LABORATORY STUDY

The effects of N-acetyl-L-cysteine on the female reproductive performance and nephrotoxicity in rats

Mona A. M. Helal
Department of Zoology, Faculty of Women for Arts, Science and Education, Ain Shams University, Cairo, Egypt

ABSTRACT
This study was designed to investigate whether the treatment with the N-acetyl-L-cysteine prior to the administration of chemotherapy drug would prevent from nephrotoxicity and the loss of reproductive performance induced from chemotherapy treatment. Female rats were divided into five equal groups of six each: 1/control group; 2/rats i.p administered saline solution; 3/rats i.p administered holoxan (50 mg/kg b.wt); 4/rats i.p administered N-acetyl-L-cysteine (160 mg/kg b.wt); 5/rats i.p administered holoxan and N-acetyl-L-cysteine at the same doses. After medications, females rats were allowed to mate with males and the pregnant rats were sacrificed on day 18 of gestation. Premating treatment with holoxan showed reduction (p<0.05) in reproductive performance. Whereas administration of N-acetyl-L-cysteine prior to treatment with holoxan and then concurrently with it modulated significantly fertility index, progesterone level, decreasing postimplantation loss, resorbed fetuses and improved fetal growth. Additionally, holoxan elevated the renal nicotinamide dinucleotide phosphate (NADPH) oxidase activity, oxidative stress, renal functions and caused histological changes in renal tissue. N-Acetyl-L-cysteine treatment reduced (p<0.05) renal tissue NADPH oxidase, nitric oxide, malondialdehyde and improved super oxide dismutase (SOD) depletion, elevated levels phosphate, total protein and calcium as well as prevented renal histological damages. N-Acetyl-L-cysteine can confer protection against nitrosative and oxidative stresses in renal tissue induced by holoxan by suppressing NADPH oxidase activation, malondialdehyde, nitric oxide and restoring SOD activity which led to the reduction of reactive oxygen species production and subsequently might effectively improve the gonadal hormone disturbance and reproductive functions.

ARTICLE HISTORY
Received 12 August 2015
Revised 4 November 2015
Accepted 22 November 2015
Published online 1 February 2016

KEYWORDS
Female fertility; kidney; N-acetyl-L-cysteine; NADPH oxidase; progesterone

Introduction
Young patients treated with chemotherapy or radiotherapy may suffer from gonadal damage, permanent, ovarian failure and consequently a menopausal state. However, young female patients are still being poorly counseled with regard to the negative impact of treatment on their fertility for fertility preservation. It has been estimated that 10% of women become infertile early in life due to a premature critical loss of oocytes which caused an accelerated rate of loss of the oocyte pool through detrimental exposures (smoking, occupational exposures or chemotherapy). Cancer treatment with DNA-alkylating agents, such as cyclophosphamide is associated with the greatest risk of female infertility. Ifosfamide is a structural isomer of cyclophosphamide. Its use has gradually become widespread due to its apparent efficacy in the treatment of some cyclophosphamide-resistant tumors, and its lower bone marrow toxicity that has enabled the use of higher doses. Nevertheless, high therapeutic doses of ifosfamide are associated with sever nephrotoxicity. Renal toxicity is reported in 5% of ifosfamide-treated patients in the form of Fanconi syndrome. This syndrome is characterized by renal glucosuria, loss of electrolytes, bicarbonate and lactate, generalized hyperaminoaciduria and low-molecular-weight proteinuria. Several mechanisms for ifosfamide-induced nephrotoxicity have been reported including oxidative stress, depletion of glutathione and inhibition of endocytosis in renal tubular proximal cells. Ifosfamide (IF) is metabolized by hepatic CYP3A4 and 2B6. IF mustard, which is produced by ring hydroxylation pathway, is the pharmacological active metabolite, whereas chloroacetaldehyde, which is produced from chlorethyl side oxidation, is largely believed to be responsible for the nephrotoxic effects of IF. Many recent studies considered statins as the potential therapeutic agent for a targeted conservative treatment of female gonadal function, fertility endometriosis in humans and reduced mortality among women affected by endometrial/ovarian malignancies. Whereas Andrisani et al. found Astaxanthin (Asta),
a photo-protective red pigment of the carotenoid family, utilized to decrease male idiopathic infertility. Studies from the authors’ laboratory have demonstrated that insufficient local concentrations of glutathione may predispose tubular cells to damage by locally produced chloroacetaldehyde. However, GSH is present in much smaller amounts in the kidney, which may render this organ more sensitive to toxic damage. N-Acetyl-L-cysteine (NAC) has been widely used in the protection against the toxic effects produced by several chemicals because of its radical scavenger properties and because NAC is a precursor of glutathione, one of the most important intracellular defenses against oxidants. Reducing oxidative stress by the supplementation of antioxidants could potentially reduce reactive oxygen species (ROS) induced damage, thus maintaining the number and quality of oocytes and follicles in women. Also, NAC improves early embryo development and helps clomiphene citrate–resistant women with polycystic ovary syndrome to ovulate successfully. In animals and in human trials, NAC is protective against cisplatin-induced ototoxicity and nephrotoxicity.

Thus, the aim of the present study was to investigate whether the treatment with the antioxidant N-acetyl-L-cysteine prior to the administration of chemotherapy drug would prevent from nephrotoxicity, loss of female reproductive performance induced from chemotherapy treatment and promote fertility preservation and also to investigate the mechanism of N-acetyl-L-cysteine in protection.

Materials and methods

Animals

Adult virgin female Wistar rats (135–138 g; 7–8 weeks old) and proven male breeders (200–250 g; 9–10 weeks old) were purchased from the Medical Research Center, Ain Shams University. All animals were allowed a one-week acclimation period before the start of experiment. All animals were housed under standard laboratory conditions (28±2°C and 12-h light/dark cycle) and were fed on standard pellet diet and water ad libitum. The animals were used according to the guidelines of the Committee on re and Use of Experimental Animal Resources, Medical Research Center, Ain Shams University. Before mating with males, female rats were divided into five equal groups of six each: 1/control group; 2/rats i.p administered saline solution (0.9% NaCl) 1 mL; 3/rats i.p administered holoxan (50 mg/kg b. wt); 4/rats i.p administered N-acetyl-L-cysteine (160 mg/kg b.wt); 5/rats i.p administered holoxan and N-acetyl-L-cysteine at the same doses.

