflammatory effects of these antibiotics may contribute to anti-adhesive properties, since sutures placed at the uterine horn in G2, G3 and G4 might also have caused a foreign body reaction.

Ellis (33) has suggested that tissue ischemia is a very critical factor for the formation of intraperitoneal adhesion. In this regard, plasma MDA concentration is dependent on the non-enzymatic oxidative lipid peroxide breakdown. MDA exerts its toxic effects through binding to the amino groups of the proteins, and to phospholipids and nucleic acids. Under conditions with higher levels of oxidative stress, a parallel increase occurs in plasma MDA levels (34). Similarly, MDA scores were similar in G2, G3, and G4 in our study, with no significant differences between G3 and G4, suggesting that both antibiotics fail to prevent ischemia-reperfusion injury in tissues.

In conclusion, tetracycline and clarithromycin are equally effective in the prevention of postoperative adhesion in this rat model.

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