The effect of Theranekron on femur fracture healing in an experimental rat model

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Objectives: The aim of this study was to investigate the radiological, biomechanical, histopathological and immunohistochemical effects of Theranekron on fracture healing in an experimental rat model.

Materials and methods: Forty-eight male albino Wistar rats were used. Four groups were formed, with 12 rats in each of Theranekron groups 1 and 2, and control groups 1 and 2. After a fracture was created in the right femur of the rats included in the study, fixation was performed with an intramedullary Kirschner wire. Theranekron was administered subcutaneously to Theranekron groups 1 and 2 at a dose of 0.3 mg/kg on days 0, 5 and 10. After radiographic analysis of the femurs of Theranekron group 1 and control group 1 rats at four weeks of the study was performed, both groups were divided into two equal subgroups (six femurs in each group). Histopathological and immunohistochemical examinations were performed in one subgroup and biomechanical examination in the other subgroup. At the end of six weeks, the rats in Theranekron group 2 and control group 2 were evaluated after applying the same procedure as in the fourth week.

Results: When the mean radiological scores of the Theranekron and control groups were compared, a statistically significant difference was found in favor of the Theranekron group at four and six weeks (p=0.028 and p=0.006, respectively). At four weeks, statistically significant higher biomechanical forces were obtained in the Theranekron group compared to the control group (p=0.030). In the histopathological evaluation, the inflammation value of the control group at four weeks was statistically significantly higher than the Theranekron group (p=0.027). The angiogenesis, osteoblast proliferation, and bone formation values of the Theranekron group were significantly higher than the control group (p=0.014, p=0.014, and p=0.005, respectively). At six weeks, the bone formation values of the Theranekron group were statistically significantly higher than the control group (p=0.021). The difference between the Theranekron group and the control group scores of the immunohistochemical evaluation were statistically significantly different at four and six weeks (p=0.006 and p=0.011, respectively).

Conclusion: Theranekron may play a role in accelerating fracture healing by reducing acute inflammation process in the early period of fracture union, increasing fracture strength, angiogenesis, osteoblast proliferation, and bone formation.

Keywords: Femur, fracture healing, rat, Theranekron.
in veterinary medicine in the treatment of various diseases of the nail, breast and reproductive system due to its many systemic effects such as antiphlogistic, demarcative, necrotizing, and epithelializing effects. In addition, it is used as an anti-inflammatory and anti-edematous agent in many infectious diseases, as well as in traumatic and necrotic diseases.\cite{6,7}

Many studies have been conducted with Theranekron in rat models. In these studies, it has been reported that the compound accelerates wound healing,\cite{8} reduces axonal and myelin damage after sciatic nerve damage, and has a neuroprotective effect.\cite{9} In addition, it has been reported that applications of Theranekron in bone defect models in rabbits may have important potential in promoting early bone healing.\cite{10,11}

To the best of our knowledge, there is no study examining the effect of Theranekron, which has been used in many studies in recent years, on a bone fracture model in the literature. In the present study, we, therefore, aimed to investigate the radiological, biomechanical, histopathological, and immunohistochemical effects of Theranekron on fracture healing in a rat model.

**MATERIALS AND METHODS**

In this experimental prospective study, a total of 48 male albino Wistar rats were used.\cite{12} The rats were not included in any experimental study previously, had an average weight of 250 g, and were fed with standard rat pellets and normal tap water *ad libitum* in an environment with an average temperature of 22 to 24°C, 40 to 60% humidity, and reverse lighting.

**Surgical procedure**

All procedures were performed under general anesthesia using intraperitoneal administration of xylazine 10 mg/kg (2% Rompun® Bayer) and ketamine (HCl) (10% Alfamine® Atafen) 75 mg/kg. Then, the right anterior thigh regions of the rats, which were assessed for deep anesthesia using the finger pinch test, were shaved and prepared with 10% povidone iodine (Betadix; Naturel, Istanbul, Türkiye). For prophylaxis against surgical site infection, cefazolin sodium (Eqizolin; Tüm Ekip İlaç A.Ş, Istanbul, Türkiye) was given intramuscularly at single dose of 15 mg/kg.

