ÖZGÜN ARAŞTIRMA ORIGINAL RESEARCH

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EVALUATION OF THE PROTECTIVE EFFECT OF RAMELTEON IN METHOTREXATE INDUCED NEPHROTOXICITY

METOTREKSAT ARACILI NEFROTOKSİSİTEDE RAMELTEONUN KORUYUCU ETKİSİNİN DEĞERLENDİRİLMESİ

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Öz

Amaç

İmmünsüpresif ve antikanser olarak kullanılan Metotreksat (MTX), böbrek dahil birçok organda ciddi toksik yan etkilere neden olmaktadır. Mtx aracılı nefrotoksisitenin mekanizmasında oksidatif stres üzerinden apoptotik yolakların aktive olması yer almaktadır. Çalışmamızda antioksidan ve antiapoptotik özellikleri iyi bilinen melatonin'in analoglarından Ramelteon'un (RML) MTX nefrotoksisitesindeki koruyucu etkilerini araştırdık.

Gereç ve Yöntem

32 adet sıçan Kontrol, MTX, MTX+RML ve RML olmak üzere 4 gruba ayrıldı. Gruplara göre 7 gün boyunca oral gavajla salin (SF) ya da RML (10 mg/kg) uygulandı, 2. gün ise gruplara göre intraperitoneal 20 mg MTX ya da aynı hacimde salin uygulandı. Deney sonunda ratlar sakrifiye edilerek böbrek dokuları Hematoksilen-Eozin (HE) boyama ile histopatolojik olarak, caspase-3 ve TNF-α boyama ile immünohistokimyasal (İHC) olarak incelendi. Ayrıca serum BUN, kreatinin düzeyleri ölçüldü ve böbrek Total oksidan ve antioksidan durum (TAS, TOS) düzeyleri çalışılarak Oksidatif stres indeksi (OSİ) hesaplandı.

Bulgular

MTX grubunda kreatinin, TOS ve OSİ düzeyleri Kontrol grubuna göre anlamlı düzeyde yüksek saptandı. HE boyamada MTX grubunda Kontrol grubuna göre anlamlı düzeyde yüksek doku hasarı, İHC boyamada cas-3 ve TNF-α boyanma düzeylerinde artış saptandı. Bu bulguların MTX+RML grubunda geriye çevrildiği saptandı.

Sonuç

RML tedavisinin, MTX'in yol açtığı nefrotoksisiteye ilişkin bulguları iyileştirdiğini gösterdik. RML, MTX nefrotoksisitesinde umut vadeden bir ilaç olabilir.

Anahtar Kelimeler: Apoptoz, İlaca bağlı nefrotoksisite, Melatonin analogları, Oksidatif stres

Abstract

Objective

Methotrexate (MTX), which is used as an immunosuppressive and anticancer drug, causes serious toxic side effects in many organs, including the kidney. Activation of apoptotic pathways through oxidative stress is involved in the mechanism of MTX mediated nephrotoxicity. In our study, we investigated

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the protective effects of ramelteon (RML), an analogue of melatonin, whose antioxidant and antiapoptotic properties are well known, on MTX nephrotoxicity.

Material and Method
32 rats were divided into 4 groups as Control, MTX, MTX+RML and RML. According to the groups, saline or RML (10 mg/kg) was administered by oral gavage for 7 days, and on the 2nd day, 20 mg of MTX or the same volume of saline was administered intraperitoneally according to the groups. At the end of the experiment, the rats were sacrificed and kidney tissues were examined histopathologically with Hematoxylin-Eosin (HE) staining and immunohistochemically (IHC) with caspase-3 and TNF-α staining. In addition, serum BUN, creatinine levels were measured, kidney Total Oxidant and Antioxidant Status (TAS, TOS) levels were studied and Oxidative Stress Index (OSI) was calculated.

Results
Creatinine, TOS and OSI levels in the MTX group were found to be significantly higher than in the control group. In HE staining, tissue damage was significantly higher in MTX group compared to the control group, and cas-3 and TNF-α staining levels were increased in IHC staining. These findings were found to be reversed in the MTX+RML group.

Conclusion
We show that RML treatment improves the findings of MTX-induced nephrotoxicity. RML may be a promising drug in MTX nephrotoxicity.

