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Does ramelteon have an ameliorative effect in MTX-induced testicular injury?

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ABSTRACT

Aims: The aim of this study was to investigate the potential protective effect of Ramelteon (RMT), which exhibits antioxidant, anti-inflammatory and antiapoptotic properties, against testicular damage induced by Methotrexate (MTX), which is widely used in the treatment of various diseases, including chemotherapy.

Methods: 32 Wistar albino rats were equally divided into four groups: Control (group I), MTX (group II), MTX+RMT (group III) and RMT (group IV). Histologic evaluation was performed using Hematoxylin and Eosin (H&E) staining, immunohistochemical analysis using TNF-alpha and Cas-3, and biochemical evaluation using TAS, TOS and OSI.

Results: Histologic analysis using H&E staining revealed a significant difference between group I and groups II and III ($p<0.05$), while no significant difference was observed between group IV and the other groups ($p>0.05$). While normal histologic structures were observed in groups I and IV, histopathologic findings were noted in groups II and III. Immunohistochemical evaluation of TNF-alpha and Cas-3 showed a significant difference between group I and groups II and III ($p<0.05$), while no significant difference was observed between group IV and other groups ($p>0.05$). The highest immunostaining intensity was observed in group II. Biochemical evaluation revealed statistically significant differences in TAS, TOS and OSI parameters reflecting oxidative stress differences between the groups ($p<0.05$).

Conclusion: Anti-inflammatory, antioxidant and antiapoptotic properties of RMT demonstrated its protective effect against MTX-induced testicular injury in rats. Histological, immunohistochemical and biochemical analyses underline the potential role of RMT as a protective agent in MTX-induced testicular injury. This research aims to contribute to the understanding of potential applications to reduce the adverse effects of MTX therapy on reproductive health.

Keywords: Methotrexate, ramelteon, rat, testicular injury

INTRODUCTION

Methotrexate (MTX, 4-amino-10-methylfolic acid), an antimetabolite and cytotoxic agent, has been widely used in the clinical treatment of malignant and non-malignant diseases since the 1950s.[1-5]

MTX, a folate antagonist, antimetabolite, and dihydrofolate reductase inhibitor possess antitumoral, antiproliferative, anti-inflammatory, antimicrobial, immunosuppressive and immunomodulatory effects.[3,6-8]

MTX exerts its antiproliferative effect by inducing cytotoxicity during active phases of cell proliferation. Therefore, it has been reported to have toxic effects not only on cancer cells but also on highly proliferative cells such as bone marrow, gastrointestinal mucosa, and spermatogenic cells.[8]

Studies have demonstrated its toxic effects on systems such as gastrointestinal, hematologic, and central nervous systems, as well as its impact on infertility due to negative effects on oogenesis and spermatogenesis.[2] Furthermore, it has been reported to cause permanent azoospermia and infertility by inducing testicular toxicity.[1,6,9]

Oxidative stress, inflammation, and apoptosis are known to play roles in the tissue toxic effects of MTX.[3] Excessive production of reactive oxygen species (ROS) can weaken enzymatic and non-enzymatic antioxidant defence systems that protect cells from various damage mechanisms.[3,4]

Previous studies have reported that MTX increases ROS production in testicular tissue, leading to toxic effects.[2] The testicular toxicity caused by MTX has been associated with structural damage (disorganization and vacuolization) in the seminiferous tubules of the testes, rapid increase in abnormal sperm morphology, reduction in sperm

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count and density, and DNA damage in sperm.[1,2,6,8,10] In addition to these, it has been reported that MTX leads to a decrease in testicular weight and hormone levels such as FSH, LH, and testosterone.[8] Numerous studies have also demonstrated apoptotic cell death as a result of testicular damage associated with MTX.[5]

Oxidative stress is a significant pathogenic factor in MTX-induced testicular damage. Excessive production of reactive oxygen species (ROS) can lead to oxidative damage, alteration of membrane structure and function, and lipid peroxidation. It is well known that testes are highly sensitive to MTX toxicity, and studies on testicular toxicity models have shown that free radical scavengers, antioxidants, anti-inflammatory cytokines, and other drugs can mitigate testicular injury. Induction of oxidative stress and inflammatory factors are recognized as important mechanisms of MTX-related testicular toxicity.[7]

