Journal of Pharmaceutical, Chemical and Biological Sciences
ISSN: 2348-7658
UGC Approved Journal
CODEN: JPCBBG
Impact Factor (GIF): 0.701
Impact Factor (SJIF): 3.905
March - May 2018; 6(1):1-10
Published on: March 18, 2018

Research Article

Role of N-acetyl-L-cysteine in the Prevention of Hepatotoxicity Induced by Patulin in Male Mice

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Received: 14 January 2018 **Revised:** 14 February 2018 **Accepted:** 21 February 2018

ABSTRACT

The present study was designed to investigate the possible preventive effect of 7 days pretreatment with N-acetyl-L-cysteine (NAC) on patulin (PAT)-induced hepatotoxicity in male mice. Obtained results showed that intraperitoneal injection (i.p.) of mice with PAT in a single dose (3.75mg/kg) significantly increased hepatic contents of malonic dialdehyde (MDA), accompanied with elevated activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum. On contrary, PAT treatment alone markedly decreased hepatic contents of antioxidant parameters including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd), glutathione-s-transferase (GST) and reduced glutathione (GSH). Serum concentrations of total protein and albumin were also decreased following administration of PAT. On the other hand, liver contents of p53, caspase-3 and Bax were significantly increased accompanied with markedly decreased hepatic concentration of Bcl-2 after injection of PAT alone, suggesting ability of PAT to induce apoptosis. Treatment with NAC (200mg/kg/day) in mice for 7 days prior exposure to PAT significantly inhibited the above mentioned adverse effects induced by PAT. In conclusion, NAC seems to be effective in the prevention of hepatotoxicity induced by the potent mycotoxin PAT.

Keyword: N-acetyl-L-cysteine; patulin; hepatotoxicity; antioxidants; apoptosis

INTRODUCTION

Mycotoxins which are produced by fungi can cause serious diseases and even death in both humans and animals. PAT, which has a lactone structure as shown in Fig. 1, is one of these mycotoxins that made by certain fungal species of the genera *Penicillium, Aspergillus* and *Bryssochamys* [1]. It may grow on a variety of foods including fruits and vegetables, mainly apples and therefore it can cause contamination for these types of food and their commercial products, such as apple products [2].

Previous studies exhibited that exposure to PAT can induce a number of toxic effects in several animal organs including liver, kidney, intestine, immune system and brain [3,4,5]. There are evidences which indicate that PAT is a genotoxic and mutagenic [4,6]. However, the genotoxicity and cytotoxicity of this mycotoxin are associated with its electrophilic properties which facilitate high interaction of it to cellular nucleophiles, such as sulfhydryl (thiol) groups. This can cause depletion of cellular sulfhydryl containing compounds such as GSH concentration and hence production of oxidative stress [7,8]. Furthermore, available data showed that administration of PAT in animal was found to produce apoptosis [9,10].

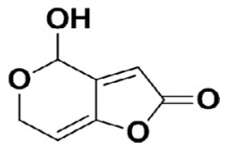

Fig. 1: Chemical Structure of PAT

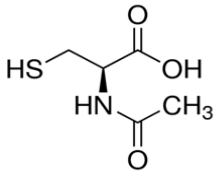

Fig. 2: Chemical Structure of NAC

NAC is considered a powerful antioxidant because it represents a good source of sulfhydryl groups (Fig. 2) which capable of scavenging the free radicals, especially radicals of oxygen. Beside this, NAC stimulates GSH biosynthesis because it is a precursor of L-cysteine, an essential component of GSH molecule. NAC was therefore recommended as a potential treatment agent for various disorders related to generation of free oxygen radicals [11]. Published data demonstrated that NAC has a hepatoprotective effect against several chemicals-induced toxicity. In both humans and animals, NAC was introduced to treat hepatotoxicity resulted from exposure to overdoses of acetaminophen [12,13,14]. Also, NAC was found to stabilize the cell membrane and to decrease the hepatocellular injury as evidenced by its ability to lower the activity of hepatic marker enzymes in the plasma (such as transaminases) and the levels of lipid peroxidation, in addition to its improvement of the antioxidant system in the liver of rats exposed to CCl_4 [15]. In view of these information, it appeared that NAC treatment may be effective in the protection against PAT-induced hepatotoxicity. Interestingly, there are no clear previous reports on the potential protective impact of NAC against PAT-induced liver injury, so we considered this point of great value and worthwhile. Based on this, present study was conducted to exploring the efficacy of NAC on the hepatic marker enzymes, non-enzymatic and enzymatic antioxidants, as well as the apoptotic markers in mice exposed to toxic dose of PAT.

