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Original article

A study on the curative effect of nobiletin on paraquat induced toxicity in rat

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ABSTRACT

This research aimed to evaluate the curative impact of nobiletin (NOB) on paraquat (PQ) induced mitochondrial impairment in rats' hepatic tissues. Twenty-four male rats were divided into control, PQ, PQ + NOB, and NOB treated groups. The alanine aminotransferase levels, aspartate aminotransferase, and alkaline phosphatase were increased in the experimental animals after exposure with PQ. A significant decrease in the level of catalase, glutathione peroxidase, superoxide dismutase, glutathione, thiobarbituric acid reactive substances, and reactive oxygen species were recorded in rats exposed to PQ. The mitochondrial TCA cycle enzyme activities, including isocitrate-dehydrogenase, succinate-dehydrogenase, alpha-ketoglutarate dehydrogenase, and malate-dehydrogenase, were reduced in PQ exposed rats. The activities of mitochondrial enzymes including NADH dehydrogenase, coenzyme Q-cytochrome reductase, succinic-coenzyme Q and cytochrome c-oxidase were significantly decreased after the PQ-exposure. PQ administration also reduced the mitochondrial membrane potential. However, the administration of mitochondria with NOB can decrease the toxic effect of the PQ in isolated mitochondria. NOB treatment potentially reduced the damaging effects of the PQ in the mitochondria isolated from hepatic tissues. Thus, the current study revealed that the NOB could attenuate PQ-induced mitochondrial damage in rats' hepatic tissues.

1. Introduction

Currently, a strong association exists between environmental pollutants and liver diseases, which provokes excellent concern for human health (Kim et al., 2019). Paraquat is a non-selective quick-acting contact herbicide that is widely used in crop cultivation and conservation systems worldwide (Li et al., 2019). In developing countries, particularly in Asia, PQ poisoning has been considered a critical problem for many years (Dinis-Oliveira et al., 2008). The typical route of PQ exposure to humans is skin and mouth; its massive exposure can kill a person within 3.5 h (Nikdad et al., 2020). PQ toxicity tends to be acute and chronic in animals and humans. The leading cause of mortality is respiratory failure, massive damage in the heart, liver, adrenal gland, central nerves, kidney, spleen, and immune system (Dinis-Oliveira et al., 2008).

Reactive oxygen species and oxidative stress are the primary mediators of PQ-induced toxicity. ROS at the physiological level, regulates the growth factors, inflammatory reactions, and cell signaling typically. Nevertheless, excessive free radical production without regulation may lead to severe injuries (Zeinvand-Lorestani et al., 2015). PQ exposure increases the levels of serum markers of the liver (El-Boghdady et al., 2017). PQ-induced oxidative stress is related to lipid peroxidation, which instigates cell membranes and various other subcellular organelles, i.e., lysosome and mitochondria (El-Boghdady et al., 2017). Mitochondria are the key site of ROS and reactive nitrogen-species production (Bhargava

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and Schnellmann, 2017). The previous study revealed that PQ exposure induces oxidative stress and causes mitochondrial impairment, which was indicated by swelling of the rat liver's mitochondrial membrane (Han et al., 2014).

Polyphenolic flavonoids have great concern nowadays due to their distinctive pharmacological properties (Wang et al., 2019; Ijaz et al., 2020). Nobiletin (NOB) is a polymethoxylated flavonoid primarily obtained from citrus fruits (Goh et al., 2019). NOB is an integral part of Chinese traditional medicine. NOB has a wide range of pharmacological features such as anti-cancer, anti-diabetes, antioxidant, and anti-inflammatory (Huang et al., 2016a). However, the potential curative effects of NOB on PQ-induced mitochondrial damage have not yet been studied. Therefore, this research was aimed to explore the curative efficiency of NOB on PQ-induced mitochondrial damage in rat liver.

2. Materials and methods

2.1. Animals

Twenty-four Sprague Dawley rats, having weight 200 ± 10 g, were procured from the breading and rearing center and placed in the Animal House of the University of Agriculture, Faisalabad. The rats were maintained in laboratory conditions (40–60% relative humidity; 24 ± 2 ℃ temperature)—the automatic light period of 12 h light and dark and offered with a standard rodent diet. Animals were treated in compliance with the international recommendations for the use and care of experimental animals.