Administration of the tested chemicals

Holoxan (sterile ifosfamide, C7H15C2N2O2P) vial (Baxter Oncology GmbH, Germany) is a synthetic analog of cyclophosphamide contains 1 g holoxan in a dry powder form. The content of each vial was freshly dissolved in sterile saline solution (0.9% NaCl) immediately before injection. Holoxan was administered at 50 mg/kg body weight, daily for five consecutive days. The dose of holoxan (50 mg kg⁻¹) is given to the rats as it is the recommended dose administered to patients with cancer daily for 5 days each cycle. N-Acetyl-L-cysteine (Sigma-Aldrich St. Louis, MO), its molecular formula is C8H16NO4S was freshly dissolved in normal sterile saline (0.9% NaCl) prior to injection. Female rats were injected (i.p.) with N-acetyl-L-cysteine at 160 mg/kg/day; 96 h (4 days) before Holoxan injection and continued with Holoxan injection (50 mg/kg/day) for 5 consecutive days before mating.

Assessment of fertility

After medications, female rats were housed with proven male breeders in ratio of three females to 1 male. Females were housed with the males for up to 10 days, or the length of two full estrous cycles. The estrous cycle is 4–5 days in the female rat. Vaginal smears were performed daily to check for the presence of sperm. The day that a copulatory plug was found and/or sperm was found in the vaginal smear was termed the first day of pregnancy. Female was then removed from the cage and house singly until day 18 of gestation. Animals were sacrificed on day 18 of gestation by decapitation and the trunk blood was collected (3 mL/rat) into nonheparinized glass test tube, centrifuged (3000 g/30 min) and the serum obtained was stored (−70°C) until used for the biochemical studies. After blood collections, kidneys were carefully removed, dissected free of the surrounding adipose tissue and weighed. One kidney from each rat was homogenized in cold potassium phosphate buffer (100 mM potassium phosphate, 2 mM EDTA, pH 7.0). The homogenate (20%) was centrifuged (3000 g, 30 min) and the supernatant was stored (−70°C) until used for physiological tests, the other kidney was fixed in 10% neutral buffered formalin for histological studies. Then, uteri were removed, the number of corpora lutea was counted and each uterine horn was examined, the number of implantation sites, resorption sites and the number of viable fetuses were counted. A resorption site was defined as an implantation site resembling a brown to greenish blood clot, with the placental tissue (early resorption) or placental and embryonic tissue (late resorption). Each fetus was removed from the
placenta, weighed and the crown rump (CR) length was recorded. Fetuses were examined for external malformation and variation. Fecundity index was calculated as described by Griffiths et al.:27

\[
\text{Fecundity index} = \frac{\text{number of pregnant females}}{\text{number of mate females}} \times 100.
\]

In order to determine the percentage of preimplantation loss, the following calculation was used:

\[
\text{Preimplantation loss} = \frac{\left(\text{number of corpora lutea} - \text{number of implantation sites}\right)}{\text{number of corpora lutea}} \times 100
\]

In order to determine the percentage of postimplantation loss, the following calculation was used:

\[
\text{Postimplantation loss} = \frac{\left(\text{number of implantation sites} - \text{number of viable fetuses}\right)}{\text{number of implantation sites}} \times 100
\]

**Determination of progesterone hormone concentrations**

For the determination of progesterone hormone levels in serum a commercially available progesterone enzyme immunoassay test kits (Bio Check, Inc., 323 Vintage Park Drive, Foster City, CA) was used. To determine the level of progesterone level per corpus luteum (CL), the level of progesterone from each individual animal was divided by the total number of CL from both ovaries of the same animal:

\[
\text{Progesterone/CL} = \frac{\text{total serum progesterone level}}{\text{total number of corpora lutea of both ovaries}}.
\]

**Biochemical studies**

Calcium and phosphorus levels in serum were determined by an enzymatic colorimetric method using commercial assay kits (Spectrum Diagnostic kits, Hannover, Germany). Quantitative determination of total protein in serum was estimated by using Vitro Scient assay kit, Hannover, Germany.

**Determination of creatinine, urea nitrogen and uric acid in serum**

Serum creatinine and uric acid were determined by an enzymatic colorimetric method using commercial assay kits (MDSS GmbH, Burckhardtsr, Hannover, Germany). Urea nitrogen level was estimated using assay kit (Linear Chemicals Inc, Spain).

**Measurement of renal nicotinamide dinucleotide phosphate (NADPH) oxidase activity**

Determination of NADPH oxidase (NADPH-OX) by using enzyme-linked immunosorbent assay (ELISA) kit (Wkea MED SUPPLIES CORP, China) according to the manufacturer’s instructions.

**Determination of nitric oxide (NO), super oxide dismutase (SOD) and lipid peroxidase (MDA) in renal tissue**

Nitric oxide assay was measured by colorimetric determination of nitrite as described by Montgomery and Dymock. Superoxide dismutase was estimated by the method of Nishikimi et al. and lipid peroxide was determined by thiobarbituric acid reaction substances (TBARS) by the method of Ohkawa et al.

**Histological examination**

After fixation in 10% neutral buffered formalin, kidney from mothers were routinely processed in ascending grades of alcohol and then xylene, embedded in paraffin wax, serially sectioned at 5 mm thickness, and stained with hematoxylin and eosin for light microscopic investigation.

**Statistical analysis**

Data were expressed as mean±SEM. One-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test was used to assess significant differences among the experimental groups (software package version 10, SPSS Inc., Chicago, IL). A \( p \) values <0.05 was considered statistically significant.