MATERIALS AND METHODS

Chemicals

PAT, 4-hydroxy-4H-furo[3,2c] pyran-2(6H), has a chemical formula $C_7H_6O_4$ (Fig. 1) and NAC, N-acetyl-L-cysteine, has a chemical formula $C_5H_9NO_3SH$ (Fig. 2). The two chemical compounds were obtained from Sigma–Aldrich, Germany. The solid powder of both PAT and NAC was dissolved in distilled water when used for treatment.

Animals and experimental design

Forty healthy male Swiss albino mice weighing 25-30 g were obtained from Helwan Animal Farm, Cairo, Egypt. The mice were maintained under controlled environmental circumstances. They were fed on commercial rodent pellet diet and water was provided *ad libitum*. Nursing and use of the animals were managed under control of the Animal Ethics Committee of Mansoura University, Egypt. After a week of acclimation, the mice were allocated into four groups, 6 animals per each, as follows:

Group I: Control mice had no chemical treatment.

Group II: Mice were given NAC alone for 7 days in a dose of 200 mg/kg/day [16].

Group III: Mice were given a single dose of PAT (3.75mg/kg) at the 7th day [17].

Group IV: Mice were given both NAC and PAT as mentioned above in group (II) and (III) respectively.

PAT was given to mice by i.p. injection, while NAC was orally introduced to used animals using gastric tube.

Blood and tissue sampling

After 24 hours of injection of PAT, mice were anesthetized by ether following overnight fasting. Then, the jugular vein of each mouse was cutted using a sharp razor blade and the blood flowed was collected into the centrifuge tube. After blood clotting (10-15 min), the tubes were centrifuged at 3000 rpm for 15 min to separate the blood sera which were kept at -20°C for biochemical analysis.

On the other hand, the animals were quickly dissected and the whole liver of each mouse was removed and cleaned. A known weight of each liver was homogenized in distilled water at 4°C

forming 10% (w/v) homogenate, and then kept at -20°C for the assays.

Biochemical analysis

Hepatic contents of MDA, GSH, SOD, CAT and GSH-Px were measured by the ELISA technique according to [18, 19, 20, 21, 22] respectively. The ELISA kit was supplied from CUSABIO BIOTECH CO., Ltd, Baltimore, Maryland, USA. The activity of GSH-Rd in the liver homogenate was determined according to the method of [23], using ELISA kit from Cell Biolabs, Inc, 7758 Arjons Drive, San Diego, CA 92126 USA. Hepatic GST activity was measured by ELISA kit from Immundiagnostik AG, Stubenwald-Allee 8a, D64625 Bensheim; as described by [24]. Serum ALT and AST activities were assayed by kinetic method according to [25], using kit from Spinreact Company, Spain. Total protein content in the serum was estimated colorimetrically using kit from Spinreact Company, Spain according to the method of [26]. Serum albumin concentration was measured colorimetrically on basis of the method of [27] using kit from Diamond Company, Egypt. The liver contents of p53 and caspase-3 proteins were quantitatively estimated using ELISA kit provided by CUSABIO BIOTECH CO., Ltd, Baltimore, Maryland, USA, as described by the method of [28,29], respectively. Bax and Bcl-2 concentrations in the liver were determined using ELISA kits (MyBioSource, San Dioego, California, USA) on basis of the procedure of [30,31], respectively.

STATISTICAL ANALYSIS

Obtained results were presented as means ±SE. Statistically significant differences between mean values were evaluated by one way analysis of variance (ANOVA) using SPSS 19.0 software considering $p \leq 0.05$ as a minimal level of significant.