2.2. Experimental design

The rats were divided into four groups (n = 6/group). Group 1: rats were treated with normal saline and labeled as control. Group 2: rats received 20 mg/kg bw of PQ. Group 3: rats were treated with PQ at 20 mg/kg and NOB at dose 25 mg/kg. Group 4: rats orally administrated with NOB at a dose of 25 mg/kg bw. Retro-orbital venous plexus was used for blood collection, and serum was separated from the blood. Rats were slaughtered on the 31st day, and the liver was removed, weighed, and rinsed in normal saline, and homogenate (10% w/v) was prepared in PBS at neutral pH. Centrifugation was carried out at 12000 × g/60 min at four ℃. The supernatant was isolated and kept at −20℃ until used for further analysis.

2.3. Isolation of liver mitochondria

The liver was isolated and chopped with a scalpel and placed in a cold mixture of mannitol comprising Ethylenediaminetetraacetic acid (0.2 mM) and 0.225 M D-mannitol, 75 mM sucrose. The liver slices were homogenized with Teflon pestle. The homogenate was continuously centrifuged at 4 °C (700 × g) for ten minutes to remove intact cells, non-subcellular components, and nuclei. The resulted supernatants were placed in a falcon. It was again centrifuged for at 7000 × g 20 min. The supernatant having mitochondria was separated from residues (Ghazi-Khansari et al., 2006).

2.4. Hepato-serum marker analysis

The assessment of liver function enzymes (ALT, AST, and ALP) was performed by following the standardized method available on Wiesbaden's diagnostic kits (Germany).

2.5. Assessment of oxidative stress and antioxidant enzymes

The activity of catalase (CAT) was measured according to Aebi, 1984 technique. Superoxide dismutase (SOD) action was evaluated by following the process of Sun et al. (1988). Glutathione peroxidase (GPx) activity was evaluated with the methodology of Flohe and Gunzler (1984). The glutathione (GSH) content was determined by the methods of Moron et al. (1979) process. TBARS level was assessed according to the procedure of Ohkawa et al. (1979). The ROS level was evaluated by ELISA kits (Shanghai Enzyme-Linked Biotech. Company Ltd., Shanghai, China) by following the manufacturer's guidelines.

2.6. Assessment of TCA cycle enzymes

Bernt and Bergmeyer (1974) method was used to determine the activities of isocitrate dehydrogenase (ICDH). α-KGDH activity was assessed by following the Reed and Mukherjee (1969) procedure. Succinate-dehydrogenase (SDH) activity was examined by the Slater and Borner (1952) technique. Mehler et al. (1948) method was followed to assess Malate dehydrogenase (MDH).

2.7. Investigation of respiratory chain complex activity in liver mitochondria

Mitochondrial respiratory chain-complex Assay kits (Suzhou-Comin Biotech. LTD, China) was used to assess the liver mitochondria's respiratory chain complexes activities.

2.8. Mitochondrial membrane potential analysis

MMP was assessed by mitochondrial uptake of a cationic-fluorescent dye (Rhodamine 123). The tubes have slightly shaken the tubes for 10 min. Tubes were shaken somewhat for 10 min at 37 ^{0}C with Rh 123 (1.5 μM) to incubate the mitochondrial suspension (0.5 mg protein ml-1). At emission (490 nm) and excitation (535 nm) wavelength, the Elmer LS-50B Luminescence fluorescence spectrophotometer was used for the estimation of fluorescence (Baracca et al. 2003).

2.9. Statistical analysis

The data tabulated as Mean ± SEM in the tables. One-way ANOVA, followed by Tukey's test, was operated to examine the data variations via Minitab Software. The level of significance was $p < 0.05$.

3. Results

3.1. Effects of NOB on serum markers in PQ-exposed rats

A significant ($p < 0.05$) increase in the level of ALT, AST, ALP was detected in the PQ intoxicated animals compared to the control group (Table 1). Concurrent treatment with PQ and NOB produced a significant ($P < 0.05$) hepatoprotective impact. It decreased the

Table 1
Effects of NOB on ALT, AST and ALP in the liver of PQ-intoxicated rats.

