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Full Length Article

Wheat germ and vitamin E decrease BAX/BCL-2 ratio in rat kidney treated with gentamicin

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ABSTRACT

The intracellular accumulation of gentamicin acts on mitochondria directly (by increasing oxidative stress) and indirectly (by increasing the proapoptotic BAX gene expression levels) and thus activates intrinsic pathway of apoptosis. This study intended to investigate the probable prophylactic effect of wheat germ as a nephro-protective agent in relation to vitamin E as a well-known natural antioxidant on a nephrotoxic model induced by gentamicin. Forty male Sprague Dawley rats were divided randomly into four groups; of 10 rats each: control group, gentamicin group (120 mg/kg/day i.p. for 15 days), vitamin E group (200 mg/kg orally for 45 days) and wheat germ group (20% of the diet for 45 days). Gentamicin injection was started on the 30th day for both groups (3) and (4). It was noticed that wheat germ significantly decreased BAX/BCL-2 ratio. The significant decrement in MDA level and the significant increment in total antioxidant capacity (TAC) level and catalase (CAT) activity of wheat germ treated rats reflecting its antioxidant activity. Additionally, the wheat germ succeeded in improvement of the kidney function of rats of this group which was manifested by amelioration of deteriorated serum creatinine, urea, sodium and potassium levels. In conclusion, the efficacy of wheat germ as anti-apoptotic and antioxidant was higher than that of vitamin E and it effectively protected against the direct and indirect toxic effects of gentamicin on kidney through regulation of BAX-BCL-2 gene expressions and further inhibition of mitochondrial cytochrome *c* translocation into cytosol.

1. Introduction

Nephrotoxicity is defined as a poisonous effect of toxic chemicals and medication on the kidneys. The nephrotoxic consequence of most drugs has been more profound in patients already suffering from renal impairment. Numerous therapeutic agents, which are used in clinical practice, have been stated to produce functional impairment and toxic injuries to the kidney. The cause for this is that the kidney is the chief organ of excretion and is exposed to huge amounts of parent and active metabolites of drugs (Ekor et al., 2010).

Gentamicin is an aminoglycoside broad spectrum antibiotic used against pathogenic gram positive and negative bacteria (Taha, 1993). Administration of gentamicin induced impairment of renal function through the generation of oxygen free radicals (Heibashy et al., 2009). Oxidative stress may occur as a result of either increased oxygen free radical liberation and/or decreased antioxidant enzyme system that protects the cell against cytotoxic free radicals (Khan et al., 2009). The search of protecting against gentamicin nephrotoxicity has concerned much attention and effort during the last decade. This protection may be achieved through decreasing aminoglycosides accumulation by the kidneys, or decreasing the lysosomal phospholipidosis induced by the cell-associated aminoglycosides and the use of antioxidants (Mingeot-Leclercq and Tulkens, 1999).

In the past few years, considerable concern has been focused on the role of naturally occurring dietary constituents for the control and management of various ailments (Connor, 2000). Medicinal plants are effective free radical scavengers. Several studies reported that medicinal plants are less toxic than synthetic antioxidants, such as butylated hydroxyanisol and butylated hydroxytoluene, which are assumed to be carcinogenic and cause cell damage (Ratnam et al., 2006). Natural antioxidants are classified as secondary plant metabolites, such as polyphenols (phenolic acids, flavonoids) and terpenoids (carotenoids). The consumption of nutrients that contain these compounds in large quantities seems to play an important role in prophylaxis against many diseases (Nawirska-Olszanska et al., 2013).

Wheat (*Triticum aestivum L.*) is one of the most vital crops. It has

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been used as a chief constituent for manufacturing of various food products. Wheat germ is the by-product of flour-milling industry. It is a unique source of highly concentrated nutrients. Wheat germ oil is rich in polyunsaturated fatty acids such as oleic, linoleic and α-linolenic acids (Rizzello et al., 2010). Wheat germ is rich in bioactive ingredients such as antioxidants, including tocopherols, phenolics and carotenoids (Gelmez et al., 2009). It is rich source of tocopherols (Schwartz et al., 2008). Wheat antioxidant activities have been widely studied. Extracts rich in antioxidant have been obtained from wheat using several solvents including ethanol, methanol, water, an aqueous ethanol solution and an aqueous methanol solution (Schwartz et al., 2008).