**Results**

**Fecundity index and reproductive performance**

To assess the influence of N-acetyl-L-cysteine and/or holoxan on fertility, female rats were mated with male rats immediately after treatment. While treatment with holoxan for five consecutive days before mating causing a significant (\( p < 0.05 \)) decrease in the percentage of females fertility index to (55.55% ± 0.17), pretreatment with N-acetyl-L-cysteine 96h before holoxan injection and continued with it for five consecutive days before mating significantly (\( p < 0.05 \)) elevated the fecundity index (+20%) to (66.66% ± 0.21) in the N-acetyl-L-cysteine + holoxan group compared to holoxan-treated group. Progesterone level significantly (\( p < 0.05 \)) reduced (∼31.45%) in the group of rat treated with holoxan alone.
and significantly (p < 0.05) reach to the nearly normal level when N-acetyl-L-cysteine was administered 4 days before holoxan injection with respect to normal control group. Moreover, the progesterone level per corpus luteum was also decreased (p < 0.05) with holoxan treatment and improved after N-acetyl-L-cysteine administered with holoxan. This suggests that the fetal loss was directly associated with corpus luteum progesterone production. Neither prior administration of holoxan alone nor combined with N-acetyl-L-cysteine led to change (p > 0.05) in the number of corpora lutea or implantation sites as compared to normal control group.

Premating treatment with holoxan alone showed reduction (p > 0.05) in the number of viable fetuses as compared to normal control group and the other treated groups. N-Acetyl-L-cysteine coadministered with holoxan protects female animals from loss of the viable fetuses. Holoxan administration prior to mating resulted in an increase in the percentage of preimplantation loss (p < 0.05) and postimplantation loss (p < 0.05) in comparison to the other groups. On the same line, the number of resorbed fetuses was elevated significantly (p < 0.05) in the groups of rats treated with holoxan alone. Administration of N-acetyl-L-cysteine with holoxan significantly (p < 0.05) reduced the percentage of preimplantation loss, postimplantation loss and the number of resorbed fetuses. Prior mating, treatment with holoxan alone (50 mg/kg body wt) for five successive days significantly (p < 0.05) reduced the average fetal body weight and length as compared to the normal control group and the other treated groups, respectively. Prior administration of N-acetyl-L-cysteine and concomitant with holoxan improved significantly (p < 0.05) fetal growth retardation (Table 1). N-Acetyl-L-cysteine-prematring treatment can protect against the loss of the female fertility and the adverse effects of prior holoxan administration on future pregnancy outcomes.

### Biochemical studies

Treatment with holoxan for five consecutive days before mating causing a significant decrease in the levels of total protein (p < 0.05), phosphorus (p < 0.01) and calcium (p < 0.05) of pregnant rats to (−23.2%) for total protein, (−61.1%) for phosphorus and (−27.7%) for calcium from control group. Pretreatment with N-acetyl-L-cysteine 9 days prior mating with males improved these levels nearly too normal control values (Figure 1).

Table 2 shows the effect of holoxan, N-acetyl-L-cysteine, and their combination on serum nephrotoxicity indices. Administration of holoxan significantly (p < 0.05) increased serum creatinine by (+44.14%), blood urea nitrogen (+11.76%) and uric acid (+27.53%) from the normal control values. Interestingly, prior mating, and concomitant with holoxan improved significantly (p < 0.05) fetal growth retardation (Table 1). N-Acetyl-L-cysteine-prematring treatment can protect against the loss of the female fertility and the adverse effects of prior holoxan administration on future pregnancy outcomes.

| Groups | Control | Saline | N-Acetyl-L-cysteine (160 mg/kg/day) | Holoxan (50 mg/kg b.wt) | N-Acetyl-L-cysteine (160 mg/kg/day) + holoxan (50 mg/kg b.wt) |
|--------|---------|--------|-----------------------------------|-------------------------|---------------------------------------------------------------|
| Progesterone (ng/ml) | 39.68 ± 4.27* | 37.48 ± 4.68* | 32.85 ± 5.54* | 27.20 ± 2.30** | 32.32 ± 5.60* |
| No of corpora lutea | 8.20 ± 0.66* | 9.00 ± 0.70* | 7.50 ± 0.50* | 8.60 ± 0.50* | 8.50 ± 0.28* |
| Progesterone/CL | 4.83* | 4.16* | 4.38* | 3.16* | 3.80* |
| No of implantation sites | 8.20 ± 0.66* | 8.80 ± 0.58* | 7.50 ± 0.50* | 8.20 ± 0.37* | 8.50 ± 0.28* |
| No of viable fetuses | 8.00 ± 0.63* | 8.00 ± 0.70* | 7.00 ± 0.01* | 6.75 ± 0.25* | 7.25 ± 0.75* |
| No of resorbed fetuses | 0.33 ± 0.02* | 0.00 ± 0.00* | 0.50 ± 0.50* | 1.25 ± 0.47* | 0.33 ± 0.33* |
| Preimplantation loss (%) | 0* | 2.22 ± 5.00* | 0* | 4.65 ± 4.00* | 0* |
| Postimplantation loss (%) | 2.43* | 9.09* | 6.66* | 17.68* | 14.70* |
| Average fetuses body weights of (g) | 1.42 ± 2.87* | 1.37 ± 1.92* | 1.41 ± 1.95* | 1.29 ± 5.83* | 1.43 ± 2.75* |
| Average fetuses body length (cm) | 2.48 ± 2.96* | 2.54 ± 2.73* | 2.46 ± 2.69* | 2.22 ± 6.28* | 2.48 ± 4.03* |

Results are expressed as mean values ± SE (n = 8).
Different superscripts mean significant differences (*p < 0.05) among means within a column.
Non-significant difference compared with control, p > 0.05.
Significant difference compared with control, p < 0.05.
administration of N-acetyl-L-cysteine with holoxan resulted in complete reversal of holoxan-induced increase in serum creatinine (−28.5%), blood urea nitrogen (−12.28%) and uric acid (−11%) with respect to the control values. From Table 2, absolute kidneys weight increased nonsignificantly (p > 0.05) by +13.83% from control group in rats treated with holoxan alone and it improved to −9.68% from control when coadministered with N-acetyl-L-cysteine. Blood urea nitrogen, serum creatinine levels and uric acid were significantly (p < 0.05) elevated in holoxan-treated group compared to normal values. Premating treatment with N-acetyl-L-cysteine and holoxan significantly improved the renal functions and uric acid nearly to normal levels.