Groups	ALT (U/l)	AST (U/l)	ALP (U/l)
Control	74.40 ± 1.92 ^{a}	94.70 ± 1.87 ^{a}	164.3 ± 1.85 ^{a}
PQ	188.5 ± 1.52^b	228.6 ± 1.80^b	291.2 ± 1.50^b
PQ + NOB	96.88 ± 1.74^c	120.1 ± 1.86^c	185.2 ± 1.79^c
NOB	69.63 ± 1.48 ^{a}	93.23 ± 2.02 ^{a}	163.5 ± 1.68 ^{a}

Values having different superscripts in a same column are significantly different.

Table 2
Effects of NOB on the mitochondrial antioxidant enzymes; CAT, SOD, GPx, GSH, ROS and TBARS in PQ-treated rats.

Groups	CAT(U/mg protein)	SOD(U/mg protein)	GSH(μM/g tissue)	GPx(U/mg protein)	TBARS (nm TBARS/min/mg tissue)	ROS (U/g tissue)
Control	3.99 ± 0.14[a]	7.18 ± 0.19[a]	19.28 ± 1.15[a]	13.28 ± 0.78[a]	8.31 ± 0.34[a]	18.25 ± 0.90[a]
PQ	1.63 ± 0.17[b]	3.77 ± 0.17[b]	7.42 ± 0.68[b]	6.21 ± 0.31[b]	19.84 ± 0.75[b]	68.57 ± 1.43[b]
PQ + NOB	3.14 ± 0.25[a]	5.32 ± 0.10[c]	17.43 ± 0.28[a]	11.18 ± 0.49[a]	13.51 ± 0.59[c]	45.12 ± 1.47[c]
NOB	3,88 ± 0.23[a]	7.23 ± 0.27[a]	20.51 ± 1.14[a]	13.61 ± 0.63[a]	8.14 ± 0.23[a]	19.25 ± 1.00[a]

Values having different superscripts in a same column are significantly different.

level of liver function enzymes ALT, AST, and ALP compared to PQ alone treated animals. The NOB alone treatment showed average serum marker values as in the control group.

3.2. Effect of NOB on oxidative stress and antioxidative capacity of the liver in PQ-exposed rats

PQ exposure resulted in a substantial ($p < 0.05$) decrease in the CAT, GPx, SOD activities, and GSH content along with increased ROS and TBARS levels in renal tissues (Table 2). Co-administration NOB with PQ elevated the CAT, SOD, GPx activities, and GSH content while substantially ($p < 0.05$) reduced the levels of ROS and TBARS compared to PQ administrated animals. The NOB alone treatment showed normal antioxidants, ROS, and TBARS values as in the control group.

3.3. Effects of NOB on activities of mitochondrial-tricarboxylic acid cycle (TCA) enzymes

As shown in Table 3, the intoxication of PQ significantly ($p < 0.05$) decreased the TCA cycle enzymes (ICDH, α-KGDH, SDH, and MDH) activities when matched with control rats. However, co-administration of NOB with PQ restored the TCA enzyme activities. Co-treated rats showed that NOB increased the TCA enzyme activities as compared with PQ treated rats. The NOB alone treatment showed non-significant changes in TCA enzyme activities as compared to the control group.

3.4. NOB effect on activities of hepatic mitochondrial respiratory chain complex

A substantial reduction ($p < 0.05$) was observed in the mitochondrial complex (I-IV) activities after PQ administration when matched with a control group (Table 4). Oral administration of NOB substantially elevated the activities of the mitochondrial complex in the liver. The restoration in the mitochondrial respiratory chain complex activities was observed in NOB + PQ treated rats compared to PQ treated rats. NOB alone treated group showed normal activities of mitochondrial respiratory chain complexes.

3.5. Effect of CAS on mitochondrial membrane potential

Rats exposed with PQ displayed a substantial ($p < 0.05$) depolarization of mitochondrial membrane potential ($\Delta\Psi m$) when compared to the control group. The cotreatment of NOB with PQ significantly restored the loss of $\Delta\Psi m$, when compared with PQ exposed rats. NOB alone treated group showed average mitochondrial membrane potential as in the control group (Table 4).