Considering all these mechanisms, there is a need for new agents with antioxidant properties to mitigate oxidative stress-induced organ damage and oxidative stress-induced inflammation caused by cancer cells and chemotherapeutic agents.[2,8,11]

RMT (RMT, (S)-N-[2-(1,6,7,8-tetrahydro-2H-indeno-[5,4-b]furan-8-yl)ethyl] propionamide, $C_{16}H_{21}NO_2$) is an orally administered hypnotic agent approved by the U.S. Food and Drug Administration (FDA) in 2005 for the treatment of chronic insomnia.[3,12,13] As a high-affinity melatonin receptor agonist, RMT is a potent and highly selective agonist for melatonin MT1/MT2 receptors believed to mediate the circadian rhythm in mammals. It also has antioxidant and anti-inflammatory effects.[3,14,15]

The aim of this study is to determine whether RMT, which has antioxidant and anti-inflammatory effects against testicular toxicity caused by acute MTX, has a protective effect through histochemical, immunohistochemical, and biochemical analyses.

METHODS

Animals

The experimental design was approved by the Süleyman Demirel University Animal Experiments Local Ethics Committee (Date: 11.09.2020, Decision No: 06/12). The study adhered to the animal research guidelines of the National Institute of Health.

Experimental Groups

A total of thirty-two male Wistar albino rats weighing 200-300 grams were included in the study. All rats were housed in standard conditions (temperature: 22-23°C, humidity: 60 ± 5%, 12-hour light/dark cycle) with access to standard laboratory chow and tap water ad libitum. The rats were randomly divided into four groups (n=8 each):

- **Control (Group I):** Received 0.1 ml saline orally for 7 days and a single intraperitoneally (IP) dose of saline on day 2.
- **MTX (Group II):** Received a single IP dose of MTX (20 mg/kg) on the second day (MTX 50 mg/ml, flk, Kocak, Turkey), and 0.1 ml saline was given orally for 7 days.[16]
- **MTX+RMT (Group III):** Received a single IP dose of MTX (20 mg/kg) on the second day and 0.1 ml RMT (10 mg/kg) orally for 7 days.[17]
- **RMT (Group IV):** Received a single IP dose of saline (0.1 ml) on the second day and 0.1 ml RMT (10 mg/kg) orally for 7 days.

Twenty-four hours after the final drug administration, rats were euthanized via intraperitoneal injection of a ketamine (90 mg/kg, Alfamine, Alfasan IBV) and xylazine (10 mg/kg, Alfazin, Alfasan IBV) combination. The testis was removed, with half stored at -20°C for subsequent analysis and the other half fixed in 10% neutral buffered formalin for histopathological and immunohistochemical evaluations of Cas-3 and TNF-alpha protein expression and biochemically, TAS, TOS and OSI values have been assessed.

Histological Analysis

Histopathological analysis: Testis tissues were fixed in a 10% buffered formaldehyde solution, dehydrated, cleared, and embedded in paraffin. Sections of 4-5 µm thickness were cut by microtome (Leica SM2000R, Germany), stained with hematoxylin and eosin (H&E), and the prepared slides were examined and imagined with a camera-equipped light microscobe with scale X20, X40 (DM500, Leica, Germany) microscopically. Seminiferous tubules, Leydig cells, and germ cells were evaluated and scored based on modified semi-quantitative criteria.

Immunohistochemical analysis: Immunohistochemical detection of Cas-3 and TNF-alpha was performed. Sections were deparaffinized, dehydrated, treated with hydrogen peroxide, blocked with Ultra-V Block, incubated with primary and secondary antibodies, followed by streptavidin peroxidase. DAB staining and hematoxylin counterstaining were carried out. Immunoreactivity was scored using a modified semi-quantitative scale.

Biochemical Analysis

Oxidative stress parameter measurement: Testis tissue samples were homogenized, TAS and TOS were measured spectrophotometrically. The OSI was calculated as TOS/TAS.

