Uranium exposure increases spermatocytes metaphase apoptosis in rats: inhibitory effect of thymoquinone and N-acetylcysteine

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Abstract. Exposure to high doses of alpha particles, the main type of radiation emitted from depleted uranium (DU), causes adverse health burden. Uranyl acetate (UA), a commercial stock prepared from DU, has a combined effect of chemical toxicity and mild radioactivity, with a danger of cumulative effects after in vivo long term exposure. In this study, we investigated the potential antioxidant, antiapoptotic and cytoprotective effects of thymoquinone (TQ) and N-acetylcysteine (NAC) against testicular toxicity in rats induced by UA,. UA caused disturbance in the testicular redox homeostasis through reducing superoxide dismutase (SOD) activity and nitric oxide (NO) and glutathione (GSH) levels relative to the control group. Interstingly, the testicular SOD activity and NO and GSH levels of UA/TQ and UA/NAC treated groups were also significantly lower relative to the control group. UA induced histopathological alterations in the blood vessels and the seminiferous tubular epithelium. A marked increase in spermatocytes metaphase apoptosis was found (stage XIII) in UA treated rats, which is probably due to difficulties in segregation of homologous-

chromosomes. This may clarify why UA exposure reduced round spermatids numbers and fertility in previous studies. To check the reason of this partial metaphase arrest, we examined the presence of damage related γ -H2AX foci in late spermatocytes of treated groups, but only insignificant increase was found in UA treated group. Supplementation of TQ or NAC reduced the numbers of apoptotic cells and improved the histological alterations in testes. Thus, TQ and NAC attenuate the adverse effects of UA on the testicular microenvironement through anti-apoptotic and cytoprotective but not antioxidant effects.

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Key words: Uranyl acetate — Testis — Metaphase apoptosis — Thymoquinone — N-acetylcysteine

Introduction

Depleted uranium (DU), a waste product of uranium (U) enrichment, is widely used in several human activities and is associated with multiple health problems (Bleise et al. 2003; Di Lella et al. 2005). U persists for long period at the bio-ecological systems because of its long half-life time, various ways of exposure, magnification through the food chain, and combined metallic and radiation toxicities (Di Lella et al. 2005; Briner 2010). Several chelating ligands have been used to hasten the urinary exertion of U and to reduce its toxicity (Jin et al. 2016; Swami and Shrivastava 2017) but unfortunately, this therapeutic strategy faced many problems including exaggeration of the DU nephrotoxicity and the *in vivo* blockage of detoxification pathways (Briner 2010; Hao et al. 2016). Unexpected lack of deleterious physiological effects was seen in rats after a chronic oral intake (for 9 months to mimic the chronic exposure of U-rich territories inhabitants, Dubliner et al., 2014). Therefore, finding agents with powerful ameliorative influences against the radiological hazards of DU with potential natural biological occurrence and wide toxic-therapeutic window represents area of increasing interest.

Testes is one of the main target organs that is exposed to DU due to the ability of UA to pass through and negatively impact the blood testis barriers (Legendre et al. 2016). Nevertheless, conflict data emerged regarding the potential reproductive health burden of UA relative to radiological activities of the compound, wide range of variations in the dose and route of exposure, and differential responses of the strain and age of challenged animals (Llobet et al. 1991; Arfsten et al. 2009; Li et al. 2009; Legendre et al. 2016). DU did not markedly influence the testicular steroidogenesis in rats, however the enriched UA significantly increased the level of circulating testosterone and upregulated the gene expression involved in its metabolism (Grignard et al. 2008). Lifelong intoxication with low doses of DU in rats from embryonic stage to adulthood through drinking water induced disturbances in the testicular steroidogenesis by acting through the hypothalamic-pituitary-testicular axis, but without causing major testicular histoarchitecture defects (Legendre et al. 2016).

Interestingly, previous histopathological examinations revealed few differences in the tubules and germ cells between uranium-exposed and control animals (Llobet et al., 1991 ; Linares et al., 2005). A significant (not dose-related) decrease in the pregnancy rate, after mating male mice exposed to UA in drinking water (10-40 mg/kg/BW) for two weeks associated with a significant decrease in spermatid number/testis have been found (Linares et al., 2005). However, long term exposure to uranium induced- reproductive toxicity through different mechanisms of action including inflammation, testicular degeneration, vacuolization of Leydig cells and spermatocytes necrosis (reviewed in Asghari et al., 2015).