The activity of NADPH oxidase was assayed in the kidney tissue homogenates. It was seen that the activity was increased significantly (p < 0.05) in holoxan group compared to control (Table 2). Whereas N-acetyl-L-cysteine alone resulted in nonsignificant change. Combination of N-acetyl-L-cysteine with holoxan decreased the level of NADPH oxidase in kidney tissue to control values.

The extent of oxidative damage was assessed by measuring the super oxide dismutase (SOD) and lipid peroxidation levels in kidney tissues (Table 2). Holoxan alone significantly reduced (p < 0.05) the level of SOD by −24.52% from control and significantly elevated (p < 0.05) the activity of lipid peroxidation in kidney tissues by (+124.27%) compared to control group. However, N-acetyl-L-cysteine prior and with holoxan completely normalized these changes. Table 2 shows the effects of premating treated with holoxan, N-acetyl-L-cysteine and their combination on the level of nitric oxide (NO) or nitrite level in kidney tissue. Holoxan resulted in a significant (p < 0.05) increase (+71.5%) in NO production in kidney tissues, whereas N-acetyl-L-cysteine alone resulted in nonsignificant change. Combination of N-acetyl-L-cysteine with holoxan decreased the level of NO production by −22.5% of control values.

**Histological examinations**

Light microscopic evaluation of the kidneys of pregnant rats in the control (Figure 2a), saline and N-acetyl-L-cysteine (160 mg/kg/day) (Figure 2c) showed regular morphology of renal parenchyma with normal structure of glomeruli and tubules in cortical and medullary regions (Figure 2b). In the renal tissue of holoxan-treated rats, there were tubular cloudy swelling, pale eosinophilic staining of the epithelial lining the renal tubules, interstitial hemorrhage and vascular congestion (Figure 2d). Vascular degeneration and nuclear pyknosis (Figure 2e) were also observed. Besides cellular debris or red cell casts in some medullary tubules were prominent (Figure 2f). In the N-acetyl-L-cysteine holoxan-treated group, despite the presence of mild vasocongestion in the parenchyma and cellular debris in the medullary tubules, vascular congestion was minimal and the renal tubules maintained a better morphology when compared with holoxan group (Figure 2g and h).

**Discussion**

**The effects on female fertility and reproductive performance**

N-Acetyl-L-cysteine administered prior to treatment with holoxan for 4 days and continued with holoxan for 5 days modulated significantly (p < 0.05) the female reproductive performance by increasing fertility index, progesterone level per CL, the number of live fetuses as well as decreasing the rate of pre- and postimplantation loss and the number of resorbed fetuses. It is possible that the toxic metabolite of holoxan caused ovarian and/or uterine damage prevents the implanted fetuses from developing.

---

**Table 2.** Levels of NADPH oxidase, nitric oxide (NO), super oxide dismutase (SOD) and lipid peroxidase (MDA) in renal tissue and levels of serum creatinine, urea nitrogen and uric acid of pregnant rats premating treated with N-acetyl-L-cysteine and/or holoxan on day 18 of gestation.

|                     | Control                    | Saline                    | N-Acetyl-L-cysteine (160 mg/kg/day) | Holoxan (50 mg/kg b.wt) + N-acetyl-L-cysteine (160 mg/kg/day) |
|---------------------|---------------------------|---------------------------|-----------------------------------|---------------------------------------------------------------|
| Kidney weight (g)   | 0.506 ± 4.64*             | 0.512 ± 1.65*             | 0.506 ± 4.41*                     | 0.576 ± 2.94*                                                |
| NADPH oxidase (Pg/ml) | 77.85 ± 0.35*            | 73.76 ± 0.34*            | 78.85 ± 1.95*                     | 84.57 ± 4.43*                                                |
| NO (μmol/L)         | 2.00 ± 0.13*              | 2.10 ± 0.14*              | 2.00 ± 0.16*                      | 3.43 ± 0.68*                                                 |
| SOD (u/gm)          | 1066.87 ± 36.27*         | 1035.00 ± 60.00*         | 1080.00 ± 15.00*                  | 805.20 ± 142.87*                                            |
| MDA (nmol/g tissue) | 140.18 ± 30.32*          | 152.95 ± 37.28*          | 140.18 ± 36.25*                   | 314.39 ± 54.39*                                             |
| Creatinine (mg/dl)  | 0.675 ± 0.11*            | 0.700 ± 0.10*            | 0.750 ± 5.00*                     | 0.973 ± 8.67*                                                |
| Urea (mg/dl)        | 34.00 ± 1.00*             | 34.00 ± 2.48*            | 34.00 ± 8.00*                     | 38.00 ± 3.54*                                                |
| Uric acid (mg/dl)   | 2.76 ± 8.61*              | 2.27 ± 0.15*             | 3.10 ± 0.50*                      | 3.52 ± 0.16*                                                 |

Results are expressed as mean values ± SE (n = 6).

Different superscripts mean significant differences (p < 0.05) among means within a column.

*Non-significant difference compared with control, p > 0.05.