After covering the surgical area in a sterile manner, a 3-cm incision was made in the lateral thigh and the femoral shaft was exposed by approaching between the vastus lateralis and rectus femoris muscle planes. A standard transverse fracture model was created in the midline of the femoral shaft with a Gigli wire. The lateral knee joint was opened from the same incision line, a Kirschner wire (K-wire) with a diameter of 1.2 mm was retrogradely delivered to the intramedullary canal from the femoral intercondyalar...
region, and an intramedullary fracture was fixed (Figure 1). The stability of the fracture line was checked and care was taken to avoid distraction in the osteotomy line (Figure 2). After bleeding control and wound irrigation, the wound was closed with 3/0 absorbable sutures (DemeCRYL; DemeTech Corp, Miami Lakes, FL, USA). No movement restriction was applied to the rats after the surgery.

**Experimental groups**

Groups was designed as described Karaduman et al. study.[14] Four separate groups were formed, with 12 rats in each group.

**Control group 1:** No medication was administered after the femoral fracture was created. The rats were sacrificed by cervical dislocation at the end of the fourth week.

**Theranekron group 1:** After the femur fracture was created, Theranekron (Richter-Pharma AG, Wels, Austria) was administered subcutaneously at a dose of 0.3 mg/kg on Days 0, 5 and 10.[15,16] The rats were sacrificed by cervical dislocation at the end of the fourth week.

**Control group 2:** No medication was applied after the femoral fracture was created. The rats were sacrificed by cervical dislocation at the end of the sixth week.

**Theranekron group 2:** After the femur fracture was created, Theranekron was administered subcutaneously at a dose of 0.3 mg/kg on Days 0, 5, and 10.[15,16] The rats were sacrificed by cervical dislocation at the end of the sixth week.

At the end of the fourth week of the experiment, all rats in control group 1 and Theranekron group 1 were evaluated. After the rats were sacrificed by cervical dislocation, their right femurs were completely resected from the knee and hip joint. The femurs were cleaned off soft tissues without damaging the callus tissue at the fracture line. After radiological imaging, the K-wires in the intramedullary canal of the femur were carefully removed without damaging the callus tissue. After radiographic analysis of the removed femurs, the femurs from control group 1 and Theranekron group 1 were divided into two equal subgroups (six femurs in each subgroup). Then, histopathological and immunohistochemical examinations were performed in one group and biomechanical examination in the other group.

At the end of the sixth week, the rats in Theranekron group 2 and control group 2 were evaluated by applying the same procedure as in the fourth week. Thus, we evaluated the effects of Theranekron on different stages of fracture healing.

**Radiological analysis**

Lateral and anteroposterior (AP) radiographs of the resected right femurs were taken. Radiographs were scored separately based on callus bridging between the fracture line of the femur at the lateral and AP planes. Radiological analysis was performed in a blind manner by two orthopedists using a five-point radiographic scoring system. Accordingly,
the following evaluation was performed; 0 = no obvious bone bridge, 1 = bone bridge of one cortex, 2 = bone bridges of two cortices, 3 = bone bridges of three cortices, and 4 = bone bridges of four cortices.\[^{17}\] Scores were calculated by averaging the scores obtained by both observers.

**Biomechanical analysis**

The bones examined for this analysis were taken from four different groups. At four and six weeks after surgery, all femurs were harvested, with a total of 23 femurs (one femur from Theranekron group 1 was excluded due to erroneous measurement) obtained for biomechanical analysis. All femurs were stored in separate sealed plastic containers at -20°C before the three-point bending test. Each femur was placed with the three-point contact on a specially produced aluminum base setup according to the ASTM D790 standards (Figure 3).\[^{18,19}\] The base was fixed in the Shimadzu testing set with a gap of ~1.6 cm. The actuator moved downwards at a rate of 1 mm/s until failure occurred. The fracture load (N) was recorded.