Shifting oxidant/antioxidant balance towards the pro-oxidant side is the possible mechanistic way lies behind the testicular UA toxicity by depletion of glutathione redox system and downregulation of inducible nitric oxide synthase gene expression (Li et al. 2005; Linares et al. 2006). TQ and NAC in this issue are highly promising candidates owing to their antioxidant and antiapoptotic properties (Kilciksiz et al. 2008; Li et al. 2015; Guida et al. 2016; Hassanein and El-Amir 2017) giving a solid-based rationality to interfere with multiple toxicological targets of DU. Both of TQ and NAC have been suggested as upstreams regulators of the antioxidant enzyme, heme oxygenase-1 (HO-1) by including its expression which suppresses inflammation and carcinogenesis (Yi and Hazell, 2005 ; Kundu et al., 2014). Because of its antioxidant and anti-inflammatory activities, TQ was found to increase the healthy sperm number and protects from testicular injury (Tüfek et al., 2015; Fouad and Jresat 2015) suggesting that it is an ideal testicular protective agent. TQ in this field is considered as a one of not fully researched radiation-modifying agents which needs further investigation to cover its protective abilities in different types of radiations and irradiated organs along with exploring its mechanistic action. A previous report showed that TQ exhibited protective effects against nitrosative damage in the brain of rats exposed to gamma radiation by reducing nitric oxide (NO) and peroxnitrite levels and NO synthase activity (Ahlatci et al. 2014). Evidence came from a study focused on T lymphocytes balance in gamma irradiated rats illustrating a marked decrease in T cell exhaustion and apoptosis following TQ supplementation by modulating the expression of pro- and anti-apoptotic markers and pro-inflammatory cytokines (Guida et al. 2016). However, the ways by which UA and gamma radiation evoked testicular toxicity have faces of both differences and similarities. Both of UA and gamma radiation induced genotoxicity, oxidative stress, and histopathological lesions in the seminiferous tubules and Leydig cells. However, UA has inflammatory properties, while gamma radiation contribute to testicular dysfunction by interfering with cellular antioxidant protective system, changing mitochondrial electron transport chain, and stimulating apoptotic pathway mediators (Aitken and Roman, 2008; Azzam et al., 2012; Hossein Asghari et al., 2015; Khan et al., 2015; Marzban et al., 2017; Szumiel, 2015).

Being an analog and a precursor for intracellular glutathione (GSH), NAC is very effective in replenishing tissue GSH content which plays a key role in enhancing the antioxidant defense mechanism against radiation injury (Chatterjee 2013; Li et al. 2015). Several types of somatic and germ cells have a high GSH content including Sertoli and peritubular cells, pachytene spermatocytes, round spermatids, and interstitial tissue (Bauché et al., 1994). NAC alleviated the radiation challenges on hippocampus, bone marrow and liver by inhibiting lipid peroxidation, stimulating redox buffer systems, and down-regulating caspase-3 gene expression (Kilciksiz et al. 2008; Demİrel et al. 2009; Li et al. 2015). However, there is no available information yet about the potential radioprotective effects of TQ or NAC on UA irradiated testis, therefore the aim of this study is to investigate this issue through monitoring the changes in the oxidant/antioxidant balance, DNA fragmentation and histopathological features in the testes of Sprague Dawley rats.

Materials and Methods

Animals and experimental groups

More than forty male Sprague Dawley rats at 6-8 weeks of age obtained from the Animal House, Faculty of Medicine, Assuit University, Assiut, Egypt, and bred under controlled conditions with 12 hr light/dark cycle, a temperature of 23 °C and a relative humidity of 55%. Food and water were provided *ad libitum*. After acclimatization period of 5 days, rats weighing 180-200 g were selected for the experiment. Rats were randomly and equally divided into four groups, 10 rats each (5 rats per cage). One group was injected intraperitoneally with distilled water and kept as a control, while the second group was injected intraperitoneally with a single dose of UA (1 mg/kg BW) (Barber et al. 2005). Because uranyl ions are the most stable types of U in solution and mammalian body fluids, U was administrated as UA (Legendre et al. 2016). The third and fourth groups were injected with the above mentioned dose of UA followed by oral administration of TQ at a dose of 20 mg/kg BW (Badr et al., 2013) and intraperitoneal injection of NAC at a dose of 100 mg/kg BW (Prakash and Kumar 2009) for 25 days. The protocol was carried out according to the National Institute of Health Guide for Care and Use of Laboratory Animals.

