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Original article

Protective effect of baicalin on methotrexate-induced mitochondrial damage in testicular tissues of rats

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ABSTRACT

Methotrexate (MTX) is commonly used as a chemotherapeutic drug against cancer but its efficacy is limited due to its side effects. Baicalin is a flavonoid that has potent antioxidant properties. This study aimed to evaluate baicalin's protective effect on MTX-induced mitochondrial damage in testicular tissues of rats. Twenty-four male Wistar rats were distributed into four groups: control, MTX, MTX + baicalin, and baicalin alone. The activities of enzymes such as catalase, superoxide dismutase, glutathione reductase and glutathione peroxidase was significantly lowered in the MTX treated group, compared to the other groups. Concomitantly, MTX group also showed elevated the levels of reactive oxygen species and thiobarbituric acid reactive substances in the testicular mitochondria. Furthermore, MTX group also showed reduced the activities of tricarboxylic acid (TCA) cycle enzymes, i.e., succinate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase, and alpha-ketoglutarate dehydrogenase. The actions of electron transport chain (ETC) complexes (I-IV), as well as mitochondrial membrane potential (MMP), were also reduced the MTX group. However, baicalin treatment reversed the damaging effect of MTX in the mitochondria of testicular tissues. The baicalin treatment potentially reduced the damaging impact of MTX in isolated mitochondria from testicular tissues. Thus, the current study revealed that the baicalin has conspicuous potential to attenuate the MTX induced mitochondrial damage in rat's testicular tissues.

1. Introduction

Methotrexate (MTX), a known folic-acid antagonist, is one of the most commonly used drugs frequently recommended to treat several types of cancers and rheumatic diseases (Jalili et al., 2020). It has been extensively used to treat inflammatory, autoimmune, and dermatological diseases (Montasser et al., 2017). It is well known that chemotherapeutic agents are not specific to cancer cells, but they also affect the healthy cells in the body (Ali et al., 2017). The major limitation of using MTX is its extreme nontargeted toxicity (Elbarbary et al., 2016). Reportedly MTX caused nephrotoxicity, suppression of bone marrow, hepatotoxicity, gastrointestinal mucosal impairment, vascular endothelial damage, hematological disturbance, pneumonia, and germ cell toxicity (Mahmoud et al., 2018). MTX exposure induces testicular toxicity by causing oxidative damage along with lipid peroxidation and disrupting membrane integrity (Wang et al., 2018; Belhan et al., 2019).

Mitochondria is the important organelle in the cells. It is also the powerhouse because it uses fatty acids, amino acids, and glucose as fuel for ATP production by oxidative-phosphorylation (Wang et al., 2010). Mitochondria also perform a vital role in various processes, including reactive oxygen species production, regulation of lipid metabolism, autophagy, and regulation of calcium homeostasis. Therefore, mitochondrial dysfunction can lead to various diseases (Friedman and Nunnari, 2014). Oxidative stress induced by MTX damages the mitochondria by prompting ROS production more than the physiological level (Paul et al., 2015). MTX induces excessive ROS production by impeding the mitochondrial

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electron transport chain complexes. It disturbs the mitochondrial membrane potential (MMP) and promotes the swelling of the mitochondrial matrix (Al Maruf et al., 2018).

Natural plant-based flavonoids are becoming more prevalent because of their exceptional pharmacological properties (Ishfaq et al., 2019). Flavonoids show hepatoprotective, anti-inflammatory, anti-allergic, anticancer, antimicrobial, and antioxidant properties (Tian et al., 2019). Among these flavonoids, baicalin is a well-known flavonoid extracted from *Scutellaria radix* roots (Hsu et al., 2016). Scutellaria, is commonly known as Baikal skullcap or Chinese skullcap, is a species of flowering plant in the family Lamiaceae. It is used in Traditional Chinese medicine (Sowndhararajan et al., 2018). It has various pharmacological properties against ailments such as inflammation, infection, oxidant, and immune dysfunction (Hsu et al., 2016). As MTX is one of the commonly used anti cancerous drug, but it causes several side effects, Therefore, baicalin was used as a pharmacological candidate to counter the side effects of MTX in the current study.