RESULTS

Table 1 shows that administration of PAT in a single dose (3.75 mg/kg) to male mice produced significance increase in the hepatic content of lipid peroxidation marker (MDA) and marked decrease in the levels of antioxidant parameters including SOD, CAT, GST, G-Px, G-Rd, and GSH, as compared to control results. However, 7 days treatment of mice with NAC prior injection of PAT significantly attenuated the adverse effects of PAT on the hepatic levels of markers of lipid peroxidation and antioxidants, in comparison with group treated with PAT alone.

Table 1: Effect of NAC on PAT-induced oxidative stress in the liver of male mice

Parameters	Control	NAC	PAT	NAC+PAT
MDA (nmol/g)	0.12±0.01	0.12±0.01	0.34±0.01ab	0.21±0.01abc
GSH (mg/g)	0.25±0.01	0.28±0.01	0.12±0.01ab	0.19±0.01abc
SOD (U/g)	0.29±0.01	0.29±0.01	0.12±0.02ab	0.19±0.01abc
CAT (µmol/g)	0.38±0.03	0.44±0.03	0.17±0.01ab	0.26±0.03abc
GST (mmol/g)	0.27±0.01	0.29±0.01	0.13±0.01ab	0.18±0.01abc
GSH-Px (mµ/mg protein)	0.36±0.02	0.35±0.02	0.11±0.01ab	0.20±0.01abc
GSH-Rd (ng/mg protein)	0.28±0.01	0.28±0.02	0.14±0.01ab	0.19±0.01abc

Values expressed as mean ± SE from 6 mice in each group.
a, b and c = Significant difference at $P \leq 0.05$ comparing to control, NAC and PAT respectively

As shown in table 2, injection of PAT in male mice produced significant increases in the serum activity of both ALT and AST, when compared to control group. On contrary, PAT significantly lowered serum contents of total protein and albumin, in comparison with obtained results of control mice. Pretreatment with NAC significantly lowered PAT-elevated activities of transaminases, and significantly increased PAT-lowered serum contents of total protein and albumin in the treated mice, as compared to mice injected with PAT alone.

Table 2: Effect of NAC on PAT-induced changes in the liver function markers in serum of male mice

Parameters	Control	NAC	PAT	NAC+PAT
ALT (U/L)	54.34±2.80	60.75±1.53	104.28±2.19ab	81.66±1.36abc
AST (U/L)	40.95±2.35	53.90±6.05	123.40±3.60ab	88.50±1.87abc
TP (g/dl)	6.18±0.26	6.55±0.12	4.34±0.11ab	5.33±0.10abc
AB (g/dl)	3.73±0.18	3.84±0.10	2.31±0.16ab	3.13±0.08abc

Values expressed as mean ± SE from 6 mice in each group.
a, b and c = Significant difference at $P \leq 0.05$ comparing to control, NAC and PAT respectively.

Obtained results in table 3 showed marked elevation in the levels of pro-apoptotic markers p53, Bax and caspase-3; and decrease in the concentration of anti-apoptotic marker Bcl-2 in the livers of mice treated with PAT, when compared to control group. However, pretreatment with NAC produced significant decrease in PAT-raised hepatic contents of p53, caspase-3 and Bax; while it enhanced PAT-declined level of Bcl-2 in the liver, in comparison with the results obtained from mice treated with PAT alone.

Table 3: Effect of NAC on PAT-induced changes in the apoptotic markers in the liver of male mice.

Parameters	Control	NAC	PAT	NAC+PAT
P53 (pg/mg protein)	0.13±0.01	0.14±0.004	0.29±0.01ab	0.24±0.004 abc
Caspase-3 (ng/mg protein)	0.18±0.01	0.19±0.01	0.27±0.01 ab	0.23±0.01 abc
Bax (ng/mg protein)	0.16±0.01	0.17±0.01	0.28±0.02 ab	0.20±0.01c
Bcl2 (ng/mg protein)	0.18±0.01	0.21±0.01	0.09±0.01 ab	0.14±0.01bc

Values expressed as mean ± SE from 6 mice in each group.
a, b and c = Significant difference at $P \leq 0.05$ comparing to control, NAC and PAT respectively.

In comparison with control results, treatment of mice with NAC alone had no significant effects on all investigated parameters (tables 1, 2 and 3).