**Histopathological examination**

Obtained femurs were fixed and decalcified in 10% buffered formalin solution. A transversal sample was taken from the fractured area of the femur and embedded in paraffin blocks after routine tissue follow-up. Tissue sections of 4 μm were taken from paraffin blocks using a rotary microtome. The tissue sections were stained with hematoxylin-eosin (HE) and immunohistochemical methods and, then, examined under a light microscope (80i; DS-RI2, Nikon, Tokyo, Japan). Fracture healing was examined in terms of inflammation, angiogenesis (vascularization), and fibrous, cartilage and bone tissue formation. The stained slides were evaluated by two specialists who were blinded to the study groups. The degree of morphological changes was evaluated by scoring as: negative (0); mild (1); moderate (2); or intense (3).\[^{20,21}\]

**Immunohistochemical examination**

Prepared tissue sections were stained with osteopontin (OPN) antibody according to the immunoperoxidase method. Tissue sections were prepared for staining, blocked with 3% H2O2, antigen retrieval solution (citrate buffer) and protein-blocking (non-immune serum), sequentially. Then, OPN primary antibody was dripped onto each tissue section and incubated overnight at +4°C and, then, incubated with biotinylated secondary antibody. Tissue sections were incubated in streptavidin-peroxidase for 20 min and, then, reacted with diaminobenzidine (DAB). After the DAB reaction, all tissues were stained with Mayer’s hematoxylin for background staining. Appropriate negative and positive controls were used to confirm the staining process. Slides used as negative controls were incubated in phosphate-buffered sodium (PBS) instead of primary antibodies. Tissue sections were evaluated, viewed, and photographed under a light microscope (80i; DS-RI2, Nikon, Tokyo, Japan). Immunohistochemical results were evaluated according to the intensity and extent of staining in the tissue as follows: negative (0); mild (1); moderate (2); or intense (3). Femoral localizations of staining were identified, and the changes in protein expression and regional differences were detected semi-quantitatively.\[^{22}\]

**Statistical analysis**

The sample size of this study was calculated using the G*Power version 3.1.9.7 software (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). Accordingly, the power of test was determined as 0.80, effect size as 0.7, and type-1 error (α) as 0.05, and (for this study, which consisted of eight subgroups in total), 48 samples in total, with a minimum of 6 rats in each subgroup. In this case, the actual power increased to 84%.

Statistical analysis was performed using the IBM SPSS version 25.0 software (IBM Corp., Armonk, NY, USA). Descriptive data were expressed in mean ± standard deviation (SD), median (min-max) or number and frequency. The conformity of the subscale scores to the normal distribution was assessed using the Shapiro-Wilk (n<50) test, and non-parametric tests were applied as the measurements were not normally distributed. The Mann-Whitney U test was used to compare the measurements according to the groups. A p value of <0.05 was considered statistically significant.

**RESULTS**

**Radiological findings**

In the radiological examination, the mean score of the control 1 group was 1.3, and the mean score in Theranekron 1 group was 2.0 at four weeks, indicating a statistically significant difference (p=0.028). In the radiological examination performed at six weeks, the mean score in the control 2 group was 2.7 and the mean score in Theranekron 2 group was 3.5; again, a statistically significant difference was found in the comparison between the groups at six weeks (p=0.006) (Table I, Figure 4).
Biomechanical findings

The results of three-point bending (biomechanical analysis) are given in Table II. At four weeks, statistically significantly higher forces were obtained in Theranekron group compared to the control group in terms of biomechanics \( p=0.030 \). At six weeks, higher results were obtained in Theranekron group than in the control group, although the difference was not statistically significant \( p=0.937 \).

|                         | Mean±SD | Median | Min-Max | \( p^* \) |
|-------------------------|---------|--------|---------|-----------|
| Theranekron group 1     | 2.0±1.1 | 1.8    | 0.0-3.5 |           |
| Control group 1         | 1.3±0.4 | 1.0    | 1.0-2.0 |           |
| Theranekron group 2     | 3.5±1.1 | 4.0    | 1.0-4.0 | 0.006     |
| Control group 2         | 2.7±1.0 | 3.0    | 0.0-3.5 |           |

SD: Standard deviation; * Significance levels according to Mann-Whitney U test results.

Histopathological findings

The inflammation in the control group 1 was statistically significantly higher than in Theranekron group 1 \( p=0.027 \). Angiogenesis, osteoblast proliferation, and bone formation levels in Theranekron 1 group were statistically higher than the control 1 group \( p=0.014, p=0.014, \) and \( p=0.005 \), respectively). Fibrosis and cartilage formation values were higher in Theranekron 1 group than in the control.
group 1, although this difference was not statistically significant (p=0.495 and p=0.147, respectively). The mean score in the histopathological evaluation for the control 2 group was higher than Theranekron 2 group only for fibrosis, showing a statistically significant difference (p<0.012). Bone formation was significantly higher in Theranekron 2 group than in the control 2 group (p=0.021). There was no statistically significant difference in the evaluation of other phases of bone healing in this period.