2. Materials and methods

2.1. Animals

In this experimental trial, sexually mature male Sprague Dawley rats (n = 24) were purchased from the breeding facility and housed in the animal house of the University. The rats were housed in cages made of steel at standard temperature (24 ± 2 ℃) and light conditions (12 h light and 12 h dark). They were fed, ad libitum with rodent chow and had free access to tap water. Animals were treated in compliance with the international guidelines for the use and care of laboratory animals.

2.2. Experimental trial

Before starting treatment, the animals were randomly distributed into four groups, with six rats each—Group I (control group) was treated with normal saline intraperitoneal (i.p.). Group II was treated with 20 mg kg^{-1} MTX and saline (i.p.) on day 1 of the treatment. Group III was treated with 20 mg kg^{-1} MTX (on day 1) + 75 mg kg^{-1} baicalin (daily). Group IV was treated with 75 mg kg^{-1} baicalin daily. The treatments of baicalin were orally administrated (through oral gavage) for seven days. Animals present in each group were given numbers on dorsal part of their bodies by using eosin solution and the identity was confirmed every time. Each group was administrated with separate sterilized and clean utensils. In this experiment, Pyrex glassware were used. After finishing the treatment, rats were sacrificed by cervical dislocation under mild anesthesia, isolated the testis, and ice-cold saline (0.9 % NaCl) was used to wash them. Ice cold isolation medium (sucrose 0.25 M) and EDTA (1 mM with 7.4 pH) were used to clean testis and blotted gently and weighed.

2.3. Mitochondrial isolation

Mitochondria from the testicular cells were segregated by the method of Mingatto et al. (1996). Briefly, the testicular tissues were removed quickly, and homogenized in the medium labeled I (70 mM sucrose, 250 mM mannitol, 50 mM Tris-HCl, 10 mM HEPES, 1 mM EDTA, 120 mM KCl, and pH 7.4). The homogenate was centrifugation for 5 min at 755×*g* to remove course debris and re-centrifuged for 15 min at 13,300×*g*. The medium labeled II (250 mM mannitol, 50 mM Tris-HCl, 70 mM sucrose, 10 mM HEPES, and pH 7.4) was used to suspend the resulting pellets and then cleaned twice using the same buffer and centrifuged at 13,300×*g* for 15 min. The acquired mitochondria pellets were suspended in medium II and used in further studies.

2.4. Analysis of biochemical parameters in testicular mitochondria

The activity of CAT was assayed using the procedure described by Aebi (1984). Similarly, the activity of SOD and GSH was determined by following procedures of Oyanagui (1984) and Shirani et al. (2019), respectively. Glutathione peroxidases activity were determined by the method of Flohe and Günzler (1984).

2.5. Analysis of reactive oxygen species and thiobarbituric acid levels in the mitochondria of testicular tissues

The amount of reactive oxygen species was estimated using the method of Keshtzar et al. (2016). The thiobarbituric acid reactive substance was assessed according to the method of Ohkawa et al. (1979).

2.6. Estimation of mitochondrial tricarboxylic acid cycle enzymes in testes

The method described by Srikantan and Krishna Murti (1955) was used to measure the level of succinate dehydrogenase (SDH). The method described by Macnicol and Jacobsen (1992) was used to estimate the levels of Malate dehydrogenase (MDH). Carlier and Pantaloni (1973) were used to estimate the Isocitrate dehydrogenase (ICDH). The α-KGDH activity was evaluated by the method described by Reed and Mukherjee (1969).

2.7. Estimation of the activity of mitochondrial respiratory chain complexes

A mitochondrial respiratory chain complex assay kit (Suzhou Comin Biotechnology ltd., China) was used to estimate the activities of mitochondrial respiratory chain complexes.

2.8. Assessment of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was measured by mitochondrial uptake of a cationic fluorescent dye (Rhodamine 123). The mitochondrial suspension (0.5 mg protein ml^{-1}) was incubated, and then the tubes were gently shaken for 10 min at 37 °C with Rh 123 (1.5 μM) at emission and excitation wavelengths of 490 and 535 nm, respectively. The Elmer LS-50B Luminescence fluorescence spectrophotometer was used to estimate levels of fluorescence (Baracca et al. 2003).

2.9. Data analysis

All results were presented as mean ± SEM. The data was statistically analyzed using Minitab software with one-way ANOVA test, followed by Tukey's test. The significance level was set at $p < 0.05$.