Our work was designed to evaluate the anti-apoptotic and antioxidant activities of wheat germ in relation to vitamin E as a common natural antioxidant on a rat nephrotoxic model induced by gentamicin.

2. Materials and methods

2.1. Chemicals

Gentamicin-sulphate (Garamycin) was obtained from Schering-Plough (U.S.A). Vitamin E® capsules (400 mg) were obtained from Pharco Corporation, Amriya, Alexandria, Egypt. Wheat germ (Golden Green) was purchased from Bon Pharma Company for pharmaceuticals and chemicals, Cairo, Egypt. Malondialdehyde (MDA), catalase (CAT), total antioxidant capacity (TAC), sodium and potassium commercial kits were purchased from Biodiagnostic Company for research kits, Cairo, Egypt. Serum creatinine and urea commercial diagnostic kits were purchased from Spinreact “Girona, Spain” and Diamond diagnostic “London, UK” Companies, respectively. Other non-mentioned chemicals used in the present study were purchased from Sigma, USA.

2.2. Animals and treatments

Forty adult male Sprague Dawley rats, weighing 120–150 g at the beginning of the experiment were used in the present study. The animals were obtained from the Egyptian Organization for Biological Products and Vaccines. The rats were kept at room temperature and exposed to natural daily light–dark cycles. Rats were fed on balanced commercial rat diet with free access of food and water. All experimental procedures were conducted in agreement with the guide for the care and use of laboratory animals and in accordance with the local Animal Care and Use Committee.

One week after acclimatization, the rats were randomly divided into 4 experimental groups of ten rats each:

Group 1 (C-group):
Rats served as control and were given normal saline daily by intraperitoneal injection.

Group 2 (G-treated group):
Rats were administered normal saline by intraperitoneal injection for 30 days, and then followed by intraperitoneal injection of gentamicin for 15 days at a dose of 120 mg/kg/day (Somdaş et al., 2015).

Group 3 (Vit. E-treated group):
Rats were administered vitamin E orally for 45 days at a dose of 200 mg/kg/day (Mehany et al., 2013); the gentamicin “120 mg/kg/day” intraperitoneal injection was started on the 30th day till the end of the experiment.

Group 4 (WG-treated group):
Rats were received wheat germ orally for 45 days at a dose of 20% of the diet (Leenhardt et al., 2008); the gentamicin “120 mg/kg/day” intraperitoneal injection was started on the 30th day till the end of the experiment.

2.3. Sampling and biochemical analysis

2.3.1. Blood sampling

Twenty-four hours after the last dose of treatments, blood samples were collected from all rats via *retro*-orbital bleeding. Blood samples were left at room temperature for 20 min to clot. The clotted blood samples were centrifuged at 1000 × *g* for 15 min for serum separation. The obtained sera were kept at −20 °C till use.

2.3.2. Specimen collection

Kidneys were excised after dissection of the animals and then washed by physiological saline. The kidney samples were divided into two parts. The first part of the kidney (0.5 g) was suspended in 5 ml ice cold phosphate buffered saline (pH: 6.8) for homogenization (*Teflon Homogenizer, India*). The kidney tissue homogenate was then centrifuged at 20,000 × *g* for 10 min at 4 °C using high speed cooling centrifuge. The supernatants were kept on −20 °C till the time of determination of oxidative/ antioxidant parameters (Lin et al., 2010). The second part of the kidney was preserved at −80 °C for molecular investigation.

2.3.3. Detection of BAX and BCL-2 gene expression by real time-polymerase chain reaction (RT-PCR)

Total RNA was isolated from kidneys using Ribozol™ RNA Extraction Reagents with the code N580 (AMRESCO, LLC Corporate Headquarters, 28,600 Fountain Parkway, Solon, OH 44,139, USA) according to manufacturer's instruction. The concentration of RNA was measured using a UV spectrophotometer “Hitachi spectrophotometer, Model U-2000, Hitachi Ltd. Tokyo, Japan”.