Keywords: Melatonin analogs, drug induced nephrotoxicity, oxidative stress, apoptosis

Introduction

Methotrexate (MTX) is a folic acid antimetabolite used as an antineoplastic in some types of cancer and immunosuppressive in autoimmune diseases such as rheumatoid arthritis and psoriasis (1). MTX acts by inhibiting the enzyme dihydrofolate reductase, which catalyzes the conversion of dihydrofolate (DHF) to tetrahydrofolate (THF) in pyrimidine synthesis. In addition to its antineoplastic, immunosuppressive, antiproliferative, and anti-inflammatory effects, MTX has serious side effects such as hepatotoxicity, nephrotoxicity, hematotoxicity, and neurotoxicity (2).

Nephrotoxicity is a serious side effect in patients using MTX (3). This effect is particularly evident in the use of high doses of MTX, but may occur in low doses, especially in patients with impaired renal function due to the use of multiple drugs (such as non-steroidal anti-inflammatory drugs) (4). Increased blood levels of MTX due to decreased renal excretion have been implicated in nephrotoxicity, which can also be observed at low doses (5). In addition, there are many studies in the literature showing that the use of MTX causes oxidative stress and causes cell damage in many tissues, including the kidney (6-8).

Melatonin (MLT) is a hormone secreted from the pineal gland that regulates the sleep/wake cycle (9). It is well known that MLT, which is synthesized in many organisms from plants to humans, has strong antioxidant properties (10). Ramelteon (RML) is a synthetic MLT analog of MLT that specifically stimulates the MT1 and MT2 receptors (11). In this study, we aimed to investigate the protective effect of RML in MTX nephrotoxicity through histopathological analysis, oxidative stress, and apoptotic pathways.

Table 1 Experimental Procedures

Groups	Applied Procedures	
Control	0.1 ml SF by oral gavage for 7 days	2nd day i.p. SF
MTX	0.1 ml SF by oral gavage for 7 days	2nd day i.p. 20 mg/kg MTX*
MTX+RML	0.1 ml 10 mg/kg RML# by oral gavage for 7 days	2nd day i.p. 20 mg/kg MTX
RML	0.1 ml 10 mg/kg RML by oral gavage for 7 days	2nd day i.p. SF

*MTX: Methotrexate 50 mg/ml flacon, Koçak, Türkiye. #RML: (Ramelda 8 mg tablet, Abdi İbrahim, Türkiye)

Material and Method

In our study, 32 Wistar Albino male rats weighing between 200-300 grams were used. Rats were randomly selected and divided into 4 groups, with 8 animals in each group. The drugs administered to the groups are given in Table 1.

All rats were anesthetized with 80-100 mg/kg Ketamine (Alfamine, Alfasan IBV, Holland) and 8-10 mg/kg Xylazine (Alfazin, Alfasan IBV, Holland) 24 hours after the last drug administration. Venous blood was taken from the vena cava inferior of the rats who underwent abdominal incision following anesthesia. Then, the kidney tissues of the rats were taken and half of them were homogenized for biochemical analysis. Total antioxidant status (TAS) and total oxidant status (TOS) values were measured from homogenized tissues, oxidative stress index (OSI) value was calculated and data were evaluated. The measurement of TAS and TOS levels and the calculation of OSI levels were performed using the methods developed by Erel et al. (12-14). The other halves of the tissues were separated by placing them in 10% formaldehyde for histopathological and immunohistochemical analyses. Hematoxylin Eosin (HE) staining was performed on the tissues and histopathological examinations were performed, as well as tumor necrosis factor-α (TNF-α) and caspase-3 (cas-3) stainings were performed immunohistochemically. Structural changes in the kidney tissue sections of the control and experimental groups were made according to the scoring of Refaiy et al. (15). A semi-quantitative evaluation method was used to determine the cas-3 and TNF-α density in kidney tissue sections stained with the immunohistochemical method (16). The blood taken into biochemistry tubes was centrifuged (3000 rpm, 10 min) and the serum specimens were separated. Blood Urea Nitrogen (BUN) and creatinine values from obtained sera were assayed on Beckman Coulter AU5800 biochemistry autoanalyzer (Beckman Coulter, Brea, CA, USA).