TAS analysis involved the reaction of Fe^{2+}-o-dianisidine with hydrogen peroxide to form OH radicals, with subsequent reaction with o-dianisidine producing dianisidyl radicals. Antioxidants suppress oxidation,

preventing colour formation. TOS analysis involved oxidation of the ferrous ion-o'dianisidine complex to ferric ion, forming a coloured complex with xylenol orange in an acidic medium. Colour intensity correlated with oxidant levels and was measured spectrophotometrically.

Statistical Analysis

Statistical evaluations of histological studies were performed using SPSS 29.0 and for semi-quantitative assessment, the Kruskal-Wallis test was applied. The non-parametric Mann-Whitney U test was used for pairwise group comparisons. For biochemical analyses, Levene's homogeneity test was conducted. Since the data did not exhibit homogeneity according to Levene's test, the non-parametric Kruskal-Wallis test was continued, followed by pairwise comparisons using the Mann-Whitney U test to determine the source of the difference between groups. Variables were presented as mean±standard deviation (SD), and p-values below 0.05 were considered statistically significant ($p<0.05$).

RESULTS

Histological results

Histological results of testis tissue sections from the control group and experimental groups were evaluated based on the grading system established by Refaiy et al.[18] In the H&E-stained testis tissue sections, significant differences were observed between the control (group I) and the experimental groups (group II - group III) ($p<0.05$), while no significant difference was found between the RMT (group IV) and the control group ($p>0.05$). When comparing the experimental groups (group II - group III - group IV) among themselves, the highest histopathological findings were observed in group II. In group III, these findings significantly decreased, while in group IV, these findings were not present. The observed histopathological findings included the following changes in seminiferous tubules: large-nucleus, vacuolization, enlargement, and degeneration of germ cells; granulomatous cells; dilatations; seminiferous tubules with reduced sperm content in their lumens; increased interstitial connective tissue between seminiferous tubules; and degeneration in Leydig cells (Figure 1, Table 1).

Table 1. Evaluation of histopathological findings between all groups

Histopathological Findings	Control Group I	MTX Group II	MTX+RMT Group III	RMT Group IV
Degeneration in Leydig cells	-	++/+++	+	-
Interstitial connective tissue	-/+	++	++/+++	-/+
Degeneration and dilatation of seminiferous tubules	-	+++	+++	-/+
Large-nucleus in germ cells	-	++	++	-
Enlargement in germ cells	-	+++	+/++	-
Degeneration in germ cells	-	+++	++/+++	-/+
Vacuolization in germ cells	-	+++	+++	-/+
Amount of sperm in the lumen	-	++	+/++	-

(-) (negative score): No structural changes, (+) (1 positive score): Light structural changes, (++) (2 positive score): Middle structural changes, (+++) (3 positive score): Serious structural changes. -:($p>0.05$), -/+:($p>0.05$), +:($p<0.05$), +/++:($p<0.05$), ++:($p<0.05$), ++/+++:($p<0.05$), +++:($p<0.05$).