2.3.3.1. c DNA synthesis. Five µg RNA was reverse transcribed using oligonucleotide (dT)18 primer (final concentration, 0.2 mM) and was denatured at 70 °C for 2 min. Denatured RNA was placed on ice and reverse transcription mixture containing 50 mM KCl, 50 mM Tris HCl (pH 8.3), 0.5 mM of deoxyribonucleotide triphosphate (dNTP), 3 mM MgCl2, 1 U/mL RNase inhibitor, and 200 units of moloney murine leukemia virus reverse transcriptase. The reaction tube was located at 42 °C for 1 h, followed by heating to 92 °C to stop the reaction.

2.3.3.2. Real-time quantitative polymerase chain reaction (RT-PCR). For real-time quantitative PCR, 5 µL of first-strand cDNA was used in a total volume of 25 µL, containing 12.5 µL 2x SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 200 ng of each primer as shown in Table 1. PCR reactions consisting of 95 °C for 10 min (1 cycle), 94 °C for 15 s, and 60 °C for 1 min (40 cycles), were done on step one plus real-time PCR system (Applied Biosystems). The data were analyzed with the ABI Prism 7500 sequence detection system software and quantified using the v1.7 Sequence Detection Software from PE Biosystems (*Foster City, CA*). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the beta-actin genes, all these steps were performed according the method described by Kenneth and Thomas (2001).

Table 1
Sequences of the primers used for amplification of mRNAs encoding BCL-2 and BAX by quantitative real-time PCR.

mRNA	Sequences (5′ → 3′)	Gene Accession No
BCL-2	Forward primer: 5′-CATGTGTGTGGAGAGCGTCAA-3′ Reverse primer: 5′-GCCGGTTCAGGTACTCAGTCA-3′	NM_016993
BAX	Forward primer: 5′-GGGACGAACTGGACAGTAACAT-3′ Reverse primer: 5′-GGAGTCTCACCCAACCACCCT-3′	NM_017059
β-actin	Forward primer: 5′-ATGAGCCCCAGCCTTCTCCAT-3′ Reverse primer: 5′-CCAGCCGAGCCACATCGCTC-3′	NM_007393

2.3.4. Oxidative/antioxidant parameters

Oxidant-antioxidant status in kidney tissue was evaluated by measuring the following parameters MDA, TAC and CAT in kidney tissue homogenate. MDA concentration was measured by method of Satoh (1978) which is depending on the reaction of thiobarbituric acid with malondialdehyde in acidic medium. The determination of TAC was achieved by the reaction of antioxidants in the sample with a defined quantity of exogenously provided hydrogen peroxide (H_2O_2) (Koracevic et al., 2001). Measurement of CAT activity is based on the reaction of catalase with a known amount of H_2O_2 according to the method described by Aebi (1984).

2.3.5. Kidney function biomarkers

The measurement of serum creatinine concentration is depending on the reaction of creatinine with sodium picrate forming a red complex in agreement with the method described by Henry et al. (1974). The determination of serum urea concentration is based on enzymatic hydrolysis of urea according to the method designated by Patton and Crouch (1977). The measurement of serum sodium concentration is dependent on the reaction of sodium ions with excess uranyl acetate and magnesium acetate agreeing with the method described by Trinder (1951). The measurement of serum potassium concentration is depending on the reaction of potassium ions with sodium tetraphenyl boron forming a colloidal solution which can be measured colorimetrically (Sunderman and Sunderman, 1958). All chemical reactions were measured by using Hitachi spectrophotometer, Model U -2000 (Hitachi Ltd. Tokyo, Japan).

3. Statistics

Statistical analysis for the results was carried out using *SPSS software, version 15 (Chicago, USA)*. One-way analysis of variance (ANOVA) followed by the *Tukey test* for post hoc comparisons were used. The results were presented as mean ± standard error of mean (SE) and the difference was considered significant at $p < 0.05$.