One-way analysis of variance (ANOVA) and post hoc Tukey tests were used to compare the results of kidney tissue TAS, TOS, OSI, and serum BUN and creatinine between groups. The significance level was accepted as $p<0.05$. Kruskal Wallis test and Mann Whitney U tests with Bonferroni correction were used to compare histopathological scores. The significance level was accepted as $p<0.0083$.

Results

Biochemical Analyzes

TOS and OSI values in kidney tissue were found to be significantly higher in the MTX group than in the other groups ($p<0.05$). On the other hand, TAS levels were found to be statistically significantly higher in the group given only RML compared to other groups ($p<0.05$, Table 2).

Serum creatinine levels were found to be significantly higher in the MTX group than in the Control group ($p<0.05$, Table 3).

Histopathological Results in Kidney Tissue Samples

In the histochemical evaluations, the kidney tissues of the control group were observed in normal histological appearance (Figure 1). In the MTX group, significant histopathological changes were observed including marked hydropic degeneration in proximal and distal tubule epithelial cells, mononuclear cell infiltrations in the perivascular and intertubular areas, vascular and glomerular congestion, tubular dilatation and degeneration of some glomeruli compared to the Control group. It was observed that glomerular degeneration, medullary congestion, and mononuclear cell infiltrations were significantly reduced in the MTX+RML group compared to the

Table 2 Oxidative stress parameters in kidney tissue

	Control (n=8) Mean ± S.D.	MTX (n=8) Mean ± S.D.	MTX+RML (n=8) Mean ± S.D.	RML (n=8) Mean ± S.D.
TAS (mmol Trolox Eq./L)	1.32 ± 0.21	1.20 ± 0.15	1.37 ± 0.53	1.64 ± 0.13$^{**,\#\#,¥}$
TOS (µmol H2O2/L)	12.09 ± 3.98	24.87 ± 8.67$^{**,¥¥,\&}$	12.43 ± 5.55	13.41 ± 4.23
OSI (Arbitrary Unit)	0.92 ± 0.25	2.09 ± 0.70$^{**,¥¥,\&\&}$	0.92 ± 0.42	0.82 ± 0.26

'*' represents statistical significance according to the control group, '#' according to the MTX group, '¥' according to the MTX+RML group, and '&' according to the RML group (single symbol for $p<0.05$, a double symbol for $p<0.001$).

Table 3 Serum BUN and creatinine results

	Control (n=8) Mean ± S.D.	MTX (n=8) Mean ± S.D.	MTX+RML (n=8) Mean ± S.D.	RML (n=8) Mean ± S.D.
Creatinine (mg/dl)	0.28 ± 0.07	0.44 ± 0.08*	0.34 ± 0.10	0.40 ± 0.12
BUN (mg/dl)	22.25 ± 1.49	24.75 ± 4.77	23.63 ± 5.10	22.88 ± 2.36

'*' represents statistical significance compared to the control group ($p<0.05$).

Table 4 Scoring results of structural changes observed in kidney tissue in control and experimental groups

	Control (n=8) Median(25-75%)	MTX (n=8) Median(25-75%)	MTX+RML (n=8) Median(25-75%)	RML (n=8) Median(25-75%)
Glomerular degeneration	1.0 (1.0-1.8)	4.0 (3.3-4.0)*	3.0 (3.0-3.0)*, #	1.0 (1.0-1.8)#,¥
Tubular dilatation	1.0 (1.0-1.8)	4.0 (4.0-4.0)*	3.0 (3.0-3.0)*	2.0 (1.0-2.0)#,¥
Mononuclear cell infiltrates	1.0 (1.0-1.0)	4.0 (4.0-4.0)*	2.0 (2.0-2.0) *,#	1.0 (1.00-1.8)#
Cortical congestion	1.0 (1.0-1.0)	4.0 (3.0-4.0) *	3.0 (2.3-3.0) *	1.0 (1.0-1.8) #,¥
Medullary congestion	1.0 (1.0-1.8)	4.0 (4.0-4.0) *	3.0 (2.3-3.0) *,#	1.0 (1.0-1.0) #,¥

'*' indicates statistical significance according to the control group, '#' according to the MTX group, and '¥' according to the MTX+RML group ($p<0.0083$).

