

Immunomodulatory Effect of Irradiated β -glucan in Diethylnitrosamine Induced Renal Toxicity

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β -GLUCANS are one of the most abundant forms of polysaccharides known as biological response modifiers which influence host's biological response and stimulate immune system. Accordingly, this study is carried out to evaluate the effect of gamma irradiated β -glucan ($I\beta$ -glucan) extracted from mushroom in modulating diethylnitrosamine (DEN)-induced immune response and mitochondrial dysfunction in renal toxicity. Rats were divided into 4 groups. Group I: control group, group II: animals received the DEN (20mg/kg) for 6 weeks, group III: was gavaged $I\beta$ -glucan (65mg/kg b. wt.) for 6 weeks, group IV: received as group II and then treated as group III. Exposure to DEN induced a change in the levels of cytokines (IFN- γ , TNF- α , IL-6) and nephrotoxicity, which was proved by significant elevated levels of creatinine and urea along with upregulation of mRNA cytochrome P450 (2E1) and downregulation in mitochondrial enzymes complex I and II. Treatment with $I\beta$ -glucans post DEN-exposure significantly improved the disturbances in all the ++ tested parameters. It could be concluded that $I\beta$ -glucan treatment exerts a potential biological effect on both mitochondrial and immune system dysfunction caused by the DEN- induced renal toxicity in rats. It is considered for further trials.

Keywords: $I\beta$ -glucan, DEN, Cytokines, Cytochrome P450 (2E1), Mitochondrial complexes.

Introduction

One of the naturally occurring polysaccharides is β -glucans (glucose polymers) and it is produced by a variety of plants, such as oat, barley, and mushroom. β -glucan is a strong immune stimulant and antagonist to both benign and malignant tumors. Also, it diminished the levels of cholesterol and triglycerides and normalizes blood sugar level (Sima et al., 2018). β -glucan could modify the biological response that can regulate the immune system by stimulating phagocytosis which is followed by the production of pro and anti-inflammatory cytokines (Vetvicka, 2011; Silveira et al., 2014; Ruthes et al., 2013 and Smeekens et al., 2015). Moreover, β -glucan is a non-toxic to the cells of the host organism by empowering the defense mechanisms of the host against disease instead of attacking the infectious agent leading to lack of any toxic or adverse effects (Zeković et al., 2005 and Sener et al., 2005). Also, beta-glucans can bind directly to specific receptors of immune cells (Vos et al., 2007).

Because of its polymeric structure, β -glucan has antioxidant and free radical scavenger properties, which is the most important mechanism proposed for the protective effects of β -glucan (Jaehrig et al., 2007). In this investigation, β -glucan was exposed to 50kGy to enhance the potential of its solubility and viscosity without changes in the functional group status as per El-Sonbaty et al. (2013), who found that both non-irradiated and irradiated β -glucan ($I\beta$ -glucan) had a similar pattern of FTIR spectra. In addition, ionizing radiation leads to the degradation of polysaccharides such as starch, cellulose, and pectin by the cleavage of the glycosidic bonds (Cho et al., 2003).

One of the imperative groups of carcinogens is nitroso compounds which frequently present in human environment and food preservation. Nitroso compounds could be converted to nitrosamine due to the effect of heat and gastric acid, also produced from metabolism of some drugs (Verna et al., 1996). Under a certain condition such as acidic pH of the stomach,

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nitrosamine is formed endogenously from nitrate (Jakszyn & Gonzalez, 2006). It was reported that diethylnitrosamine (DEN) causes oxidative stress during the metabolism that prompts cytotoxicity, mutagenicity and carcinogenicity (Pradeep et al., 2007 and Farombi et al., 2009). By the action cytochrome p_{450} enzymes, DEN activated metabolically to produce reactive electrophiles that increase the level of oxidative stress and cell injury (Ezz et al., 2017). The cell injury induced by DEN was achieved through its metabolized end product (ethyl radical), which induces DNA damage that plays a role in carcinogenesis (Bansal et al., 2000).