Morphological changes related to regeneration in the femoral fracture areas of the control and Theranekron group rats are summarized and scored in Table III and Figure 5 (A-F).

### Immunohistochemical findings

Osteopontin expression varied according to the tissue and cell type studied, as well as the type of ossification at four weeks. The OPN expression was weak in both groups, particularly in the fibrous tissue areas. While a mild immunoreaction was observed in the transchondral regions and newly proliferating osteoblasts of the control femurs (Figure 6a), this was more prominent in the Theranekron group (Figure 6b). While the staining was prominent in

### TABLE II

| Group                | Mean±SD  | Median | Min-Max       | p*     |
|----------------------|----------|--------|---------------|--------|
| Theranekron group 1  | 50.9±10.9| 55.1   | 29.8-58.6     | 0.030  |
| Control group 1      | 31.2±17.5| 37.7   | 2.2-46.9      |        |
| Theranekron group 2  | 54.2±30.0| 44.8   | 27.2-104.2    | 0.937  |
| Control group 2      | 42.3±7.6 | 44.8   | 29.2-49.5     |        |

SD: Standard deviation; * Significance levels according to Mann-Whitney U test results.

### TABLE III

|                         | Theranekron group 1 | Control group 1 | p*     |
|-------------------------|---------------------|-----------------|--------|
|                         | Mean±SD  | Median | Min-Max | Mean±SD  | Median | Min-Max |        |
| **4th week**            |          |        |         |          |        |         |        |
| Inflammation            | 1.2±0.4  | 1.0    | 1.0-2.0 | 1.8±0.4  | 2.0    | 1.0-2.0 | 0.027  |
| Angiogenesis            | 2.3±0.5  | 2.0    | 2.0-3.0 | 1.3±0.5  | 1.0    | 1.0-2.0 | 0.014  |
| Fibrosis                | 2.3±0.8  | 2.5    | 1.0-3.0 | 2.0±0.9  | 2.0    | 1.0-3.0 | 0.495  |
| Osteoblast proliferation| 2.3±0.5  | 2.0    | 2.0-3.0 | 1.3±0.5  | 1.0    | 1.0-2.0 | 0.014  |
| Cartilage formation     | 2.5±0.8  | 3.0    | 1.0-3.0 | 1.8±0.8  | 2.0    | 1.0-3.0 | 0.147  |
| Bone formation          | 2.5±0.6  | 2.5    | 2.0-3.0 | 1.2±0.4  | 1.0    | 1.0-2.0 | 0.005  |
| Severity of immunoreactivity | 2.3±0.5  | 2.0    | 2.0-3.0 | 1.2±0.4  | 1.0    | 1.0-2.0 | 0.006  |
| **6th week**            |          |        |         |          |        |         |        |
| Inflammation            | 0.0±0.0  | 0.0    | 0.0-0.0 | 0.3±0.5  | 0.0    | 0.0-1.0 | 0.138  |
| Angiogenesis            | 0.8±1.0  | 0.5    | 0.0-2.0 | 1.5±0.6  | 1.5    | 1.0-2.0 | 0.201  |
| Fibrosis                | 1.0±0.9  | 1.0    | 0.0-2.0 | 2.5±0.6  | 2.5    | 2.0-3.0 | 0.012  |
| Osteoblast proliferation| 1.7±1.4  | 2.0    | 0.0-3.0 | 1.7±0.8  | 1.5    | 1.0-3.0 | 0.868  |
| Cartilage formation     | 1.8±1.5  | 2.5    | 0.0-3.0 | 2.2±0.8  | 2.0    | 1.0-3.0 | 0.932  |
| Bone formation          | 3.0±0.0  | 3.0    | 2.0-3.0 | 2.0±0.9  | 2.0    | 1.0-3.0 | 0.021  |
| Severity of immunoreactivity | 2.5±0.6  | 2.5    | 2.0-3.0 | 1.3±0.5  | 1.0    | 1.0-2.0 | 0.011  |