4. Results

4.1. Relative expression of BAX and BCL-2

Fig. 1. showed that measurement of kidney BAX and BCL-2 gene expression levels in rats of all tested groups revealed that gentamicin administration significantly increased BAX and decreased BCL-2 expression levels as compared to C group at $p < 0.05$. Interestingly wheat germ showed anti-apoptotic effect as it significantly decreased BAX and increased BCL-2 gene expression levels at $p < 0.05$ as compared to G-treated group and this influence was slightly similar to that of vitamin E. Wheat germ significantly ameliorated the altered BAX/BCL-2 ratio induced by gentamicin at $p < 0.05$ and this effect was similar to that of vitamin E.

Table 2
Changes in MDA level, TAC level and CAT activity in the various studied rat groups.

	MDA (nmol/g tissue)	TAC (mmol/L)	CAT (U/g tissue)
C-group	11.57 ± 0.202	2.27 ± 0.018	0.66 ± 0.022
G-treated group	13.80 ± 0.374[a]	1.98 ± 0.020[a]	0.26 ± 0.015[a]
Vit. E-treated group	11.29 ± 0.184[b]	2.14 ± 0.057[a,b]	0.55 ± 0.002[a,b]
WG-treated group	11.40 ± 0.245[b]	2.12 ± 0.024[a]	0.67 ± 0.001[b,c]

Values are represented as mean ± standard error of mean.
[a] $p < 0.05$ versus normal control group.
[b] $p < 0.05$ versus G-treated group.
[c] $p < 0.05$ versus Vit. E-treated group.

4.2. Oxidative/antioxidant markers

Table 2 revealed that wheat germ significantly decreased the elevated renal MDA level as compared to G-treated group. Gentamicin significantly decreased renal TAC level and CAT activity in comparison with C-group. Wheat germ could return both renal TAC level and CAT activity to their normal pattern when compared to control and G-treated groups at $p < 0.05$. The ability of wheat germ to keep MDA and TAC levels within normal is similar to that of vitamin E but this effect was higher concerning the CAT activity.

4.3. Kidney function biomarkers

Fig. 2. showed that wheat germ significantly ameliorated the elevated serum creatinine levels in comparison with G-treated group at $p < 0.05$. These effects were more obvious than that of vitamin E.

Fig. 3. showed that gentamicin significantly elevated serum urea levels as compared to C-group. Wheat germ administration significantly lowered this elevated level and became non-significant in comparison with C-group at $p < 0.05$. This effect was similar to that of vitamin E.

Fig. 4. showed that wheat germ significantly increased the serum sodium levels as compared to G-treated group and became non-

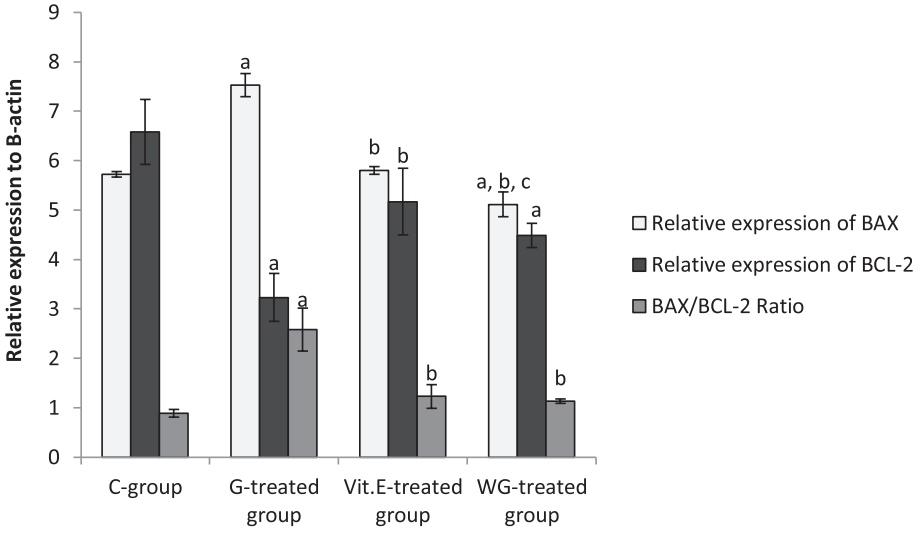

Fig. 1. Relative expression levels of BAX and BCL-2 and BAX/BCL-2 ratio in the various studied rat groups. Values are represented as mean ± standard error of mean. [a] $p < 0.05$ versus normal control group. [b] $p < 0.05$ versus G-treated group. [c] $p < 0.05$ versus Vit. E-treated group.