4. Discussion

Mitochondria is a cellular powerhouse that plays a vital role in producing oxidative energy for the cell, which is essential for oxidative phosphorylation (Devkar et al., 2016). The mitochondria are the primary site for ROS production. Excessive ROS attacks on the mitochondria result in the dysfunction of mitochondria and the respiratory chain (Zhou et al., 2020). A previous study indicated that PQ exposure induced ROS and triggered oxidative damage (El-Boghdady et al., 2017). PQ induced redox reaction resulted in GSH and NADPH reduction, which ultimately resulted in the mitochondria's dysfunction (Gawarammana and Buckley, 2011). NOB is a polymethyl flavonoid with various pharmacological properties viz., antioxidant, hepatoprotective, and neuroprotective effects (Huang et al., 2016a). Therefore, this research was conducted to assess NOB's ameliorative effects on PQ-induced hepatic mitochondrial damages in Sprague Dawley rats.

Comparing the normal range and measured data of serum markers (like ALT, AST, and ALP) can help in the diagnosis of liver disease (Faheem et al., 2019). ALT, AST, and ALP are markers linked with parenchyma cells of the liver. When the cell membrane of

Table 3
Effects of NOB on TCA cycle enzymes (α-KGDH, ICDH, SDH MDH) activities in the renal mitochondria of PQ-administered rats.

Groups	ICDH (units/min/mg of protein)	α-KGDH (units/min/mg of protein)	SDH (units/min/mg of protein)	MDH (units/min/mg of protein)
Control	933.91 ± 12.33[a]	265.1 ± 6.8[a]	92.82 ± 3.56[a]	660.51 ± 8.7[a]
PQ	441.1 ± 10.7[b]	84.91 ± 4.88[b]	28.40 ± 1.94[b]	274.8 ± 7.66[b]
PQ + NOB	773.0 ± 10.0[c]	194.1 ± 9.6[c]	64.64 ± 2.59[c]	445.0 ± 7.66[c]
NOB	956.1 ± 6.5[a]	271.7 ± 15.16[a]	98.21 ± 3.85[a]	672.8 ± 13.8[a]

Values having different superscripts in a same column are significantly different.

Table 4
Effects of NOB on the mitochondrial ETC complexes activities (Complex I-IV) and mitochondrial membrane potential (MMP) of PQ-treated rats.

Groups	Complex-I (NADH dehydrogenase)	Complex-II (Succinate-dehydrogenase)	Complex-III (Coenzyme Q-cytochrome reductase)	Complex-IV (Cytochrome *c* oxidase)	MMP %
Control	40.85 ± 1.74[a]	81.07 ± 1.42[a]	0.84 ± 0.01[a]	293.14 ± 10.21[a]	77.33 ± 2.67[a]
PQ	16.66 ± 0.61[b]	35.73 ± 1.09[b]	0.25 ± 0.02[b]	147.13 ± 9.98[b]	32.68 ± 0.99[b]
PQ + NOB	29.61 ± 1.00[c]	61.86 ± 1.37[c]	0.57 ± 0.01[c]	266.9 ± 6.61[a]	66.08 ± 1.84[c]
NOB	39.64 ± 1.61[a]	84.78 ± 1.85[a]	0.86 ± 0.01[a]	296.08 ± 8.27[a]	79.85 ± 2.5[a]

Values having different superscripts in a same column are significantly different.

hepatic cells is injured, these markers usually exist in the cytosol are escaped out into the bloodstream. Thus, these markers' serum levels are used to identify liver injury (Carobene et al., 2013). Serum markers (ALT, AST, and ALP) level increase in PQ administrated rats verifies that the morphological durability of hepatic tissues has been impaired as enzymes generally located in the cytoplasm were discharged into the bloodstream. Previous investigations revealed that elevation in hepatic serum markers indicates increased oxidative damage in hepatic tissues, associated with liver dysfunction (Ali et al., 2017). However, co-treatment with NOB ameliorated the PQ-persuaded liver toxicity by reducing the serum ALT, AST, and ALP levels.

Our results showed that PQ treatment reduced the CAT, SOD, GPx activities, and GSH content while increasing the TBARS and ROS levels in mitochondria isolated from the hepatic tissues. In homeostasis, the generation and elimination of ROS balance are indispensable, regulated by antioxidant enzymes (Latif et al., 2020). GSH and SOD are usually considered fundamental indicators of OS (Yang et al., 2018). SOD converts O_2 into hydrogen peroxide (H_2O_2), then CAT and GPx convert H_2O_2 into water (Yang et al., 2018). Intracellular ROS performs a significant role in various biological functions because the ROS acts as a signaling molecule and is regulated by biphasic machinery (Wang et al., 2016).