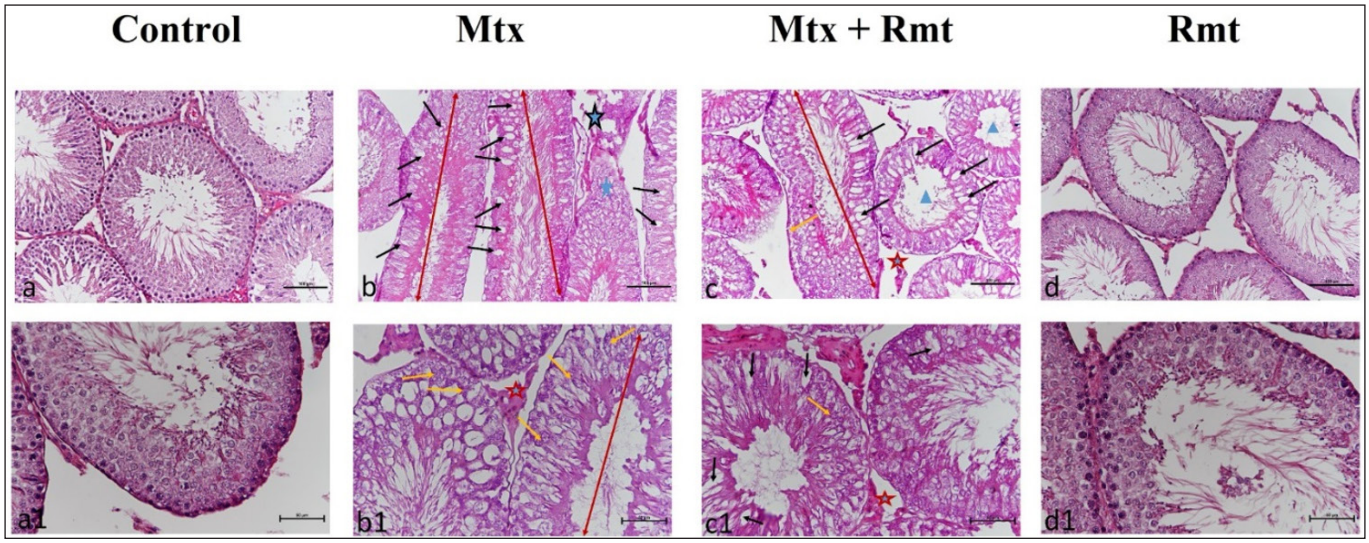

Figure 1: Histopathological findings in testis tissues belonging to control and experimental groups: a - a1, control group; normal histological structure of seminiferous tubules, Leydig cell, and interstitial area. b - b1, MTX group; lots of histopathological findings; degeneration and dilatation of seminiferous tubules (red arrows), vacuolization in germ cells (black arrows), increase of interstitial area (black star), granulomatous cells in seminiferous tubules (blue star), degeneration of Leydig cells (red star), degeneration of germ cells (yellow arrows), a few of sperm in the lumen (blue triangles) c - c1, MTX+RMT group; mild histopathological findings, d - d1, RMT group; normal histological structure. (a, b, c, d, H- E, x20 - a1, b1, c1, d1, H-E, x40).

Immunohistochemical Results

Immunohistochemical staining of testis tissue sections showed significant differences between the control group (group I) and the experimental groups (group II - group III) ($p<0.05$), while no significant difference was found between the RMT group (group IV) and the control group ($p>0.05$). When comparing the experimental groups (group II - group III - group IV) among themselves, the highest immunostaining was observed in group II. In group III, immunostaining decreased significantly compared to group II, while the lowest immunostaining was observed in group IV, which was equally comparable to the control group (Figure 2, Table 2). No significant differences were observed in the degrees of staining among TNF-alpha and Cas-3 antibodies.

Table 2. Degrees Staining of TNF-Alpha, Cas-3

Staining Levels			
Control Group I	MTX Group II	MTX+RMT Group III	RMT Group IV
+/-	+++	++	+/-

Biochemical Results

Oxidative stress parameters in the testicular tissue were compared among all groups using the Kruskall-Wallis test, and statistically significant differences were observed among groups in terms of TAS, TOS, and OSI parameters ($p=0.006$, $p=0.019$, $p=0.005$, respectively) (Figure 3). To understand from which group the difference originated, pairwise comparisons were conducted using the Mann-Whitney U test (Figure 4). When comparing the Control and MTX groups, there were significant differences in all three parameters (TAS, TOS, and OSI) ($p=0.001$, $p=0.007$, $p=0.002$, respectively). These data, in accordance with the materials and methods, reveal that the systemic toxicity induced by MTX leads to a depletion of TAS and an elevation of TOS levels in testicular tissue. There were no significant differences in all three parameters between the Control and RMT groups ($p=0.105$, $p=0.574$, $p=0.798$, respectively). This indicates that the investigated RMT preparation alone did not have a significant impact on the oxidative and antioxidative systems, either positively or negatively. Similarly, no statistically significant difference was found between the Control and MTX + RMT groups ($p=0.382$, $p=0.505$, $p=0.083$, respectively). These data reflect the positive effects of RMT for all three parameters. When administered alone, MTX-induced increases in TOS and decreases in TAS levels were brought back to control levels upon co-administration with RMT. Statistically significant differences were observed in all three parameters between the MTX and MTX + RMT groups ($p=0.010$, $p=0.003$, $p=0.005$, respectively). In the MTX-treated group, the increased oxidative stress and depleted TAS levels displayed an opposing trend in the group that received RMT alone, similar to the control group. When comparing the MTX and MTX + RMT groups, a significant difference was only observed in TAS ($p=0.021$). Although there was a decrease in TOS in the MTX + RMT group in terms of other parameters, this difference was not statistically significant ($p=0.065$). There were no significant differences in all three parameters between the RMT and MTX + RMT groups. This suggests that RMT exhibits a protective effect, resulting in a restorative effect that brings the MTX + RMT group closer to control levels.