The mitochondrial respiratory enzyme activities (electron transport chain) comprise 5 complexes: NADH-ubiquinone reductase (complex I), succinate-Q reductase (complex II), cytochrome c oxidoreductase (complex III), cytochrome c oxidase (complex IV) and ATP synthase (complex V). The protons are moving into the inter membrane space and combine with substrates of the Complexes I, III and IV, creating the gradient that drives ATP synthase (complex V) (Fulda et al., 2010). Cellular energy was generated by the process of oxidative phosphorylation (OXPHOS) in the mitochondrial cell as adenosine triphosphate (ATP), which contains hundreds of them (Andersson et al., 1998). Complex I is the largest of the five enzyme super complexes in the mitochondrial electron transport chain. In spite of the fact that complex I plays an initial step of the oxidative phosphorylation pathway, it is not understood partly because of the number of its sub-units and their potential collaborations (Perfeito et al., 2012).

The more important aspects of mitochondrial OXPHOS for disease pathogenesis are: (i) energy production, (ii) generation of reactive oxygen species (ROS). (ii) Modulation of programmed cell death (apoptosis). OXPHOS proteins incorporate the electron transport chain (ETC) components; The ETC oxidizes hydrogen derived from the oxidation of organic acids for example, pyruvate and fatty acids with atomic oxygen to generate water (Shoffner & Wallace, 1995). Hence, the main objective of this study is to evaluate the immunomodulatory effect of the irradiated β -glucan on DEN-induced renal impairment.

Materials and Methods

Chemical

Diethylnitrosamine was purchased from Sigma Aldrich's. Louis, Mo. USA.

Extraction of β -glucan

β -glucan was extracted from edible mushroom *Agaricus bisporus* fungi according to the method published by Hunter et al. (2002).

β -D-glucan irradiation

β -glucan powder was exposed to a single dose of γ -radiation (50 KGy) at room temperature ($25 \pm 2^\circ\text{C}$) performed by Co^{60} gamma cell-40 at a dose rate of 10 k Gy/hour at the National Center for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Cairo, Egypt. The irradiated β -glucan sample was stored at 4°C (El-Sonbaty et al., 2013).

Experimental animals

The animals were housed in plastic cages, kept under normal temperature, pressure, humidity, good ventilation and illumination conditions. Rats were supplied with water and standard granular chow and *libitum* and handled according to the rules and regulations of the National Centre of Radiation Research and Technology (NCRRT) Committee for Experimental Animals.

Experimental design

40 Male Swiss albino rats were categorized into four main groups as follows: Group I: normal control. Group II: rats received DEN (20mg/kg b.wt.) five times per week for 6 weeks orally. Group III: animals were given β -glucan (65mg/kg b.wt. daily) for 6 weeks. Group IV: animals received the same treatment as group II and then treated with β -glucan as group III.

Blood and tissue sampling

All animals were anesthetized with ether after 24h of the last treatment. Blood samples were withdrawn by heart puncture, using heparinized syringes. Blood was centrifuged and the separated plasma was used for the investigations. Kidney was excised and frozen in liquid nitrogen and stored at -80°C until being used.

Determination of urea and creatinine levels in serum

Levels of urea and creatinine were determined colorimetrically using commercially available

kits (Bioclin, Santacoloma, Spain).

Determination of cytokines levels tumor necrosis factor- α (TNF- α), interleukin-6 levels (IL-6) and interferon-gamma (INF- γ)

Plasma was used for the determination of TNF- α , IL-6 and INF- γ using ELISA kits for rat (Glory Science Co., Ltd., USA). The measurements were performed according to the catalog instruction guidelines.

Detection of mitochondrial enzymes in renal tissue homogenate

Isolation of mitochondria

kidney samples were homogenized and minced in ice-cold extraction buffer (250mM sucrose, 20mM Hepes, 10mM KCl, 1.5mM MgCl₂, 1mM EDTA, 1mM EGTA, 1mM DTT and 0.1 mM phenylmethylsulphonyl fluoride, pH 7.4) in the presence of the protease inhibitor cocktail. Following a gentle homogenization with a Teflon pestle motorized with an electronic stirrer, homogenates were centrifuged at 800g for 10min at 4°C to pellet the nuclei and cell debris. The supernatants were then spun twice at 16000g for 20min at 4°C to pellet the mitochondria, and the final supernatants were collected as nuclei-free, mitochondria-free cytosolic protein fractions (Sui et al., 2004).