On the other hand, once the free radical production is exceeded and overwhelms the cells' natural defense mechanism, it may cause cellular damage (Kouretas et al., 2013). The OS can damage a wide range of macromolecules, i.e., lipids, protein, and sugar (Kouretas et al., 2013). Nikdad et al. (2020) stated that OS is the primary cause of PQ-induced toxicity in the liver's mitochondria as it is supported by the formation of lipid peroxidation (LPO) and reduction in CAT and SOD activities. However, the NOB administration significantly decreased the ROS and TBARS levels by restoring antioxidant enzymes' activities. Our results are consistent with previous experimental observations of Huang et al. (2016a). They revealed that NOB possesses antioxidant properties as it is beneficial in reducing the ROS levels by raising antioxidant enzyme activities.

The present study revealed that PQ reduced the TCA cycle enzyme activities in the mitochondria isolated from the liver. It is a fact that certain enzymes (α-KGDH, ICDH, SDH, and MDH) present in mitochondria catalyze the various substrates by the TCA-cycle, producing reducing-equivalents. These reducing-equivalents are directed within the respiratory-chain for ATP production through oxidative-phosphorylation (Josephine et al., 2007). In the TCA cycle, the ICDH enzyme is the vital producer of NADPH and performs an essential part in the defensive mechanism to counter the OS-induced injury (Vedi et al., 2014). The high ICDH activity can lead to decreased mitochondrial glutathione regeneration, subsequent in a noticeably high confrontation of the enzymes to OS (Jo et al., 2001). SDH is the essential enzyme in mitochondria that controls ATP production. It is a member of the thiol (SH) group containing enzymes vulnerable to free radicals (Murugesan and Manju, 2013). Reduced mitochondrial enzyme activities influence the oxidation of the mitochondrial-substrate, which results in reduced substratum-oxidation and a reduced rate of transport of reducing equivalents, thus destroying cellular energy (Morimoto et al., 2000). PQ property of TCA was considered as an effective method in the interference of cell metabolism. After the PQ exposure, disruption in the TCA cycle's ETC and dysfunction was noticed (Anandhan et al., 2017). However, in this research, the cotreatment of NOB with PQ reversed the activities of these enzymes. A decrease in ROS production might be the reason for the restoration of these enzyme activities.

The present study indicated that PQ exposure reduced the mitochondrial complex (I-IV) activities of the ETC. Mitochondria are the primary site of ROS formation. ETC's disorder of mitochondrial inner membrane contributes to excessive production of ROS and subsequent OS following mitochondrial dysfunction (Sorrentino et al., 2018). Mitochondria are the main target for toxicity (Khiati et al., 2014) because they perform an essential function in detoxification. Previous studies showed that intracellular ROS accumulation is the main reason for the damage of mitochondrial ETC (Cheraghi et al., 2019). Once the transmission of the mitochondrial ETC is blocked, ATPs' synthesis is affected, and this condition is due to mitochondrial damage (Schwarz et al., 2014). The previous investigation revealed the mitochondrial role in PQ intoxication (Huang et al., 2016) because PQ inhibits complexes' activities in mitochondria (Choi et al., 2008). However, cotreatment with CAS substantially reversed the activities of ETC complexes.

The present investigation revealed that PQ induced significant depolarization of mitochondrial membrane potential ($\Delta\Psi m$). Increased ROS may alert the constituents of mitochondria, provoking MMP collapse, oxidative phosphorylation uncoupling, and ultimately splitting membrane (Ahmadian et al., 2018). The experimental study of Nikdad et al., 2020 revealed that PQ causes the loss in mitochondrial membrane potential. The present study showed that the loss of $\Delta\Psi m$ could be reversed by oral administration of NOB by increasing the ETC complexes' activities.

5. Conclusion

Our results indicated that NOB administration exhibited protective effects against PQ-induced adverse effects on liver function markers, mitochondrial antioxidant enzymes, TCA cycle enzymes, ETC complexes, and mitochondrial membrane potential. NOB maintained hepatic mitochondrial functions by decreasing ROS and TBARS levels and protecting TCA cycle enzymes, ETC complexes' activities.

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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