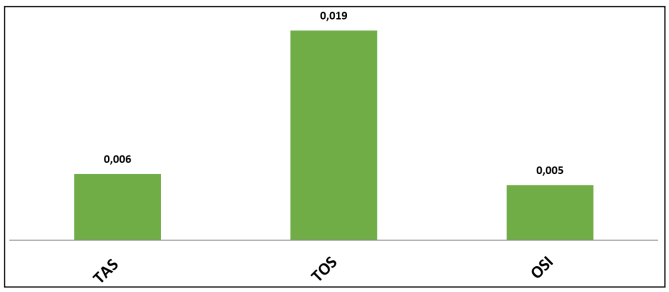

Figure 3. Levels of difference between groups in TAS, TOS, and OSI parameters as determined by Kruskal-Wallis Test

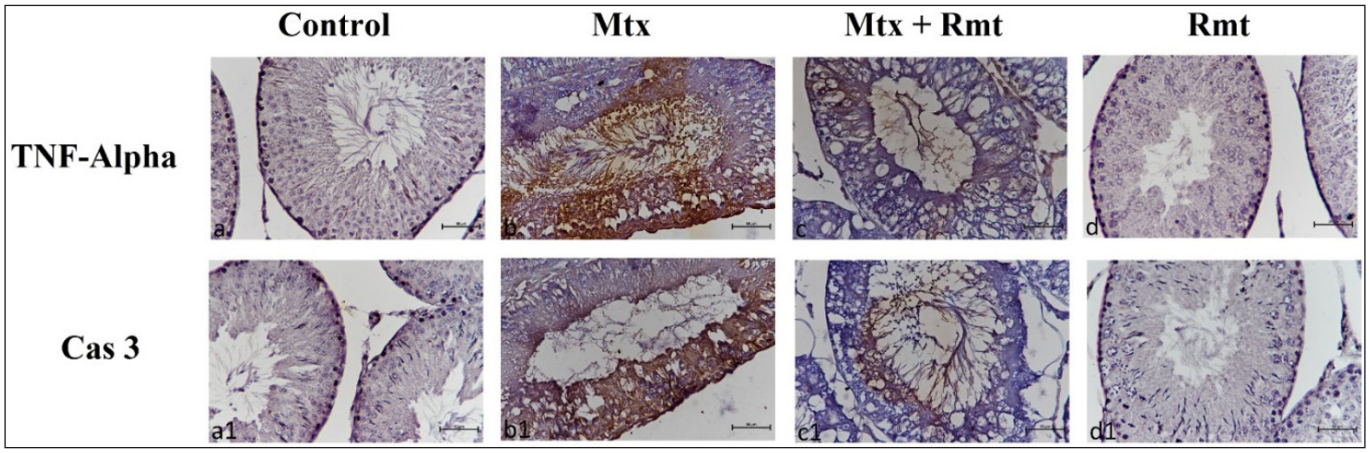

Figure 2. TNF-alpha, and Cas-3 immune stainings in testis tissues belonging to control and experimental groups. a- a1, control group; no positive staining, b - b1, MTX group; positive staining, c - c1, MTX+RMT group; mild positive stainings, d - d1, RMT group; no positive staining, immun staining, x40

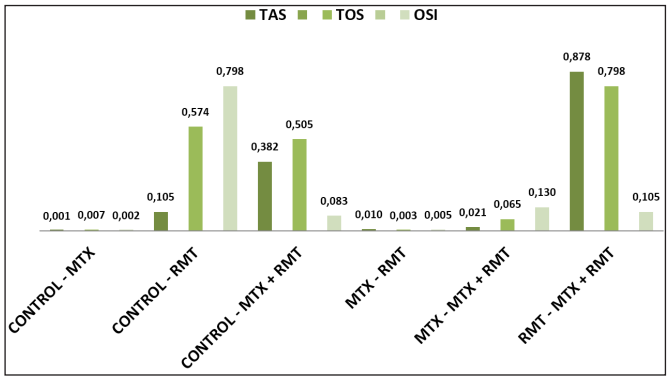

Figure 4. Levels of difference between pairwise groups in TAS, TOS, and OSI parameters as determined by Mann-Whitney U Test