DISCUSSION

PAT is a potent mycotoxin which is produced by certain fungal species, specially *P. expansum*. These microorganisms grow on fruits such as apples, citrus fruits, pears and cherries [32,33]. PAT, therefore, can find in commercial food products of these types of foods, particularly apple products, and can cause serious adverse effects to humans. This health problem for humans, actually, needs many studies to determine the mechanisms of PAT toxicity and hence to find out appropriate ways for prevention of PAT toxicity. In the current study, we examined the hepatotoxic effect of PAT in mice and the potential preventive effect of pretreatment with NAC against toxicity of this mycotoxin. For induction of acute toxicity of PAT, route of i.p. injection was selected but not oral one, although it considered more close to the real exposure to PAT, because i.p. injection has high bioavailability and injuries, while oral rout takes longer duration period for production of PAT toxicity, usually weeks to months and this practically is considered a problem [4].

Statistical analysis of the laboratory data of the current experiment showed that administration of PAT in male mice caused a significant elevation in the hepatic content of MDA corresponding to markedly decreased hepatic antioxidant parameters including GSH, SOD, CAT, GST, GSH-Px and GSH-Rd. This finding indicates that PAT caused oxidative hepatocellular injury in the treated mice, and add support that PAT is a toxic compound and hence eating of various fruits and food products contaminated with this mycotoxin can cause hepatotoxicity. Present findings closely resemble to the results obtained by several animal studies demonstrating the toxic effect of PAT in different organs, including the liver, kidney and brain [4,5,34,35]. In the study of [4], i.p injection

of PAT in a dose of 1mg/kg, once time, in mice caused marked elevation in reactive oxygen species level accompanied with significant inhibition in the activities of GSH, SOD and CAT in the liver. Also, *in vitro* studies PAT increased reactive oxygen species and lipid peroxidation product, MDA [36,37]. In addition, [38] found that inhibition of both SOD and CAT could play a role in PAT-induced oxidative damage in certain types of human cells *in vitro*. Moreover, studies on dermal toxicity of PAT showed that, in the presence of promoter, PAT in a single topical application (400 mmol) led to tumor formation along with marked rising in lipid peroxidation product and decrease in activity of antioxidant enzymes including CAT, SOD and GSH-Rd in treated mice [35].

Considering GSH, it has been reported that the depletion in this endogenous antioxidant is often associated with increases in reactive oxygen species and lipid peroxidation, which represent major factors for the pathogenesis of cytotoxicity induced by many mycotoxins [39,40]. In case of PAT, GSH depletion could be attributed to the capability of PAT, as an electrophilic chemical, to react with cellular electrophilic compounds containing sulfhydryl groups such as GSH, as well as thiol-containing proteins [41,42]. In the present study, obtained finding of reduced level of GSH and increased concentration of MDA in the livers of PAT-treated mice could provide an additional evidence of implication of declined hepatic content of GSH in the mechanism of hepatotoxicity following exposure to PAT.

In addition to reduced liver content of GSH, finding of decreased hepatic activity of the antioxidant enzymes including SOD, CAT, GST, GSH-Px and GSH-Rd following exposure to PAT could also provide evidence of incidence of oxidative stress and hence hepatotoxicity. In fact, the antioxidant device can protect the biological systems such as hepatocytes from toxicity of free radical and reactive oxygen species by several cellular defense mechanism pathways. The SOD represents the first line for protection against reactive oxygen species by catalyzing the conversion of the superoxide anion ($O_2^{\cdot -}$) to the hydrogen peroxide (H_2O_2) which is further decomposed by CAT into H_2O and O_2 [43]. Also, H_2O_2 can be eliminated by a reduction reaction which is catalyzed by GSH-Px using GSH as reducing substrate which is oxidized to glutathione disulfide (GSSG), $2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$ [44]. For restoring the normal cellular concentration of reduced GSH, the enzyme of GSH-Rd catalyzes the reduction of GSSG to the GSH via NADPH-dependent mechanism, $GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$.