Significant difference compared with control, p < 0.05.
Figure 2. (a–h) Micrographs (hematoxylin and eosin staining) of kidney tissues of pregnant rats on day 18th of gestation. a-b/control kidney: regular structure of the cortical region: glomerulus (g), proximal (PX) and distal convoluted tubules (D) (200 X). b/normal structure of medullary region (100 X). c/ N-acetyl-l-cysteine (160 mg/kg/day) treated kidney: similar organization as the control group with normal glomerulus and tubules (200 X). d-f/Holoxan (50 mg/kg b.wt) treated kidney: d/tubular cloudy swelling (c), pale eosinophilic staining of the epithelial lining the tubules, interstitial hemorrhage (H) and vascular congestion (arrow) (200 X); e/vacuolar degeneration (arrows) and nuclear pyknosis (arrow heads) (200 X); f/red cell casts in some tubules of the medullary region(arrows) (200 X). g-h/N-acetyl-l-cysteine (160 mg/kg/day) + Holoxan (50 mg/kg b.wt)-treated kidney: the regions of the cortex (g, 200 X) and medulla (h, 100x) maintained a better morphology when compared with holoxan group.
leading to the increased post-implantation loss. Cyclophosphamide has been found to induce adverse effects on oxidant status of the rats’ ovaries.\textsuperscript{32} Infertility results from a direct toxic effect of the drug on the gonads or an indirect effect on the pituitary gland\textsuperscript{33} as\textsuperscript{34} has been reported a depressing effect of cyclophosphamide in the hypothalamic pituitary axis in cycling rhesus monkeys. Moreover, the toxic metabolite, phosphoramide mustard (PM) of cyclophosphamide has been shown to be active ovotoxic\textsuperscript{35} and in vitro\textsuperscript{36} and may cause DNA double-strand breaks in oocytes, which could lead to permanent changes in gamete health and increased risk for fertility problems or unhealthy offspring.\textsuperscript{37} In addition, chloroacetaldehyde (CAA), which is produced by the side-chain oxidation of ifosfamide in renal tubular cells,\textsuperscript{38} is responsible for severe glutathione (GSH) depletion and malondialdehyde (MDA) accumulation.\textsuperscript{9} 

\textit{N}-Acetyl–L-cysteine prevented this damage when administered before and concurrently with holoxan by decline the intracellular antioxidant levels and increased activity of the enzyme responsible for recycling and restoring glutathione to normal levels, protecting cells from oxidative stress\textsuperscript{39} and from maternal inflammation.\textsuperscript{40} \textit{N}-Acetyl–L-cysteine is proved effective in inducing or augmenting ovulation, number of mature follicles, serum E2, serum progesterone and endometrial thickness in polycystic ovary patients.\textsuperscript{41} In the same line, recent study showed that inositol supplementation in preconceptional period, in early phase of pregnancy reduces the risk of developing gestational diabetes mellitus in patients, and improves their metabolic status. In addition to, all women at risk of folate-resistant embryo neural tube defects assuming inositol from the periconceptional period until late pregnancy are reported to have healthy newborns without any significant complications linked to inositol supplementation.\textsuperscript{42} The present study showed that the function of the corpora lutea was affected by prior holoxan exposure, as the levels of progesterone significantly reduced as compared to the other groups. Holoxan did not alter the number of oocytes ovulated, as evidenced by the same number of corpora lutea found in all groups. This is in agreement with previous studies which demonstrated that cisplatin did not alter the number of follicles; rather it affected the function of the follicle.\textsuperscript{43,44} In the present study, holoxan administration led to significant increase in fetal resorption that may be due to the reduction in progesterone hormone and the direct effects of the toxic metabolite of holoxan on the embryo. In this concern,\textsuperscript{45} pointed out that reduction in each of luteinizing hormone (LH) prolactin and progesterone after treating the female rats with cisplatin on day 6 may be responsible for embryonic resorption because continuation of gestation depends primarily on this hormone system. Fetal growth retardation and decrease number of viable fetuses might be explained on the basis of incomplete formation of the placenta and degeneration of the trophoblast and decidual cell, which play an important role in the transmission of nutrients to the embryo.\textsuperscript{46} \textit{N}-Acetyl–L-cysteine reduced tissue injury and endothelial cell apoptosis by enhancing antioxidant enzymes and/or inhibiting lipid peroxidation.\textsuperscript{47} NAC also improved microcirculation and tissue oxygenation that may facilitate tissue regeneration in the degenerative diseases.\textsuperscript{48} In fact, NAC suppressed inflammatory cytokines in amniotic fluid and placenta in LPS-treated rats.\textsuperscript{40} Recently, D-Chiro-inositol is effective in improving ovarian function and metabolism of patients affected by polycystic ovarian syndrome due to significant improvements in the metabolic (glucose profile and serum glucose to insulin ratio) and hormonal profiles (LH, FSH, androgen levels).\textsuperscript{49,50}

\textbf{The effects on renal tissue}

Holoxan (50 mg/kg/day) altered renal functions by elevating kidney weight, tissue NADPH, serum creatinine and urea and reduced serum phosphate, total protein and calcium levels. At the same time, it produced remarkable oxidative damage as shown by the depletion of tissues SOD levels, elevated lipid peroxide (MDA) and NO contents. Moreover, it led to detectable morphological damage in renal tissue. The same results in rats were observed by.\textsuperscript{21}\textsuperscript{22}\textsuperscript{51}\textsuperscript{52} IFO at a dose 50 mg/kg/day for five days in rat induced a Fanconi syndrome and oxidative damage in the renal and bladder tissues.\textsuperscript{21} Nephrotoxicity induced by IFO may due to the oxidative stress and free radical generation such as NO, local depletion of GSH, reduction in the level of renal SOD\textsuperscript{53} and increased in MDA levels indicating the enhancement lipid peroxidation as a result of impaired antioxidant defense mechanism\textsuperscript{22,23} Membrane peroxidation can lead to changes in membrane fluidity and permeability and also to enhanced rates of protein degradation, with these eventually leading to cell lysis.\textsuperscript{54} Mohrmann et al.\textsuperscript{55} have shown that exposure of LLC-PK1 cells to IFO metabolites acrolein or chloroacetaldehyde significantly impairs several sodium-dependent transport systems and the sodium/proton antiport system. Both acrolein- and chloroacetaldehyde-induced renal injury is associated with lipid peroxidation and suppression activation of complex I in the mitochondrial respiratory chain resulting in decreased levels of GSH and ATP, and can induce cell death.\textsuperscript{56,57} It has been reported that necrosis is the predominant form of cell death by chloroacetaldehyde in human proximal tubular cell lines.\textsuperscript{58}
Furthermore, chloroacetaldehyde caused elevations of intracellular free Ca2+ by an inhibitory action on Na+/Ca2+ exchanger, which ultimately led to the disruption of membrane integrity.59 Nitric oxide (NO) has an important role in modulating oxidative stress and tissue damage.60 Excessive NO production can be cytotoxic, the result of NO’s reaction with reactive oxygen and nitrogen species, leading to peroxynitrite anion formation, protein tyrosine nitration, hydroxyl radical production61 and mitochondrial dysfunction.62 NADPH oxidase has been shown to be one of the most powerful prooxidants in both the vasculature and the kidney. NADPH oxidase contributes importantly to renal cortical oxidative stress, inflammation, renal damage and dysfunction and increased in arterial pressure.63 Previous study showed that the primary source of ROS generation in the renal cortex is NADPH oxidase.64 NADPH oxidase is a cytoplasmic enzyme consisting of at least one catalytic, transmembrane-spanning NOX subunit, which produces ROS by transferring electrons from NADPH to molecular oxygen.65