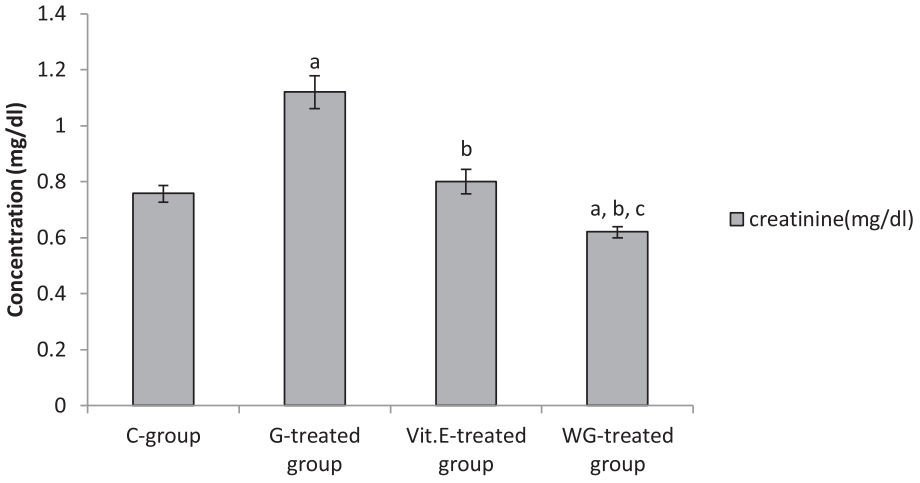

Fig. 2. Changes of serum creatinine concentration in the various studied rat groups. Values are represented as mean ± standard error of mean. [a]$p < 0.05$ versus normal control group. [b]$p < 0.05$ versus G-treated group. [c]$p < 0.05$ versus Vit. E-treated group.

significant as compared to that of the C-group at $p < 0.05$. The ameliorating effect of wheat germ is more pronounced than that of vitamin E. Gentamicin significantly increased the serum potassium levels as compared to C-group while wheat germ significantly decreased these high levels in comparison with G-treated group at $p < 0.05$. The ameliorating influence of wheat germ is more pronounced than that of vitamin E.

5. Discussion

Cytochrome *c* translocation into the cytosol is a key feature in gentamicin induced renal apoptosis as it activates caspase 9 which in turn enhances caspase 3 dependent apoptosis (Bratton et al., 2001). Intracellular accumulation of gentamicin triggers the translocation of cytochrome *c* through its direct and indirect action on mitochondria (Morales et al., 2010) and thus activates the intrinsic apoptotic pathway.

The interactions between three functionally and structurally distinct subgroups of the BCL-2 protein family, on the outer mitochondrial membrane, set apoptosis. These family members are BH3 (the BCL-2 homology 3) proteins which transmit signals to initiate apoptosis, the pro-survival cell guardians for instance BCL-2 itself and finally, the pro-apoptotic effector proteins BAX (BCL-2–associated X protein) and BAK (BCL-2 antagonist/killer) (Czabotar et al., 2014).

Cytotoxic stresses activate BH3-only proteins causing BAX and BAK stimulation. The altered permeability of the outer membrane of mitochondria as a result of oligomers formed by BAX and BAK leads to release of apoptogenic cytochrome *c* (Kluck et al., 1997). Cytochrome *c* binds apoptotic protease-activating factor 1 (APAF1), and causes its oligomerization and thus activates an initiator caspase 9. Caspase 9 cleaves and initiates executioner caspase 3 and 7, causing apoptosis. BCL-2 proteins inhibit the previous steps by binding BH3-only proteins and so inactivated BAX or BAK (Czabotar et al., 2014).