SD: Standard deviation; * Significance levels according to Mann-Whitney U test results.
FIGURE 5. (a) Control, 4th week. In the fracture area, prominent purulent (Pr) exudate is observed in the areas close to the center of the femur, and angiogenesis (Ag) and fibrosis (F) are observed just at the periphery of this inflammatory exudate. H.E. Bar; 200 µm. (b) Control, 4th week. Angiogenesis (A), fibrosis (F), proliferation of diffuse chondroblastic (CHB) cells, cartilage (chondrocyte) and osteoid tissue formation are observed in the areas close to the center of the femur in the fracture area. H.E. Bar; 500 µm. (c) Theranekron, 4th week. Angiogenesis (A), mild infiltration of inflammatory cells and fibrosis (F) are observed in the areas close to the center of the femur in the fracture area, while proliferation of diffuse chondroblastic (CHB) cells (cartilage tissue formation) is observed in the periphery. H.E. Bar; 500 µm. (d) Theranekron 4th week. Angiogenesis (Ag), fibrosis (F) and prominent proliferation of osteoblastic cells (OSB) are observed in the periphery of newly formed osteoid (OSD) tissue at the fracture site. H.E. Bar; 100 µm. (e) Control, 6th week. While fibrosis (F), angiogenesis (Ag) and mildly purulent (Pr) exudate are observed in the fracture area near the center of the femur; significant proliferation of osteoblastic cells, formation of bone trabeculae (TB) with a small amount of bone marrow, and proliferation of chondroblastic (CHB) cells (cartilage tissue formation) are observed in more peripheral areas. Note that the regeneration is not yet completed. H.E. Bar; 500 µm. (f) Theranekron, 6th week. Bone trabeculae, including bone marrow, are observed at the fracture site. Note that the regeneration is completed. H.E. Bar; 500 µm.
The effect of Theranekron on fracture healing in rats

The cytoplasm of the cells, it was weak in the nuclei.
It was determined that the OPN expression at six
weeks was significantly higher in the Theranekron
group, similar to the fourth week, compared to
control group (Figure 6c, d). The differences between
the scores of the Theranekron and control group
were statistically significant at four and six weeks
(p=0.006 and p=0.011).

The scoring of the immunohistochemical findings
detected in the transchondral regions and proliferating
osteoblasts in the femoral fracture regions of the
control and Theranekron groups is shown in Table III.

DISCUSSION

The results of this comprehensive preliminary
study, in which the effects of Theranekron on
fracture healing were investigated by creating a
femur fracture model in rats, demonstrated that the
Theranekron-treated group showed more effective
fracture healing radiologically, biomechanically,
histopathologically and immunohistochemically
compared to the control group.

From radiological examination, we observed that
fracture healing was statistically significant better
at four and six weeks in the Theranekron groups.
We believe that local hematoma, one of the fracture
healing stages, stimulates angiogenesis through
its stimulating effect on mesenchymal tissue and
cytokines, accelerating the osteogenesis stage.

In our biomechanical study, which was conducted
using the most commonly used three-point bending
test[24,25] to measure the strength of bone callus tissue,
it was observed that the fracture strength of the
Theranekron group was statistically significantly
higher at four weeks than in the control group.
On the other hand, although the fracture strength
was higher in Theranekron 2 group at six weeks,
the difference between the two groups was not
statistically significant.

On histopathological examination, there were
significant differences between the groups in terms
of the intensity of morphological changes such as
inflammation, angiogenesis, fibrosis, osteoblast
proliferation, cartilage and bone tissue formation.
In particular, it was noted that inflammation was significantly lower in the Theranekron group compared to the control group. This suggests that the inflammatory process was completed earlier in the Theranekron group, contributing to faster bone healing. Thus, it was observed that other findings related to bone healing such as angiogenesis, fibrosis, osteoblast proliferation, and cartilage and bone formation were more prominent in the Theranekron group.