In the present work, female rats injected with N-acetyl-L-cysteine (160 mg/kg/day) four days before holoxan and continued with holoxan injection (50 mg/kg/day) for five consecutive days before mating markedly reduced the severity of renal dysfunction induced by ifosfamide with a significant decrease in elevations of serum creatinine, urea and uric acid as well as a reduced elevation of renal tissue No, MDA and NADPH oxidase. Moreover, NAC significantly improved the ifosfamide-induced SOD depletion, elevated the lowered calcium, phosphate and total protein and prevented morphological damages in renal tubules and glomeruli. N-Acetylcysteine (NAC) is the possible role of in protection against IF toxicity in male rats.24 N-acetylcysteine is an ideal candidate for replenishing tissue GSH levels through two major protective mechanisms: it acts as precursor for GSH synthesis (sulfhydryl source for GSH synthesis)66 and nucleophile that scavenges reactive oxygen species such as O2*, H2O2 and OH-67 and conjugates with reactive metabolites rendering them less toxic.68 The underlying mechanisms governing the tissue-specific effects of NAC on SOD protein expression might be due to tissue-specific effectiveness of NAC in reducing the production of superoxide.69 N-Acetylcysteine had caused a 91% decrease in glomerular necrosis.63

In conclusion, antioxidant NAC can confer protection against nitrosative and oxidative stresses in renal tissue induced by holoxan by suppressing renal NADPH oxidase activation, MDA, nitric oxide and restoring or enhancing SOD enzyme activity. Thus, inhibition of NADPH oxidase elevation level and activation led to subsequent reduction of ROS production and subsequently might effectively improve the gonadal hormone disturbance, sexual and reproductive functions.

Acknowledgements
The author thanks all staff members of the Medical Research Center, Ain Shams University for making all facilities available for the author’s research.

Declaration of interest
The author reports no conflicts of interest. The author alone is responsible for the content and writing of the paper.

References
1. Kishk EAF, Ali MHM. Effect of a gonadotropin-releasing hormone analogue on cyclophosphamide-induced ovarian toxicity in adult mice. Arch Gynecol Obstet. 2013;287:1023–1029.
2. Hoyer PB, Sipes IG. Assessment of follicle destruction in chemical-induced ovarian toxicity. Annu Rev Pharmacol Toxicol. 1996;36:307–331.
3. Tomao F, Spinelli GP, Panici PB, Frati L, Tomao S. Ovarian function, reproduction and strategies for fertility preservation after breast cancer. Crit Rev Oncol Hematol. 2010;76:1–12.
4. Yazawa Y, Takagi T, Asakura S, Suzuki K, Kano Y. Effects of 4-hydroperoxy ifosfamide in combination with other anticancer agents on human cancer cell lines. J Orthop Sci 1999;4:231–237.
5. Rossi R, Godde A, Kleinebrand A, et al. “Unilateral nephrectomy and cisplatin as risk factors of ifosfamide-induced nephrotoxicity nephrotoxicity: Analysis of 120 patients.”. J Clin Oncol. 1994;12:159–165.
6. Pratt CB, Meyer WH, Jenkins JJ, et al. Ifosfamide, Fanconi’s syndrome, and rickets. J Clin Oncol. 1991;9:1495–1499.
7. Drube J, Schiffer E, Mischak H, et al. Urinary proteome pattern in children with renal Fanconi syndrome. Nephrol Dial Transplant. 2009;24:2161–2169.
8. Yaseen Z, Michoudet C, Baverel G, Dubourg L. Mechanisms of the ifosfamide-induced inhibition of endocytosis in the rat proximal kidney tubule. Arch Toxicol. 2008;82:607–614.
9. Springate JE. Ifosfamide metabolite chloroacetaldehyde causes renal dysfunction in vivo. J Appl Toxicol. 1997;17:75–79.
10. Vitagliano A, Noventa M, Gizzo S. Emerging evidence regarding statins use as novel endometriosis targeted treatment: Real “magic pills” or “trendy” drugs? Some considerations. Eur J Obstet Gynecol Reprod Biol. 2015;184:125–126.
11. Vitagliano A, Noventa M, Quaranta M, Gizzo S. Statins as anticancer agents: Molecular targeted actions ahead of print]. doi: 10.1177/1933719115584446.
12. Gizzo S, Quaranta M, Nardelli GB, Noventa M. Lipophilic statins as anticancer agents: Molecular targeted actions.
and proposal in advanced gynaecological malignancies. *Curr Drug Targets*. 2015;16:1142–1159.

13. Andrisani A, Dona G, Tibaldi E, et al. Astaxanthin improves human sperm capacitation by inducing lyn displacement and activation. *Mar Drugs*. 2015;13:5533–5551.

14. Aleksa K, Halachmi N, Itso S, Koren G. A tubule cell model for ifosfamide nephrotoxicity. *Can J Physiol Pharmacol*. 2005;83:499–508.