Gentamicin indirectly affects mitochondria via increasing BAX levels (Horibe et al., 2004) and that was confirmed by our results which revealed that gentamicin significantly increased BAX and decreased BCL-2 expression levels which indicate apoptosis. Pfannenstiel et al. (2009) declared that BCL-2 family members have been linked to aminoglycoside cytotoxicity.

The interaction of vitamin E function and oxidative stress-induced cell apoptosis and the signal transduction pathways underlying this are the matter of concern (Sen et al., 2000). Our results have shown that vitamin E significantly reduced the effects of gentamicin on BAX and BCL-2 expression levels and these results are in agreement with Almeida et al. (2000).

Pre-treatment with wheat germ successfully increased BCL-2 and

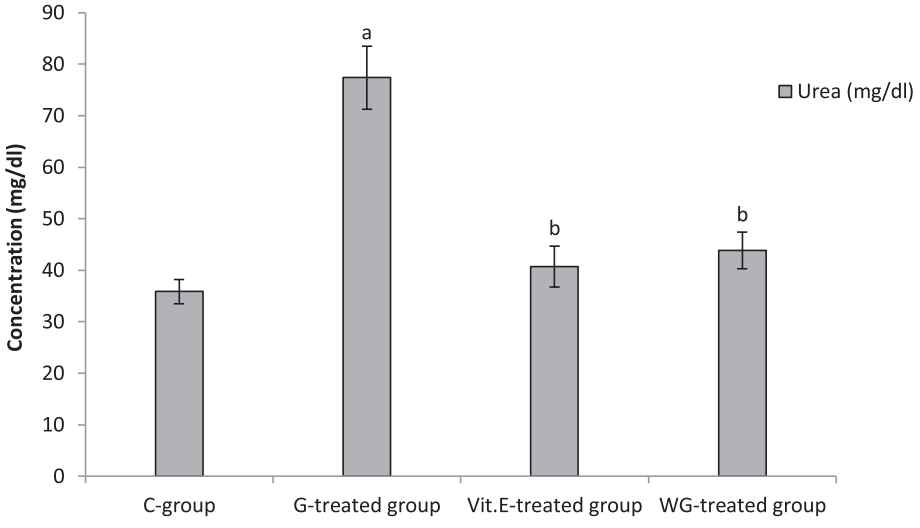

Fig. 3. Changes of serum urea concentration in the various studied rat groups. Values are represented as mean ± standard error of mean. [a]$p < 0.05$ versus normal control group. [b]$p < 0.05$ versus G-treated group. [c]$p < 0.05$ versus Vit. E-treated group.

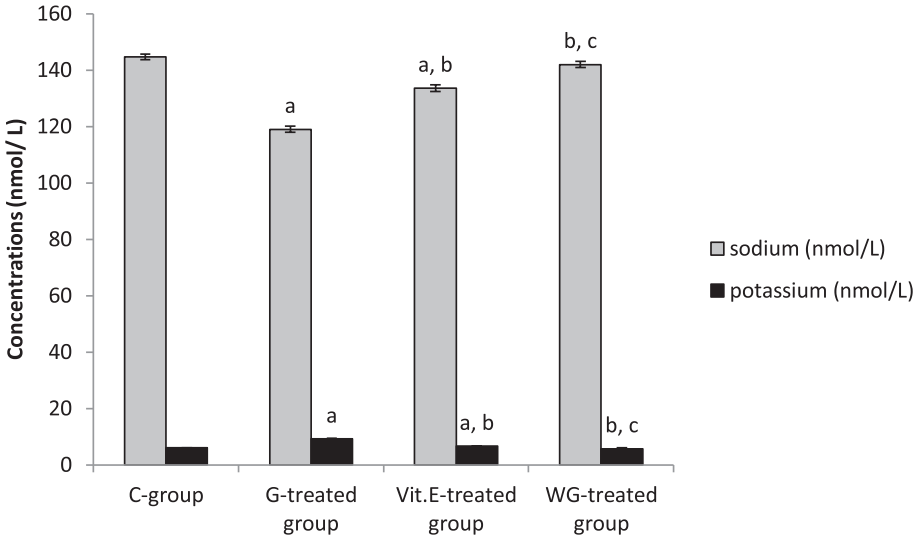

Fig. 4. Changes of serum sodium and potassium levels in the various studied rat groups. Values are represented as mean ± standard error of mean. [a]$p < 0.05$ versus normal control group. [b]$p < 0.05$ versus G-treated group. [c]$p < 0.05$ versus Vit. E-treated group.

decreased BAX gene expression levels and these results are in accordance with Mohamed and Ahmed (2014). The fate of cells to die or survive is subject to on the equilibrium between survival and apoptosis signaling (Banua et al., 2011).