RNA extraction and cDNA synthesis

To investigate the changes in mRNA expressions of CYP_{450 2E1}, complex I (NADH-ubiquinone reductase), and complex II (succinate-ubiquinone oxidoreductase), total RNA was isolated from 100mg kidney using TRIzol reagent

(Life Technologies, USA) in accordance to the manufacturer's instructions. RNA integrity was confirmed by 1% agarose gel electrophoresis and stained with ethidium bromide. First strand complementary DNA (cDNA) synthesis was performed by reverse transcriptase (Invitrogen) according to the manufacturer's protocol using 1µg of total RNA as the template.

Quantitative real time PCR

qRT-PCR was performed using an optical 96-well plate with an ABI PRISM 7500 fast sequence detection system (Applied Biosystems, Carlsbad, California) and universal thermal cycling conditions (95°C for 10min, 40 cycles of 95°C for 15sec and 60°C for 60sec). Each 10µl reaction contained 5µl SYBR Green Master Mix (Applied Biosystems), 0.3µl gene specific forward and reverse primers (10µM), 2.5µl cDNA and 1.9µl nuclease-free water. The sequences of PCR primer pairs used for each gene are shown in Table 1.

Gel electrophoresis

Ten µl of PCR product was analyzed on 2% agarose gel with ethidium bromide staining and the product was visualized on ultraviolet transilluminator, then gel documentation was performed. PCR products were semi-quantified using a gel documentation system (Bio Doc Analyze) supplied by Biometra, Germany. The relative expression of the studied genes was calculated using the comparative threshold cycle method. All values were normalized to the GAPDH genes (Kushnareva et al., 2002).

TABLE 1. Primer sequences used for RT-PCR .

Gene	Primer sequence	GenBank accession No.
Cytochrom P ₄₅₀ (E1)	F: 5'-ATGGATGCTGTGGTGCATGA-3' R: 5'CTCTGGTTGCTTCATGGGGT-3'	>NM_031543.1
NADH: ubiquinone oxidoreductase (Complex I)	F: 5'- CACTGGTGGATTGTCCCTCC -3' R: 5'- AGAAGCTTGTGTGCATCCGA -3'	>NM_001106426.1
Succinate Dehydrogenase (Complex II)	F: 5'- GCCATGAACATCAACGGAGG-3' R: 5'- GGTCCCTCGATGGATTGCAGA- 3'	>NM_001100539.1
β-actin	F: 5'TGTTGTCCCTGTATGCCTCT-3' R: 5'TAATGTCACGCACGATTTC-3'	>NM_031144.3

Determination of DNA fragmentation

For determination of genomic DNA fragmentation, rat kidney was rapidly removed, washed of blood, frozen in liquid nitrogen, and ground to powder. The powdered tissue was transferred to a 50-ml centrifuge tube with extraction buffer (10mmol/L tris-HCl [pH 8.0], 0.1mol/L EDTA [pH 8.0] and 0.5% SDS, was first incubated for 1h at room temperature and then digested in the same buffer with 200µg/ml proteinase K (Sigma) at 50°C overnight. An equal volume of phenol equilibrated with 1mol/L tris buffer (pH 8.0) was then added, and the tube was placed on a roller apparatus for 1 hour. After the two phases were separated by centrifugation at 5000g for 30min at room temperature, the viscous aqueous phase was transferred to a clean 50-ml tube, and the extraction was repeated with an equal volume of phenol/chloroform. After the second extraction, the aqueous phase was transferred to a new 50-ml tube and the DNA precipitated by addition of 0.1 vol 3mol/L sodium acetate and 2 vol 100% ethanol. DNA precipitate was collected by centrifugation at 5000g for 20min at room temperature, rinsed with 70% ethanol, and finally resuspended in 0.5ml extraction buffer in a 1.5-ml micro centrifuge tube until dissolved. The concentration of DNA in each sample was measured using spectrophotometry (260nm). To detect DNA fragmentation, 10µg of each DNA sample was electrophoretically fractionated on 1.5% agarose gel with 0.5µg/ml ethidium bromide. The DNA in the gel was visualized and photographed under UV light (Okamura et al., 2000).

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by post-hoc analysis using SPSS. The data were expressed as mean ± standard error (SE). Differences were considered

statistically significant at $P < 0.05$.

Results

Effect of $I\beta$ -glucan on DEN induced inflammation

In the present study, plasma pro-inflammatory cytokines (IFN- γ , IL-6 and TNF- α) levels were increased significantly ($P < 0.05$) in the DEN treated rats (20mg/kg b. wt.) compared to both the control and irradiated β -glucan group, as shown in Fig 1. However, administration of $I\beta$ -glucan attenuates toxicity effect of DEN in IFN- γ , IL6 and TNF α level in (DEN+ $I\beta$ -glucan) group as compared to DEN group; $I\beta$ -glucan alone did not change those cytokine levels significantly.