Table 5 Cas-3 immunostaining grades in kidney (Mean)

	Control	MTX	MTX +RML	RML
Degree of Staining	-/+	+++	+	-/+

Table 6 TNF-α immunostaining grades in kidney (Mean)

	Control	MTX	MTX +RML	RML
Degree of Staining	-/+	+++	+	-/+

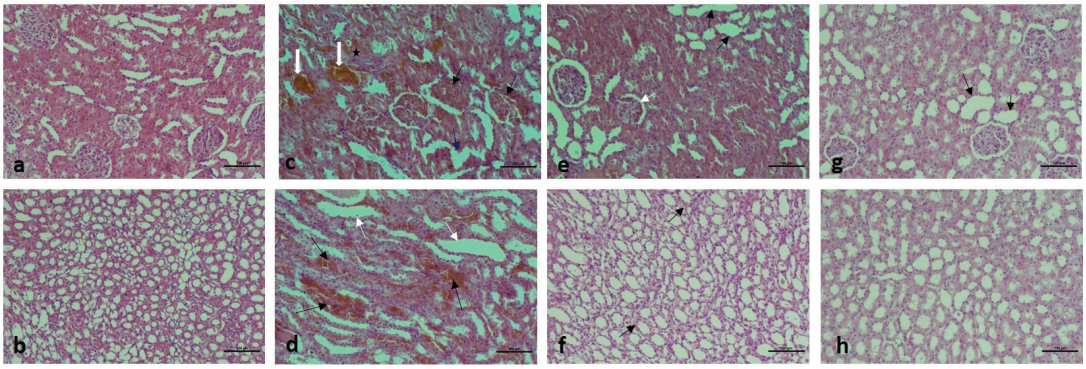

Figure 1:
Renal cortex (a), medulla (b) region of the control group are observed in normal histological structure (H-E, ax10, bx10). The rat kidney cortex region of the MTX group (c) Glomerular degeneration (black thin arrow), vascular congestion (white thick arrow), tubular dilatation (blue thin arrow), and inflammatory cell infiltrations (black star) are clearly observed (H-E, x10). In the rat kidney medulla region (d) of the MTX group, vascular congestion (black thin arrow), tubular dilatation (white thin arrow) are clearly observed (H-E, x10). Rat kidney cortex region of the MTX +RML group (e) Glomerular degeneration (white thin arrow), tubular dilatation (black thin arrow) are observed at low levels (H-E, x10). A low level of vascular congestion (black thin arrow) is observed in the rat kidney medulla region (f) of the MTX +RML group (H-E, x10). The rat kidney cortex (g), medulla (h) region of the RML group is observed similar to the control group (H-E, x10).

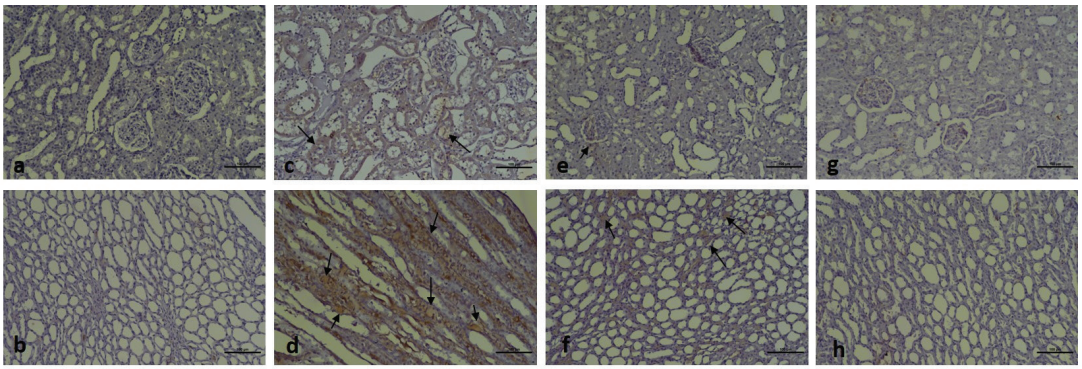

Figure 2:
No staining is observed in the kidney cortex (a), medulla (b) region of the rats of the control group (cas-3 immunostaining, x10). Intense staining is observed in the tubules of the rat kidney cortex (c), medulla (d) region of the MTX group (cas-3 immunostaining, x10). A low level of staining is observed in the kidney cortex (e), medulla (f) region of the rat in the MTX+RML group (cas-3 immunostaining, x10). A very low level of staining is observed in the kidney cortex (g), medulla (h) region of the rat in the RML group (cas-3 immunostaining, x10). Stained areas are seen in orange to brown.