In addition to its indirect action, gentamicin directly acts on the mitochondria and produces oxidative stress, which was manifested in this study by the high level of renal MDA and the decreased renal TAC level and CAT activity and these observations are in accordance with those obtained by Varzi et al. (2007). Previously, it has been revealed that free radicals are capable of inducing DNA strand breaks and promutagenic DNA adducts, such as 8-oxodeoxyguanosine (8-oxodG) and malondialdehyde-deoxyguanosine (M1G). This DNA damage leads to BH3-only protein activation with subsequent stimulation of BAX and cytochrome *c* translocation and initiation of mitochondrial pathway of apoptosis causing cell death (Anarkooli et al., 2008).

ROS induces cellular injury and necrosis via lipid peroxidation and protein modification (Edson and Terrell, 1999). ROS production in lysosomes seems to be a key pathogenic event for gentamicin toxicity leading to apoptosis. The generation of ROS caused by gentamicin may induce peroxidation of the lysosomal membranes and their permeability (Zdolsek et al., 1993).

The lysosomal content bears highly active proteases named cathepsins which are capable of producing cell death. Intracellular gentamicin accumulation enhances the generation of superoxide anion and hydrogen peroxide by mitochondria in the renal cortex. They activate nuclear factor kB, which has a key role in the inception of the inflammatory process. It induces the expression of proi-nflammatory cytokines (Markewitz et al., 1993).

These factors decrease the glomerular filtration rate (Nenad et al., 2008) and cause a direct tubular injury (Varzi et al., 2007) which was detected in our study by a marked rise in the serum creatinine, urea and potassium levels and the decreased serum sodium level. The alterations of kidney function biomarkers confirmed kidney injury.

Vitamin E is the chief endogenous antioxidant which reacts with ROS preventing free radical chain reactions to protect the membranes (Kadkhodaee et al., 2004). In this study, vitamin E significantly reduced MDA level and increased TAC level and CAT activity that are agreeing with the results reported by Liu et al. (2008) who declared that vitamin E is considered as a strong antioxidant. Moreover, vitamin E supplementation ameliorated the changes of serum levels of creatinine, urea, sodium and potassium observed in G-treated group and that was explained by Abdel-Naim et al. (1999) who reported that vitamin E may play an important role as a nephron-protective agent against gentamicin-induced renal impairment because of its lipophilic nature with minimum toxicity and potent antioxidant property.

Wheat germ effectively protects the mitochondrial membrane through its powerful antioxidant activity which was noticed in this study by the considerable decrease in the level of MDA and the high TAC level and CAT activity which are in accordance with those of Alessandri et al. (2006). Therefore, the previous alterations in kidney function indicators were ameliorated because of wheat germ antioxidant properties and these results coincide with the data reported by Saleh et al. (2010).

Our study revealed that the antioxidant properties of wheat germ are more potent than that of vitamin E. The improvements observed in the oxidant-antioxidant parameters of WG-treated group may be explained by the impact of mechanisms aiming the reduction or inhibition of free radicals that lead to the lipid peroxidation of cell membranes as reported by Karabacak et al. (2011) who declared that wheat germ antioxidant activities may arise from the individual and synergistic properties of antioxidant vitamins, phenolic and polyphenols compounds present in its structure.

Our study concluded that wheat germ has a reno-protective effect better than that of vitamin E and this action is thought to be not only through its direct action as an antioxidant agent, but also through its indirect anti-apoptotic action by decreasing BAX/BCL-2 ratio.

Conflict of interest

The authors affirm that they have no conflicts of interest concerning this article.

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