3. Results

3.1. Effect of MTX and baicalin on mitochondrial antioxidant enzymes activity:

MTX exposure significantly ($p < 0.01$) reduced the activities of antioxidant enzymes (CAT, SOD, GSH, and GPx) in comparison with the control group. Co-treatment of baicalin with MTX substantially ($p < 0.01$) restored the activities of antioxidant enzymes compared to MTX-treated rats. Baicalin alone administration showed non-

significant changes in antioxidant enzyme activities compared to the control animals (Table 1).

3.2. Effect of MTX and baicalin on ROS and TBARS production in testicular mitochondria

The results explained that the exposure of MTX substantially ($p < 0.05$) escalated the ROS and TBARS levels in testicular mitochondria in comparison to control animals (Table 2). Moreover, ROS and TBARS levels were substantially ($p < 0.05$) decreased after baicalin co-treatment with MTX compared to the MTX intoxicant rats. Baicalin alone treatment showed non-significant changes in antioxidant enzyme activities compared to the control animals.

3.3. Effect of MTX and baicalin on activities of TCA cycle enzymes

Data presented in Table 3 shows that MTX substantially ($p < 0.001$) reduced the activities of TCA cycle enzymes (ICDH, α-KGDH, MDH, and SDH) as compared to control rats. Co-administered baicalin substantially ($p < 0.001$) elevated TCA cycle enzyme activities compared to MTX intoxicated rats. Baicalin alone treatment showed established activities of TCA cycle enzymes as in the control group.

3.4. Effect of MTX and baicalin on testicular mitochondrial respiratory complexes activities:

As shown in Table 4, a substantial ($p < 0.05$) decrease was observed in activities of the mitochondrial complex of the respiratory chain (I-IV) after MTX exposure in comparison to the control group. Co-administration of baicalin substantially elevated the activities of the respiratory chain's mitochondrial complex in testicular mitochondria compared to MTX intoxicated rats. Baicalin alone treatment showed non-significant variations in mitochondrial respiratory chain complex activities compared to control animals.

3.5. Effect of MTX and baicalin on mitochondrial membrane potential

The rats treated with MTX showed a substantial ($p < 0.01$) depolarization of $\Delta\Psi m$ in comparison to the control group (Table 4). However, baicalin co-treated substantially ($p < 0.01$) augmented the loss of $\Delta\Psi m$ compared to MTX administrated rats. Baicalin alone treatment showed normal values of $\Delta\Psi m$ as compared to control rats.

4. Discussion

Mitochondria are multifunctional organelles. Its primary function is the formation of ATP by oxidative phosphorylation (Junge and Nelson, 2015; Fisher et al., 2019). In addition, mitochondria extensively participate in the calcium homeostasis of cells and regulate cellular functions (Santulli et al., 2015). Mitochondria is the organelle that generates ROS; on the other hand, it is the organelle most vulnerable to oxidative stress if the level of ROS exceeds the normal physiological range. The elevated production of ROS causes the mitochondrial membrane to be highly susceptible to oxidative damage (Calabrese et al., 2005). With the increased cancer rate worldwide, various chemotherapeutic agents such as MTX have been extensively increased. At the same time, MTX usage is also linked with severe side effects on multiple body organs, including testicular tissues (Oyanagui, 1984). Therefore, germinal cell protection is the primary intention during chemotherapy (Pınar et al., 2018; Aslankoc et al., 2020). Baicalin is a bioflavonoid having diverse pharmacological effects such as antiplatelet, antioxidant, anticancer, and anti-inflammatory (Srinivas, 2010).

Table 2
Baicalin effect on ROS and TBARS levels in testis of MTX administrated rats.

Groups	Thiobarbituric acid (nm TBARS/min/mg tissue)	Reactive oxygen species (U/g tissue)
Control	7.24 ± 0.49[c]	16.21 ± 0.83[c]
MTX	26.35 ± 0.94[a]	65.85 ± 1.07[a]
MTX + Baicalin	12.6 ± 0.58[b]	30.17 ± 1.04[b]
Baicalin	7.21 ± 0.53[c]	16.19 ± 0.97[c]

Means that do not share similar letters are significantly different from each other.