Another important finding of our study is that the OPN expression in the Theranekron group was more pronounced compared to the control group. Osteopontin is an extracellular matrix protein named after the bone bridge.\(^{[20]}\) It is produced in bones and regulates bone mineralization and is believed to play a complementary role in cellular responses to mechanical stimulation.\(^{[27,28]}\) In an experimental study on distraction osteogenesis, OPN expression was detected at low levels in fibroblast-like cells and at very high levels in areas of transchondral ossification. Specifically, proliferating pre-osteoblasts have been reported to express OPN.\(^{[29]}\) It has been reported that various stages of the regeneration process in bone tissue, such as neovascularization, callus formation and remodeling, can be significantly disrupted in the absence of OPN.\(^{[30]}\) While immunohistochemistry of OPN expression in the transchondral regions and proliferating osteoblasts was observed to be weaker in the control group rats, it was observed to be more pronounced in the Theranekron group in our study. Due to the fact that the healing process was completed earlier in the fracture areas and OPN expression was found to be significantly higher in the Theranekron group rats compared to the control group, we believe that Theranekron accelerates the regeneration process by stimulating proliferation and OPN expression in osteoblasts during the healing process of broken bones.

On examination of previous studies in the literature about Theranekron, the effects of Theranekron on bone fracture models have not been investigated; however, the beneficial effects of the compound have been discussed in studies on different tissues. Sardari et al.\(^{[6]}\) reported that Theranekron significantly stimulated epithelialization in full-thickness wounds during the first 14 days of healing in cows. Oryan et al.\(^{[31]}\) reported that Theranekron decreased inflammation, edema and necrosis, and increased tissue maturation, thereby accelerating tendon healing according to biomechanical, histopathological, and radiological examination in a rabbit study on tendon healing. Dolapcioglu et al.\(^{[15]}\) also reported in a prospective, randomized experimental study on rats that Theranekron significantly accelerated recovery in endometriosis. Bigham-Sadegh et al.\(^{[11]}\) presented in their study on the rabbit radius bone defect model that Theranekron reduced inflammation and fibrosis, and accelerated the bone repair process by increasing angiogenesis and collagen tissue profiling. In another study on the wound healing effect of Theranekron, the wound epithelialization time was shorter and the mean wound contraction percentage was significantly higher.\(^{[8]}\) In a study on the nerve healing effect of Theranekron, the authors reported that the nerve repair process was accelerated. The findings of this study reported that Theranekron decreased axonal and myelin damage after sciatic nerve injury and increased the neuroprotective effect.\(^{[9]}\) In a recent study, Sencar et al.\(^{[7]}\) reported that the combination therapy of Theranekron and alphalipoic acid supported structural recovery and could be considered as an effective treatment protocol following peripheral nerve injury. In another recent study, Theranekron, as an apoptosis-inducing agent, was possibly shown to be an excellent alternative therapeutic drug for the treatment of malignant neoplasms.\(^{[32]}\)

Nevertheless, this study has some limitations. The effects of different doses of Theranekron on fracture healing were unable to be examined. In our study, primarily the effects of Theranekron on fracture were investigated. Further studies should be conducted to investigate varying doses and efficacy of Theranekron. Another limitation of our study is that we did not use micro-computed tomography for radiological evaluation. Instead of this analysis, biomechanical tests were performed and the study was supported by histopathological and immunohistochemical evaluation.

In conclusion, our study results show that Theranekron reduces the acute inflammatory process and increases fracture strength, and has effects on angiogenesis, osteoblast proliferation and bone formation in rats, particularly in the early stages of fracture union. We believe that this result should be supported by further studies to be carried out on the management of a difficult process such as fracture healing, and may have potential in treating fractures with union problems.

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**Ethics Committee Approval:** The study was carried out at Van Yüzüncü Yıl University Experimental Medicine Application and Research Center. Necessary permissions were obtained.
The effect of Theranekron on fracture healing in rats

from Van Yüzüncü Yıl University Experimental Animals Ethics Committee before the study (No: 2021/01-09).

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Researched literature and conceived the study, performed data analysis, and wrote the paper: N.G.; Searched the literature and contributed to the writing of the article: S.Ö.; Was involved in animal experimental work and gained ethical approval: T.T.; Performed the biomechanical analysis: S.K.; Were performed the histological investigation: Ö.F.K., Z.Y.; Was involved in experimental work and contributed to the design of the study: A.K.; All authors reviewed and edited the manuscript and approved the final version of the manuscript.

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