Effect of $I\beta$ -glucan on DEN induced alteration in mitochondrial respiratory enzymes

As deduced from Fig. 2, DEN induced a significant reduction in mitochondria enzymes gene transcripts namely complex I and II concomitant with a significant elevation in mRNA of cytochrome p_{450} gene expression compared to both the control and irradiated β -glucan group. While the administration of $I\beta$ -glucan alone or post DEN treatment showed a significant elevation in mitochondrial complex's (I, II) gene expressions compared to DEN group, but the level of cytochrome p_{450} gene transcript significantly overexpressed in respect to DEN group.

Effect of $I\beta$ -glucan on DEN induced DNA fragmentation

Agarose gel electrophoresis in Fig. 3 shows DNA fragmentation. Lane1: DNA marker with 100bp (M). Lane 4 shows apoptotic strand breaks/streaking DNA in DEN group, while lane 2, 3 and 5 show intact undamaged DNA (control, glucan and DEN+ $I\beta$ -glucan) respectively. $I\beta$ -glucan indeed reduced DEN induced DNA fragmentation.

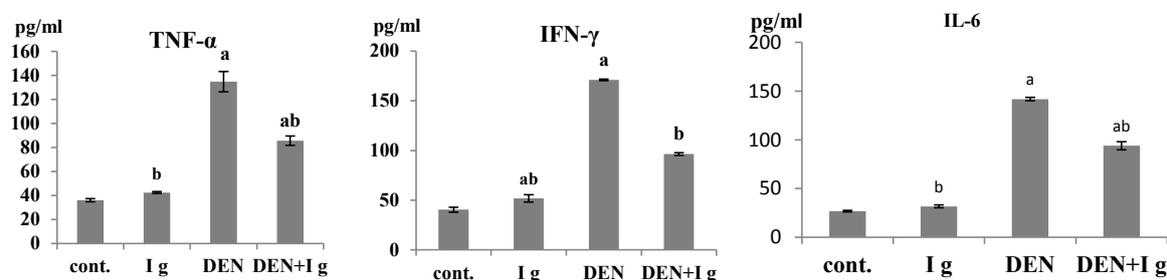


Fig. 1. Effect of $I\beta$ -glucan on plasma inflammatory cytokines (IFN- γ , IL6 and TNF- α), a= significantly different from control group, b= significantly different from DEN group. Control (Cont.) group; $I\beta$ -glucan administrated group (I g); DEN administrated group; group administrated with DEN and $I\beta$ -glucan (DEN+ I g).

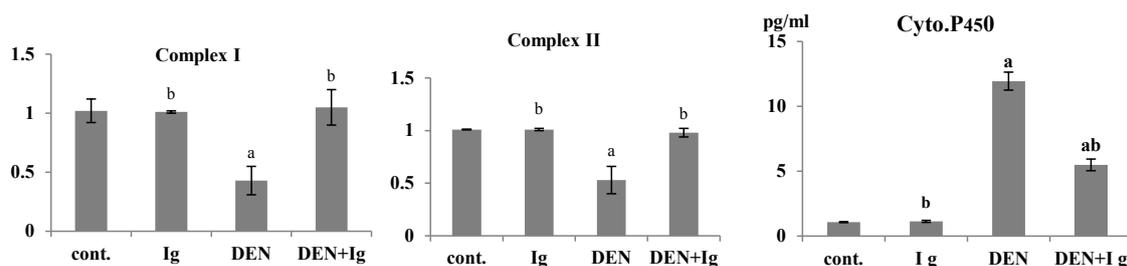


Fig. 2. Effect of Iβ-glucan on mitochondrial enzymes, a= significantly different from control group, b= significantly different from DEN group. Control (Cont.) group; Iβ-glucan administrated group (Ig); DEN administrated group; group administrated with DEN and Iβ-glucan (DEN+Ig)