The current investigation revealed that MTX induction elevated ROS and TBARS while reducing the activities of antioxidants (SOD, GSH, and GPx) in mitochondria of rat testicular tissues. ROS plays a vital role in regulating various biological functions modulating the activities of enzymes by the redox reaction cycle (Wang and Hai, 2016). Antioxidant enzymes such as GSH, SOD, GPx, and CAT protect male reproductive organs against excessive ROS production (Yuluğ et al., 2013). Endogenous antioxidant GST catalyzes the GSH conjunction into endogenous and exogenous electrophiles, GSH oxidizes into glutathione disulfide by GPx, SOD converts oxygen (O_2) into molecular oxygen and hydrogen peroxide (H_2O_2), which convert into water and O_2 by CAT (Ghaznavi et al., 2016; Mehrzadi et al., 2016). Observation has shown that MTX disrupts testes by decreasing the antioxidant status of the cells, which reveals the damaging effect of free radicals (Sayılmaz et al., 2016; Belhan et al., 2017). When testicular mitochondria are impaired, it causes cellular dysregulation and obstruction in energy metabolism, leading to cell death and sperm dysfunction (Zhang et al., 2019; Zhang et al., 2018). Lipid peroxidation is known as oxidative degradation of lipids. Lipid peroxidation disrupts cells membrane and also retards physiological function (Wong-Ekkabut et al., 2007). The elevation in the level of lipid peroxidation and ROS by elevating the activities of antioxidant enzymes including SOD, GSH, and GPx in mitochondria of rat testis in a group with a co-treatment of MTX with baicalin.

In the present investigation MTX administration significantly lowered the activity of TCA cycle enzymes, including SDH, MDH, IDH, and NADH-dehydrogenase, compared to control rats. In cells, the TCA cycle is the primary route for oxidative phosphorylation that satisfies all biosynthesis, bioenergetics, and redox balance requirements. The TCA cycle is the central part of energy production and macromolecules synthesis. TCA cycle is composed of var-

Table 1
Effect of baicalin treatment on catalase, superoxide dismutase, Glutathione and Glutathione peroxidase activities in isolated mitochondria from MTX intoxicated rat testicular tissues.

Groups	Catalase (U/mg protein)	Superoxide dismutase (U/mg protein)	Glutathione reductase (μM/g tissue)	Glutathione peroxidase (U/mg protein)
Control	2.58 ± 0.14[a]	3.59 ± 0.32[a]	25.47 ± 0.71[a]	10.19 ± 0.73[a]
MTX	1.13 ± 0.08[b]	1.18 ± 0.22[b]	13.62 ± 0.67[c]	4.96 ± 0.21[b]
MTX + Baicalin	2.24 ± 0.07[a]	3.01 ± 0.13[a]	20.5 ± 0.442[b]	8.74 ± 0.22[a]
Baicalin	2.59 ± 0.12[a]	3.63 ± 0.29[a]	25.73 ± 0.77[a]	10.25 ± 0.54[a]

Means that do not share similar letters are significantly different from each other.

Table 3
Effect of baicalin on activities of TCA cycle enzymes in testicular mitochondria of MTX treated rats.

Groups	Isocitrate dehydrogenase (units/min/mg of protein)	Alpha-ketoglutarate dehydrogenase (units/min/mg of protein)	Succinate dehydrogenase (units/min/mg of protein)	Malate dehydrogenase (units/min/mg of protein)
Control	803.59 ± 5.04[a]	263.15 ± 4.95[a]	90.03 ± 2.97[a]	612.39 ± 6.45[a]
MTX	370.72 ± 9.88[c]	99.01 ± 5.135[c]	28.54 ± 1.60[c]	143.56 ± 7.93[c]
MTX + Baicalin	684.33 ± 5.84[b]	211.62 ± 7.22[b]	71.51 ± 1.72[b]	539.13 ± 8.02[b]
Baicalin	808.19 ± 8.19[a]	272.48 ± 7.67[a]	90.60 ± 3.35[a]	620.53 ± 6.06[a]

Means that do not share similar letters are significantly different from each other.

Table 4
Baicalin effect on activities of mitochondrial electron transport chain complexes activities and mitochondrial membrane potential of MTX intoxicated rats.