15. Lohr JW, Willsky GR, Acara MA. Renal drug metabolism. *Pharmacol Rev*. 1998;50:107–141.

16. Ornaghi F, Ferrini S, Prati M, Giavini E. The protective effects of N-acetyl-L-cysteine against methyl mercury embryotoxicity in mice. *Toxicol Sci*. 1993;20:437–445.

17. Huang J, Okula M, McLean M, Keefe DL, Liu L. Telomere susceptibility to cigarette smoke-induced oxidative damage and chromosomal instability of mouse embryos in vitro. *Free Radic Biol Med*. 2010;48:1663–1676.

18. Nasr A. Effect of N-acetyl-cysteine after ovarian drilling in clomiphene citrate-resistant PCOS women: A pilot study. *Reprod Biomed Online*. 2010;20:403–409.

19. Dickey DT, Muldoon LL, Kraemer DF, Neuwelt EA. Protection against cisplatin-induced otoxicity by N-acetylcysteine in a rat model. *Hearing Res*. 2004;193:25–30.

20. Nisar S, Feinfeld DA. N-acetylcysteine as salvage therapy in cisplatin nephrotoxicity. *Ren Fail*. 2002;24:529–533.

21. Sehiri O, Sakarcan A, Velloglu-Ogunc A, et al. Resveratrol improves ifosfamide-induced Fanconi syndrome in rats. *Toxicol Appl Pharmacol*. 2007;222:33–41.

22. Sener G, Sehiri O, Yegen B, Cetinel S, Gedik N, Sakarcan A. Melatonin attenuates ifosfamide-induced Fanconi syndrome in rats. *J Pineal Res*. 2004;37:17–25.

23. Chen N, Alekse K, Woodland C, Rieder M, Koren G. N-Acetylcysteine prevents ifosfamide-induced nephrotoxicity in rats. *Br J Pharmocol*. 2008;153:1364–1372.

24. El-Sisi A, El-Syaad M, El-Desoky K, Moussa E. Protective effects of alpha lipoic acid versus N-acetylcysteine on ifosfamide-induced nephrotoxicity. *Toxicol Ind Health*. 2013;12:1–11.

25. Naya M, Deguchi T, Yasuda M. Effects of N-acetyl-L-cysteine on teratogenicity of 5-fluorouracil in mice. *Congenital Anomalies*. 1993;33:77–84.

26. Kim JC, Shin JY, Yang YS, et al. Evaluation of developmental toxicity of amitraz in Sprague-Dawley rats. *Arch Environ Contam Toxicol*. 2007;52:137–144.

27. Griffiths JC, Borzelleca JF, St Cyr J, Griffiths JC, Borzelleca JF, St Cyr J. Lack of oral embryotoxicity/teratogenicity with D-ribose in Wistar rats. *Food Chem Toxicol*. 2007;45:388–395.

28. Yeh J, Su Kim B, Peresie J. Reproductive toxic effects of cisplatin and its modulation by the antioxidant sodium 2-mercaptoethanesulfonate (mesna) in female rats. *Reprod Biol Insigh*. 2011;5:17–27.

29. Montgomery HA, Dymock JF. The determination of nitrite in water. *Analyst*. 1961;86:414–416.

30. Nishikimi M, Roa N, Yogi K. Measurement of superoxide dismutase. *Biochem Biophys Res Comm*. 1972;46:849–854.

31. Ohkawa N, Ohishi W, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem*. 1979;95:351–358.

32. Yener N, Sinanoglu O, Ilter E, et al. Effects of spirulina on cyclophosphamide-induced ovarian toxicity in rats: Biochemical and histomorphometric evaluation of the ovary. *Biochem Res Int*. 2013;2013:764262.

33. Maclean F, Lee A. Drug-induced sexual dysfunction and infertility. *Pharm J*. 1999;262(7047):780–784.

34. Pfeiffer D, Mattison D, Nikolarakis K. Ovarian failure after chemotherapy due to toxic effects on the ovary and hypothalamus-pituitary axis. *Acta Endocrinol*. 1986;3:7.

35. Plowchalk DR, Mattison DR. Phosphoramide mustard is responsible for the ovarian toxicity of cyclophosphamide. *Toxicol Appl Pharmacol*. 1991;107:472–481.

36. Desmeules P, Devine P. Characterizing the otoxicity of cyclophosphamide metabolites on cultured mouse ovaries. *Toxicol Sci*. 2006;95:500–509.

37. Petrillo S, Desmeules P, Truong T, Devine P. Detection of DNA damage in oocytes of small ovarian follicles following phosphoramide mustard exposures of cultured rodent ovaries in vitro. *Toxicol Appl Pharmacol*. 2011;253:94–102.

38. Woodland C, Itso S, Granvil CP, Wainer IW, Klein J, Koren G. Evidence of renal metabolism of ifosfamide to nephrotoxic metabolites. *Life Sci*. 2000;68:109–117.

39. Zembron-Lacny A, Szyzska K, Szygula Z. Effect of cysteine derivatives administration in healthy men exposed to intense resistance exercise by evaluation of pro-antioxidant ratio. *J Physiol Sci*. 2007;57:343–348.

40. Beloosesky R, Gayle D, Amid D, et al. N-Acetyl-cysteine suppresses amniotic fluid and placenta inflammatory cytokine responses to lipopolysaccharide in rats. *Am J Obstet Gynecol*. 2006;194:268–273.

41. Badawy A, State O, Abdelgawad S. N-Acetyl cysteine and clomiphene citrate for induction of ovulation in polycystic ovary syndrome: A cross-over trial. *Acta Obstet Gynecol Scand*. 2007;86:218–222.

42. Noventa M, Vitagliano A, Quaranta M, Borgato S, Abdulrahim B, Gizzo S. Preventive and therapeutic role of dietary inositol supplementation in periconceptional period and during pregnancy: A summary of evidences and future applications. *Reprod Sci*. 2015. [Epub ahead of print]. doi: 10.1177/1933719115594018.