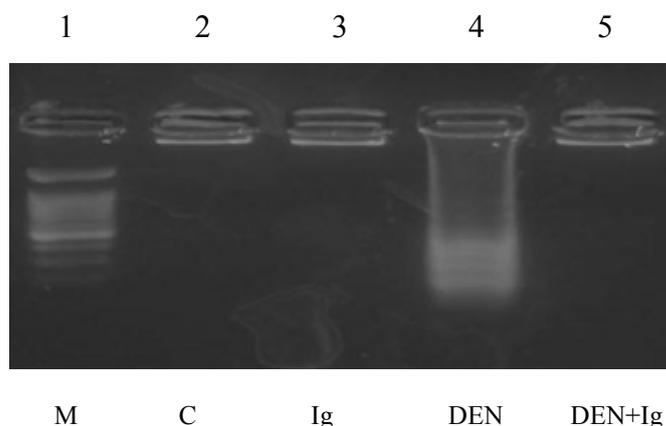


Fig. 3. Effect of Iβ-glucan on DNA fragmentation.

Effect of Iβ-glucan on DEN- induced kidney dysfunction

Figure 4 shows the status of urea and creatinine levels in the control and all groups. DEN induced a renal toxicity as shown in a 2 fold increase in the urea and creatinine levels as compared to control group. This increase of urea and creatinine levels due to DEN challenge was significantly decreased on post treatment with Iβ-glucan for 4 weeks.

Discussion

In the current study, the proinflammatory cytokines; IFN-γ, TNF-α, IL-6 revealed high levels in the DEN-administered group, which coincide with Horras et al. (2011). Toxicant generated inflammatory mediators, which stimulate the migration, infiltration of leukocytes and aggravates the primary injury. On the other hand, the oral administration of Iβ-glucan alleviates the alteration in plasma levels of IFN-γ, TNF-α and IL-6. It was found that Iβ-glucan enhanced the immuno- modulators, which might be may be ascribed to the effect of its constituents

that are able to scavenge free radicals (Azab & El-Dawi, 2005; Silva et al., 2015 and Silva et al., 2017). Additionally, Pillai et al. (2013) have demonstrated that β-glucan promote the repair of DNA of human lymphocytes and restored the TNF-α generation (Gao et al., 2003).

The major source of ROS and oxidative damage is the mitochondria (Fatemeh & Jalal, 2013). The generation ROS is associated with several procedures such as autophagy, differentiation, metabolic adaption and immune cell activation (Sena & Chandel, 2012). Jin et al. (2014) revealed that the basic neonatal genetic program; mitochondrial complex I is essential for the postnatal metabolic adaptation to counteract inflammation. In the current investigation, the dysfunction in the expression of respiratory chain enzymes complexes; complex I and II was observed in DEN treated group which might increase the levels of ROS. These respiratory deficiencies could be attributed to the impairment of metabolic pathways upstream to the oxidative phosphorylation, such as the different membrane transport systems.

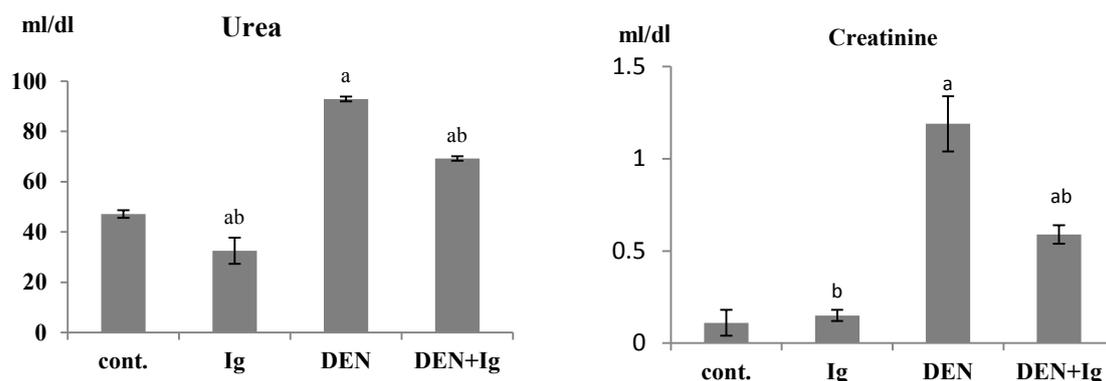


Fig. 4. Effect of I β -glucan on plasma urea and creatinine; a= significantly different from control group, b= significantly different from DEN group [Control: (Cont.) group, I β -glucan administrated group: (Ig), DEN administrated group: group administrated with DEN and I β -glucan (DEN+ Ig)].