Groups	Complex-I (NADH dehydrogenase)	Complex-II (Succinate-dehydrogenase)	Complex-III (Coenzyme Q-cytochrome reductase)	Complex-IV (Cytochrome *c* oxidase)	Mitochondrial membrane potential (%)
Control	46.33 ± 1.86[a]	88.21 ± 2.24[a]	0.92 ± 0.03[a]	364.92 ± 6.5[a]	85.26 ± 1.07[a]
MTX	17.62 ± 1.16[c]	28.62 ± 1.73[c]	0.38 ± 0.11[b]	128.41 ± 4.5[c]	29.79 ± 1.56[c]
MTX + Baicalin	33.88 ± 1.59[b]	72.21 ± 1.44[b]	0.72 ± 0.02[a]	258.50 ± 5.9[b]	68.73 ± 1.50[b]
Baicalin	47.28 ± 1.93[a]	89.67 ± 3.41[a]	0.93 ± 0.03[a]	372.03 ± 5.8[a]	85.52 ± 2.02[a]

Means that do not share similar letters are significantly different from each other.

ious biochemical reactions, which permit the aerobic organism to oxidize the fuel, which results in energy production. The TCA cycle produces electron acceptors through different biochemical reactions, channeling to ETC (Anderson et al., 2018). Mitochondria possess different enzymes i.e., a-KGDH, ICDH, SDH, and MDH that catalyze the various substrates by the TCA cycle, producing reducing-equivalents. These reducing equivalents are channeled to the respiratory-chain for ATP production through oxidative-phosphorylation (Josephine et al., 2007). Our results are inconsistent with the study of Singh et al. (2012), as they reported that MTX treatment disrupted the mitochondrial functions by causing a considerable loss in the enzymes of the TCA cycle in the mitochondria of cardiac tissues of rats. However, baicalin co-treatment effectively elevated TCA cycle enzymes' activities in testicular tissues' mitochondria.

The present study revealed that MTX exposure substantially reduced the activities of ETC complexes (I-IV) compared to control rats. Mitochondrial dysfunction results in infertility (Ramalho-Santos et al., 2009). In the mitochondrial inner membrane, ETC produces ATP as the final product necessary for cellular functions and sperm maturation (Rajender et al., 2010). Primarily the ETC comprises four major complexes; complex I (NADH dehydrogenase), complex II (succinate ubiquinone oxidoreductase), complex III (ubiquinone cytochrome *c* reductase), and complex IV (cytochrome *c* oxidase) (Zorov et al., 2014). These enzymes are essential to transfer the electron and establish a proton gradient to regulate ATP synthesis (Duchen, 2004). Destruction of any enzyme of ETC can halt ATP production, which may lead to metabolic collapse (Cuperus et al., 2010). A previous investigation by Kolli et al. (2014) supports our results that MTX causes mitochondrial dysfunction by decreasing the activities of ETC complexes in the enteritis of a rodent model. MTX-induced mitochondrial dysfunction might be due to excessive ROS production that may inhibit ETC complexes' activities (I-IV). However, baicalin co-administration alleviated the variations in ETC complexes (I-IV) which may be attributed to its antioxidant potential.

In the current investigation, $\Delta\Psi$m was significantly reduced in MTX intoxicated rats compared to control rats. $\Delta\Psi$m is usually produced by the proton gradient necessary for forming ATP (Zorova et al., 2018). Oxidative stress is potentially linked with the dissipation of $\Delta\Psi$m (Zorov et al., 2014). Normal $\Delta\Psi$m is indispensable to maintaining mitochondrial oxidative phosphorylation (Zorova et al., 2018); however, $\Delta\Psi$m reduction stimulates the mitochondrial impairment, which may eventually lead to cellular apoptosis (Zamzami et al., 1995). MTX-induced variation in $\Delta\Psi$m can affect ATP production and disturb mitochondria's everyday activities. However, loss of $\Delta\Psi$m was restored after baicalin co-treatment, possibly by increasing the ETC complexes' activities in mitochondria of rat testicular tissues.

5. Conclusion

In conclusion, the findings of our study showed that baicalin administration exhibited a remarkable protective effect against MTX damaging effects on mitochondrial biochemical parameters, TCA cycle enzymes, ETC complexes (I-IV), and $\Delta\Psi$m. Baicalin improved the activities of antioxidant enzymes and reduced the levels of ROS and TBARS. It protected the mitochondria by restoring the depleted activities of TCA cycle enzymes and ETC complexes and fixing the $\Delta\Psi$m. The study can be very useful to treating the patients suffering from male infertility. However, its underline molecular mechanism is still need to be explored in future study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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