DISCUSSION

MTX, extensively used in tumour chemotherapy,[7] structurally resembling folic acid and being able to inhibit folate-dependent enzymes, suppresses the synthesis of tetrahydrofolate required for the formation of purines and pyrimidines, crucial for cell replication, by binding to dihydrofolate reductase. Suppression of purine and pyrimidine synthesis results in DNA damage and subsequently leads to apoptosis.[4]

As a folate antagonist, MTX effectively suppresses cellular growth. Hence, it finds applications in various clinical conditions including autoimmune diseases like psoriasis and rheumatoid arthritis, non-neoplastic and neoplastic diseases like graft-versus-host disease, multiple sclerosis, keratoacanthomas, psoriatic arthritis, systemic lupus erythematosus, acute lymphoblastic leukaemia, non-Hodgkin lymphoma, breast cancer, head and neck malignancies and dermatomyositis, as well as ectopic pregnancy.[1-4,10,11,19,20]

Studies have shown that chemotherapy drugs can lead to side effects in various organs, including the reproductive system, causing conditions such as azoospermia, testicular damage, sex hormone dysfunction, and infertility in both human and animal models due to their antiproliferative properties.[19]

Previous studies on MTX have demonstrated negative effects on the male reproductive system, causing irregularities and vacuolization in the seminiferous epithelium, reduced sperm counts, sperm DNA damage, decreased testicular weight, and diminished seminal vesicle and prostate gland sizes.[19] Our study also exhibited notable histopathological changes, especially in the group treated with MTX. Various histopathological findings were observed, including large-nucleus germ cells with vacuolated appearance, enlargement, and degeneration; granulomatous cells; dilations; seminiferous tubules with low sperm content in their lumens; increased interstitial connective tissue; and Leydig cell degeneration. Conversely, these findings were alleviated in the MTX + RMT group, suggesting the positive impact of RMT on the damage caused by MTX.

Oxidative stress and inflammation play a significant role in tissue damage due to various causes, including drug toxicities. An imbalance between the production of high levels of reactive free radicals and the antioxidant defence systems results in oxidative stress. Consequently, the body experiences elevated oxidant radicals and reduced antioxidant molecules. To assess the overall oxidant and antioxidant status, markers such as TOS, TAS, and OSI have been developed.[4]

MTX's adverse effects have been associated with increased reactive oxygen species (ROS) in the testes. Furthermore, MTX induces testicular germ cell apoptosis, leading to increased apoptotic cell counts and oxidative stress due to enhanced apoptotic indices, and mRNA expression of caspase-3, caspase-8, and caspase-9.[19]

In our study, all groups were compared, and statistically significant differences were found among groups in terms of TAS, TOS, and OSI parameters. These findings indicate that systemic toxicity induced by MTX increases TOS levels in testicular tissue. When MTX was administered alone, the increased TOS and decreased TAS levels were restored to control levels in the MTX+RMT group. This outcome highlights the positive effects of RMT.

CONCLUSION

This study demonstrates the potential protective effect of RMT on the damage induced by MTX, owing to RMT's anti-inflammatory, antioxidant, and antiapoptotic properties. As a high-affinity melatonin receptor agonist, RMT is used in insomnia treatment and has potential applications in anticancer therapy. The study could serve as a reference for future research, investigating the protective effects of RMT on chemotherapeutic agent-induced damage.

ETHICAL DECLARATIONS

Ethics Committee Approval: The experimental design was approved by the Süleyman Demirel University Animal Experiments Local Ethics Committee (Date: 11.09.2020, Decision No: 06/12). The study adhered to the animal research guidelines of the National Institute of Health.

Informed consent: This project is an animal experiment, so informed consent has not required.

Referee Evaluation Process: Externally peer reviewed.

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

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Author Contributions: All the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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