Hepatocellular membrane damage is typically reflected by increased serum aminotransferases activity, particularly ALT. In the present study, PAT administration in mice caused elevation in the serum activities of ALT and AST. This result confirmed that PAT is a hepatotoxic mycotoxin and has the ability to produce hepatocyte injury. The mechanism of this liver injury seemed to be due to PAT-increased lipid peroxidation product and reduced both GSH and antioxidant enzymes in the liver of treated mice. Hepatotoxic effect of PAT was further confirmed by obtained finding of lowered serum concentrations of total proteins and albumin, which reflects inhibition of liver synthetic function. Thus, injection of PAT in mice could induce oxidative stress which resulted in hepatic cell injury and dysfunction. Of previous studies, PAT was found to depress synthesis of proteins in culture of liver cancer tissue [45]. Also [46] observed damage in the DNA in rat liver slices exposed to PAT and suggested that PAT could induce genotoxicity and subsequently affects the functions of liver. Following repeated administration of PAT (0.2 mg/kg), [47] recorded time dependent hepatocellular damage as reflected by increased serum activity of transaminases. Also, PAT (1mg/kg) induced severe hepatotoxicity in male mice as indicated by markedly elevated serum ALT and AST activities [4]. In recent studies, both rats administered fresh apple juice contaminated with PAT and mice injected with a single dose of PAT (3.73 mg/kg) showed significant increase in serum activity of ALT and AST [10,48].

In the present study, induction of oxidative tissue damage in the livers of mice treated by PAT may reflect that this mycotoxin could induce genotoxicity. It has been documented by several previous reports that PAT can induce DNA damages as manifested by DNA strand breaks [49], chromosome aberrations [37] and micronuclei formation [50]. Thus, PAT appeared to be cytotoxic and genotoxic mycotoxin, and may cause cell death. In this line, [48] observed degeneration and necrosis in hepatocytes of rats received apple juice contaminated by PAT. On

the other hand, there are several previous studies which provided evidence exhibits the implication of apoptosis in the PAT-induced toxicity [8,49,51]. However, apoptosis is a cellular program which regulated cell death and plays dual role in biological operations, as physiologic or pathologic process. It has been known that both p53 and caspase-3 acts as a pro-apoptotic proteins which play an important role in cell cycle control and apoptosis, and its enhancement is often associated with the activation of apoptosis process [52,53]. On the other hand, the ratio of Bax to Bcl-2 determines the induction or the inhibition of apoptosis, since Bax is a pro-apoptotic factor, while Bcl-2 is an anti-apoptotic factor [54,55]. In this context, current study showed that administration of PAT in mice enhanced the apoptotic activity in the liver. Obtained results showed increased levels of pro-apoptotic markers p53, Bax and caspase-3 accompanied with decreased anti-apoptotic marker Bcl-2 in the liver of male mice treated with PAT. This finding closely resemble to the results documented by several previous studies [8,9,10,49,51]. Of these studies, [10] reported that treatment of mice with similar dose of PAT (3.75mg/kg) induced apoptosis as indicated by observed increase in p53, Bax, cytochrome C and caspase-3; and decrease in Bcl-2 expressions. These apoptotic markers changes led [9] to suggest that PAT-induced apoptosis could be mediated via mitochondrial pathway, and Bax is an essential factor in regulating this pathway. Deleterious changes in redox status and production of oxidative stress observed in the current study could also suggest incidence of mitochondrial damage which might be implicated in the apoptotic process induced by PAT.