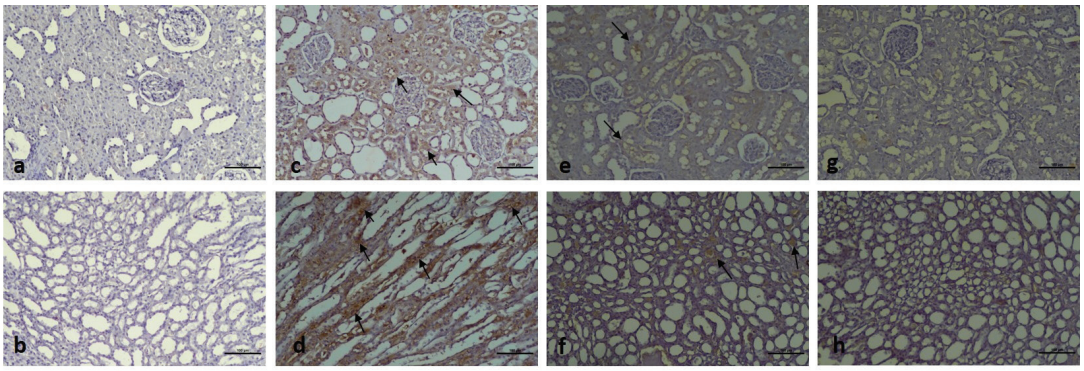

Figure 3:
No staining is observed in the kidney cortex (a), medulla (b) region of the rats of the control group (TNF-α immunostaining, x10). Intense staining is observed in the tubules of the rat kidney cortex (c), medulla (d) region of the MTX group (TNF-α immunostaining, x10). A low level of staining is observed in the kidney cortex (e), medulla (f) region of the rat in the MTX+RML group (TNF-α immunostaining, x10). A very low level of staining is observed in the kidney cortex (g), medulla (h) region of the rat in the RML group (TNF-α immunostaining, x10). Stained areas are seen in orange to brown.

MTX group (Table 4). A normal histological structure was observed in the RML group, similar to the control group (Figure 1).

Immunohistochemical Results in Kidney Tissue Samples

Cas-3 Immune Staining in Kidney Tissue Samples

It was observed that cas-3 was stained at a very low level or absent in the kidney tissues of the rats in the control group. In the MTX group, very intense staining was observed in the cortex and medulla regions of the tubules. In the MTX+RML group, cas-3 staining was observed at a low level. On the other hand, in the RML group, cas-3 staining was observed to be very low or absent, similar to the control group. (Figure 2, Table 5).

TNF-α Immune Staining in Kidney Tissue Samples

TNF-α staining in the kidney tissues of the rats in the control group was either very low or absent. Intense staining was observed in the cortex and medulla regions of the kidney tissues of the rats in the MTX group. In the kidney tissue samples of rats in the MTX+RML group, TNF-α staining was observed at a low level. On the other hand, in the RML group, cas-3 staining was observed to be very low or absent, similar to the control group, in the kidney tissues (Figure 3, Table 6).

Discussion

In our study, we investigated the possible protective effects of RML, which is used in the treatment of sleep disorders and is known to have antioxidant properties, on kidney toxicity caused by MTX use. We showed that RML reduces the increased oxidative stress due to MTX, decreases the increase in serum creatinine levels, and also improves the pathological findings developing in the kidney tissue. In addition to these results, we found that RML also reduced cas-3 and TNF-α staining intensities, which are associated with renal damage. It is well known that oxidative stress causes the activation of transcription factors through various signaling pathways, changes gene expression, and leads the cell to apoptosis (17). Many studies have shown that oxidative stress is also involved in the mechanism of MTX-mediated nephrotoxicity. Heidari et al. showed that MTX administration at doses of 20-30 mg/kg for 6 days decreased TAS and glutathione levels while increasing lipid peroxidation and reactive oxygen species (6). Similarly, Devrim et al. (2005) showed that malondialdehyde levels, which is one of the markers of lipid peroxidation in kidney tissue, increased after i.v. MTX administration for 7 weeks (7).