Moreover, it was recorded that the administration of DEN induces increment in the expression of CYP2E₁, mitochondrial which prompts ROS production, oxidative stress, and mitochondrial DNA damage that leads to cell damage³⁴. This is in agreement with the results of the current study which may be attributed to the contribution of CYP2E1 in the DEN metabolism (Bansal et al., 2010). The activation of DEN by cytochrome P₄₅₀ enzymes family generates free radicals (Briede et al., 2004). In the current study, administration of I β -glucan significantly suppress the over expression of CYP2E1 which could be supported by the contribution of receptors on the cell membrane that activate signaling pathways and transcriptional factors (Angeli et al., 2006; Hashimoto et al., 2002 and Okamoto et al., 2004). Therefore, the protective effect of β -glucan could be explained by its inhibitory effect on the CYP2E1.

As a consequence of DEN- generated ROS, the present study found that DEN administration significantly induced a renal injury which was evident by the increased concentration of markers related to the kidney function such as creatinine and urea. These findings are in agreement with the studies of Atakisi et al. (2013) and Pashmforoosh et al. (2015). The sharp increase in urea and creatinine levels have also been ascribed to the highly reactive free radicals produced by DEN, which initiates lipid peroxidation of the cell and endoplasmic reticulum membranes (Khan et al., 2001 and Vitaglione et al., 2004).

However, the treatment of I β -glucan markedly

diminished the levels of urea and creatinine as shown in the results of the present study. The nephronprotective effects of I β -glucan may be attributed to its immunomodulatory effects. Therefore, the protective effect of I β -glucan against DEN toxicity could be attributed to their antioxidant and anti-inflammatory efficacy, especially β -glucan, which had low molecular weight, high solubility and low viscosity (Byun et al., 2016).

Conclusion

In conclusion, I β glucan, seemed to attenuate the cytotoxic effect DEN by its immunomodulatory effect associated with its free radical scavenging activities. Moreover, the I β -glucan prevented mitochondrial disruption. Further studies are underway to elucidate the molecular mechanisms involved in I β -glucan protection .

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التأثير المناعي لجزيء البيتاجلوكان المشع ضد السمية الكلوية المستحثة بداي-إثيل نيتروزامين

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البيتاجلوكان هي واحدة من السكريات الأكثر وفرة والمعروفة بمعدلات المناعة التي تؤثر على الاستجابة البيولوجية وتحفز الجهاز المناعي. ولذلك تهدف هذه الدراسة إلى تقييم تأثير البيتاجلوكان المشع المستخلص من فطر عيش الغراب ضد التأثير المناعي و الخلل الميتوكونديري في السمية الكلوية المستحثة بداي إثيل نيتروزامين. تم تقسيم الجرذان إلى 4 مجموعات، المجموعة الأولى: المجموعة الضابطة، المجموعة الثانية: تلقت الحيوانات داي إثيل نيتروزامين 20مج/كجم من وزن الجسم لمدة 6 أسابيع، المجموعة الثالثة: تلقت الحيوانات البيتاجلوكان 65مج/كجم من وزن الجسم لمدة 6 أسابيع، المجموعة الرابعة: تم معالجتها مثل المجموعة الثانية ثم عولجت مثل المجموعة الثالثة. لقد تسبب التعرض لداي إثيل نيتروزامين في إحداث تغيير في مستويات السيتوكينات IL6 ، TNF-α ، IFN-γ و إحداث السمية الكلوية، والذي ثبت حدوثه بارتفاع مستويات الكرياتينين واليوريا إلى جانب زيادة في التعبير الجيني لإنزيم السيتروكروم P450 (2E1) ونقص في التعبير الجيني لإنزيمات الميتوكوندريا المعقدة الأول والثاني. العلاج بالبيتاجلوكان المشع مع التعرض لداي إثيل نيتروزامين أدى إلى تحسن ملحوظ في جميع الدلالات المختبرة. يمكن الاستنتاج أن العلاج بالبيتاجلوكان المشع له تأثيراً بيولوجياً محتملاً على كل من خلل الميتوكوندريا وجهاز المناعة الناجمة عن السمية الكلوية المستحثة بداي-إثيل نيتروزامين في الجرذان. مع الأخذ في الاعتبار بمزيد من التجارب.