Maintenance of normal cellular thiols levels could add support to the protection of hepatocytes against attack by free radicals and reactive intermediates of toxic chemicals. Thus, NAC, which contains sulfhydryl group, could represent a potential treatment option for different hepatic disorders resulted from production of free oxygen radicals inducing oxidative injury. The drug (NAC) itself is a powerful antioxidant and can act as a scavenger of free radicals. Furthermore, NAC could contribute to elevation of GSH biosynthesis since it represents a good precursor of L-cysteine [11]. For these reasons, present work was designed to determine the possible preventive impact of NAC against hepatotoxicity induced by the potent mycotoxin PAT. Interestingly, obtained results showed that treatment of male mice with NAC (200mg/kg) for 7 days before exposure to PAT led to decreased the hepatic content of MDA, accompanied with increased hepatic antioxidant parameters including GSH, SOD, CAT, GST, GSH-Px and GSH-Rd, when compared to PAT group. Modulation of hepatic levels of GSH and antioxidant enzymes by pre-treatment with NAC in the current study could be related to the antioxidant activity of NAC and possibility of its contribution to enhancement of GSH biosynthesis [56]. In this context, there are no detailed information on the beneficial effect of NAC on PAT toxicity in mice. However, present results could be explained in view of published data on the effect of NAC on non-PAT toxicity in animals. It has been reported that, NAC treatment was found to improve mitochondrial content of GSH and scavenged reactive oxygen species and reactive nitrogen species in the liver of mice treated with acetaminophen [57]. This was further supported by [14] who recorded improvement in GSH levels and mitochondrial function, and attenuation of necrotic areas and DNA damage in mice treated with NAC at 1.5 h after administration of a single overdose of acetaminophen. Moreover, oral administration of NAC significantly decreased the contents of MDA and hydroperoxides and increased the levels of GSH, SOD, CAT and GSH-Px in both liver and kidney of rats intoxicated with CCl_4 [15]. Also, [58] demonstrated the ability of NAC to ameliorate acrylamide-induced oxidative tissue damage in several organs including liver in rats, as reflected by decreased lipid peroxidation and enhanced GSH content in the investigated organs. Recently, administration of NAC in the pesticides carbosulfan-induced hepatic oxidative stress in rats was found to decrease the concentration of MDA and to increase the amount of GSH in the liver [59]. In one study on the mechanism of PAT genotoxicity, [8] showed that pretreatment of mice with NAC significantly attenuated PAT-induced DNA damage and lipid peroxidation via increasing cellular content of GSH in the hippocampus.

Decline in PAT-induced oxidative stress in mice by pretreatment with NAC in the current study provides evidence that NAC is a potential

hepatoprotective agent which could reduce liver injury and hence ameliorate its function. In this concern, present finding showed that treatment of mice with NAC prior exposure to PAT significantly lowered the elevated serum activities of ALT and AST, as compared to group treated with PAT alone. Moreover, the same treatment regimen of NAC significantly increased the concentrations of serum total proteins and albumin, as compared to results of mice treated with PAT alone. This finding implies that NAC, which improved the antioxidant capacity, tends to prevent hepatocellular injury induced by PAT and preserves the integrity of the plasma membranes leading ultimately to blocking of the leakage of cellular transaminases enzymes and improvement of the synthetic function of the liver. Available data in this regard demonstrated the ability of NAC to decrease the elevated plasma activities of hepatic marker enzymes such as AST and ALP in rats intoxicated with either CCl_4 or acetaminophen [15,14]. Additionally, [58] reported that, administration of NAC significantly decreased the high serum levels of both ALT and AST, as well as, LDH in rats treated with acrylamide. Recently, [16] recorded dose dependent decline in the serum activities of both ALT and AST after long-term administration of NAC in CCl_4-treated rats.

In the current study, pretreatment with NAC was effective against PAT-induced apoptosis. Obtained results showed decreases in the pro-apoptotic markers (p53, Bax and caspase-3) and increase in anti-apoptotic marker (Bcl-2) in mice treated with NAC prior administration of PAT, as compared to the group treated with PAT alone. In this line, [58] observed that NAC treatment inhibited leukocyte apoptosis produced by administration of acrylamide in rats. Also, *in vitro* studies, NAC was found to regulate apoptosis in melanoma cells [60]. It seems that, the protective impact of NAC against PAT-induced apoptosis might be explained on basis of its antioxidant activity and its ability to improve the cellular levels of GSH and other antioxidant parameters. In conclusion, NAC appeared to be effective in the protection against PAT hepatotoxicity, so it could be considered as a hepatoprotective agent.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Cite this article as:
Mamdouh R. F. El-Sawi, Faried A. E. Hemieda, Sameh Shabana, Rasha G.S. Khmalj. Role of N-acetyl-L-cysteine in the Prevention of Hepatotoxicity Induced by Patulin in Male Mice. J Pharm Chem Biol Sci 2017; 6(1):1-10.