In the development of MTX-mediated nephrotoxicity, mechanisms such as the decrease in NADPH level as a result of the effects of MTX on nucleotide metabolism and consequently the decrease in reduced glutathione level, the increase in NADPH oxidase enzyme activity, the activation of apoptotic pathways as a result of inflammation and oxidative stress-mediated mitochondrial damage have been held responsible (8). In addition, it has been reported that MTX and its main metabolite, 7-OH-MTX, cause tubular obstruction by causing crystal deposition in the renal tubules, which leads to impaired excretion of MTX (18).

Cas-3 is an enzyme that is a member of the serine-aspartate proteases (CASPASE) family that catalyzes the degradation of many key cellular proteins during apoptosis. Cas-3 can be induced via both the intrinsic pathway of the mitochondrial damage-mediated apoptotic pathway and the TNF-mediated extrinsic pathway (19). Therefore, cas-3 is used as an important marker of apoptosis. In parallel with our findings, Mahmoud et al. showed that cas-3 expression increased in rats receiving MTX, and this increase was reversed by ferulic acid (20). Similarly, Osman et al. examined the renal NF-κB/Keap1/HSP-70/cas-3 axis in MTX-induced nephrotoxicity and found that cas-3 expression increased in the group receiving MTX (8).

TNF-α is a critical cytokine involved in both inflammation and apoptosis (21). Campbell et al. showed that renal obstruction stimulates apoptosis by increasing the level of TNF-α in the kidney (22). Türk et al., on the other hand, showed that MTX increased TNF-α levels in the kidney, again in line with our findings (23).

It is well known that MLT has antioxidant and antiapoptotic properties as well as anti-inflammatory and immunomodulatory effects (24). In recent years, many experimental studies have been conducted to show that it can be used as a preventative against the toxic effects of different xenobiotics. (25-27). Although there are a limited number of studies in the literature evaluating the effects of MLT on MTX nephrotoxicity, we could not find a study that specifically evaluated the effects of RML on MTX nephrotoxicity, which has a stimulating power of MLT receptors that is approximately 10 times greater than MLT (28). Jahovic et al. found that reduced glutathione levels in the liver and kidneys were decreased, MDA levels and myeloperoxidase activities were increased in MTX toxicity, and showed that these effects were reversed by MLT (29). Similarly, Abraham et al.

showed that pre-treatment with MLT is protective in MTX nephrotoxicity by measuring the activities of various antioxidant enzymes and histopathological examination of the kidney. (30). Oğuz et al. demonstrated that MLT administered together with lycopene in MTX toxicity reversed renal damage by histopathological examinations, and also by analyzing inflammatory markers in kidney tissue (TNF-α, IL-1β, Nitric oxide, ceruloplasmin) (31).

Conclusion

Through histopathological findings and biochemical measurements, we showed that RML reduces MTX nephrotoxicity, and we explained this improvement by RML's reducing effect on oxidative stress and suppressing of apoptotic pathways. In conclusion, RML may be a promising drug that can prevent the development of nephrotoxicity in patients using MTX.

Conflict of Interest Statement
The authors have no conflicts of interest to declare.

Ethical Approval
All experiments are conducted in accordance with the Turkish Ministry of Health, the Declaration of Helsinki, the Recommendations for Animal Care and Experiments of the Council of European Communities Directive (86/609/EEC), and the Care and Use of Laboratory Animals Guidelines accepted and published by the United States National Institutes of Health (NIH). Süleyman Demirel University Experimental Animal Center and Animal Experiments Ethics Committee approved all experimental procedures in this study. (Date: 11/09/2020, Issue: 06/11).

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Availability of Data and Materials
Data available on request from the authors.

Authors Contributions
HIB: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing-original draft.

DB: Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Writing-review & editing.

HKD: Conceptualization; Investigation; Validation.

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