43. Singh K, Davies M, Chatterjee R. Fertility in female cancer survivors: Pathophysiology, preservation and the role of ovarian reserve testing. *Hum Reprod Update*. 2005;11:69–89.

44. Chung MK, Han SS, Kim JC, Chung MK, Han SS, Kim JC. Evaluation of the toxic potentials of a new camptothecin anticancer agent CKD-602 on fertility and early embryonic development in rats. *Regul Toxicol Pharmacol*. 2006;45:273–281.

45. Bajt ML, Aggarwal SK. An analysis of factors responsible for resorption of embryos in cisplatin-treated rats. *Toxicol Appl Pharmacol*. 1985;80:97–107.

46. Kurebe M, Asaoka H, Moriguchi M. Toxicological studies on a new cephamycin, MT-141. IX. Its teratogenicity test in rats and rabbits. *Japanese J Antibiot*. 1986;3:7.

47. Stanislaus R, Glig A, Singh A, Singh I. N-acetyl-L-cysteine ameliorates the inflammatory disease process in experimental autoimmune encephalomyelitis in Lewis rats. *J Autoimmun Dis*. 2005;2:4.

48. Cuzzocrea S, Mazzon E, Costantino G, et al. Beneficial effects of N-acetylcysteine on ischaemic brain injury. *Br J Pharmacol*. 2000;130:1219–1226.

49. Laganà AS, Barbaro L, Pizzo A. Evaluation of ovarian function and metabolic factors in women affected by...
polycystic ovary syndrome after treatment with D-Chiro-Inositol. Arch Gynecol Obstet. 2015;291:1181–1186.

50. Vitagliano A, Quaranta M, Noventa M, Gizzo S. “Empiric” inositol supplementation in normal-weight non insulin resistant women with polycystic ovarian disease: From the absence of benefit to the potential adverse effects. Arch Gynecol Obstet. 2015;291:955–957.

51. Nenad S, Dragan M, Slavimir V. Glomerular basement membrane alterations induced by gentamicin administration in rats. Exp Toxicol Pathol. 2008;60:69–75.

52. El-Sisi AE, El-Syaad ME, El-Desoky KJ, Moussa EA. Protective effects of alpha lipoic acid versus N-acetylcysteine on ifosfamide-induced nephrotoxicity. Toxicol Ind Health. 2015;31:97–107.

53. Lei S, Liu Y, Liu H, Yu H, Wang H, Xia Z. Effects of n-acetylcysteine on nicotinamide dinucleotide phosphate oxidase activation and antioxidant status in heart, lung, liver and kidney in streptozotocin-induced diabetic rats. Yonsei Med J. 2012;53:294–303.

54. Garcia JJ, Reiter RJ, Guerrero JM, et al. Melatonin prevents changes in microsomal membrane fluidity during induced lipid peroxidation. FEBS Lett. 1997;408:297–300.

55. Mohrmann M, Kupper N, Schonfeld B, Brandis M. Ifosfamide and mesna: Effects on the Na/H exchanger in renal epithelial cells in culture. Renal Physiol Biochem. 1995;18:118–127.

56. Kehrer JP, Biswal SS. The molecular effects of acrolein. Toxicol Sci. 2000;57:6–15.

57. MacAllister S, Martin-Brisac N, Lau V, Yang K, O’Brien P. Acrolein and chloroacetaldehyde: An examination of the cell and cell-free biomarkers of toxicity. Chem Biol Interact. 2013;202:259–266.

58. Schwerdt G, Gordjani N, Benesic A, et al. Chloroacetaldehyde- and acrolein-induced death of human proximal tubule cells. Pediatr Nephrol. 2006;21:60–67.

59. Benesic A, Schwerdt G, Mildenberger S, Freudinger R, Gordjani N, Gekle M. Disturbed Ca2+-signaling by chloroacetaldehyde: A possible cause for chronic ifosfamide nephrotoxicity. Kidney Int. 2005;68:2029–2041.

60. Goligorsky M, Brodsky A, Noiri E. Nitric oxide in acute renal failure: NOS versus NOS. Kidney Int. 2002;61:855–861.

61. Klahr S. The role of nitric oxide in hypertension and renal disease progression. Nephrol Dial Transplant. 2001;16:60–62.

62. Hardelander R. Melatonin and its metabolites as anti-nitrosating and anti-nitrating agents. J Exp Int Med. 2011;1:67–81.

63. Tian N, Moore RS, Phillips WE, et al. NADPH oxidase contributes to renal damage and dysfunction in Dahl salt sensitive hypertension. Am J Physiol Regul Integr Comp Physiol. 2008;295:1858–1865.

64. Wang D, Chen Y, Chabrashvili T, et al. Role of oxidative stress in endothelial dysfunction and enhanced responses to angiotensin II of afferent arterioles from rabbits infused with angiotensin II. J Am Soc Nephrol. 2003;14:2783–2789.

65. Chen S, Meng X, Zhang C. Role of NADPH oxidase-mediated reactive oxygen species in podocyte injury. BioMed Res Int. 2013;2013:839761.

66. Chen N, Aleksa K, Woodland C, Rieder M, Koren G. The effect of N-acetylcysteine on ifosfamide-induced nephrotoxicity: In vitro studies in renal tubular cells. Transl Res. 2007;150:51–57.

67. Aruoma OI, Halliwell B, Hoey BM, Butler J. The antioxidant action of N-acetylcysteine: Its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Radic Biol Med. 1989;6:593–597.

68. Tylicki L, Rutzowski B, Horl WH. Antioxidants: A possible role in kidney protection. Kidney Blood Press Res. 2003;26:303–314.

69. Guo Z, Xia Z, Jiang J, McNeill JH. Downregulation of NADPH oxidase, antioxidant enzymes, and inflammatory markers in the heart of streptozotocin-induced diabetic rats by N-acetyl-L-cysteine. Am J Physiol Heart Circ Physiol. 2007;292:H1728–H1736.