Samples collection

At the end of the experiment, after 25 days of injecting UA, rats from the different groups were killed by cervical dislocation. Testes were quickly removed and one testis fixed in 10% neutral buffered formalin for histological investigation. The other testis was stored at -20 °C to be used for determination of oxidant/antioxidant markers. To prepare 10% w/v homogenate, testis was homogenized in 0.1 M phosphate buffer (pH 7.4) using IKA Yellow line DI homogenizer (18 Disperser, Germany). The homogenates were centrifuged at 8000 rpm for 15 minutes at 4 °C, and the supernatant cytosols were kept frozen at -20 °C for the subsequent biochemical assays.

Biochemical measurements

In the supernatant of testicular homogenate, total protein (TP) concentration was determined by the method of (Lowry et al. 1951). Lipid peroxidation products (LPO) as thiobarbituric acid reactive substances (TBARS) were estimated according to the method of (Ohkawa et al. 1979). NO was measured as nitrite concentration colorimetrically using the method of (Ding et al. 1988). The activity of superoxide dismutase (SOD) was determined according to its ability to inhibit the autoxidation of epinephrine in alkaline medium according to the method of (Misra and Fridovich 1972). Glutathione (GSH) content was determined using the method of (Woolliams et al. 1983).

Histopathological examination

Specimens from the testicular tissues were fixed in formalin buffer, dehydrated in a series of ethyl alcohol, cleared in xylene, and then embedded in paraffin. Four micron sections were cut, stained with haematoxyline and eosin (HE), and examined by the light microscope.

TUNEL assay

TUNEL assay was done to check apoptosis in 5-7-um paraffin embedded testis (Ahmed et al. 2013) according to the manufacturer's protocol (In Situ Cell Death Detection Kit, POD; Roche Diagnostics GmbH, Germany). The kit also includes Converter-POD:anti fluorescein antibody, Fab fragment conjugated with peroxidase (POD), thus it was possible to visualize the apoptotic signal with DAB staining and also in fluorescent color.

Immunofluorescence and Immunohistochemistry

In order to check whether Uranium exposure induces persistent damage in testicular cells or not, immunostaining was performed as described earlier (Ahmed et al. 2017). Rabbit anti γ -H2AX (dil1:500, Millipore) and mouse anti 53BP1 (1:300, Novus) primary antibodies were used in the immunostaining experiments, and were diluted in PBS, 0.1% Tween 20, 0.2% BSA, and 1% Milk powder. The 2^{ry} antibodies used were HRP goat anti-rabbit (dil1:500, DAKo) or goat anti-rabbit (488, dil1:500, Alexa flour), and Cy3 goat anti-mouse (Alexa Flour 546). Preparations were mounted in Vectashield (Vector Labs) containing 0.5 mg/mL DAPI (Sigma) to stain nuclear DNA.

Statistical analysis

Data were represented as means \pm standard error of means (SEM). The results were analyzed by one-way analysis of variance (ANOVA) followed by Duncan posttest using SPSS program version 16 (SPSS Inc., Chicago, USA). Differences of *P*<0.05 were considered to be statistically significant. One-way ANOVA had been used to examine the differences between the various experimental groups using the difference in group as an independent factor and the change in the assessed parameter as a dependent factor.

Results

Oxidant/antioxidant balance

In the present study, UA caused a marked disturbance in the free radicals-antioxidants balance in testes of rats manifested by significant reductions in SOD activity and NO and GSH levels as compared with the control group. However, there were insignificant changes in LPO levels in the UA intoxicated group versus the unexposed one. Surprisingly, the studied therapeutic strategies failed to modulate the testicular oxidant/antioxidant balance in the UA irradiated rats, and the testicular SOD activity and NO and GSH levels of the treated groups still significantly lower than those of the control group (Table 1).

Histopathological examination

The testicular tissues of control rats showed normal seminiferous tubules displaying the different stages of spermatogenesis (Fig. 1A). Examination of the histopathological sections shows that UA caused alterations in the blood vessels and the stages of spermatogenesis. Vascular alteration included interstitial edema with pale acidophilic material (Fig. 1A). The blood vessels were congested (Fig. 1B). Degenerative changes extended to necrosis were observed in some seminiferous tubules (Fig. 1D). Histological examination of the testicular tissues of TQ treated group showed normal appearance (Fig. 1E), while the testes in NAC treated group revealed normal appearance with minor changes as congestion and edema (Fig. 1F). The histopathological lesions score of different groups are listed in table 2.

Uranium exposure increases spermatocytes metaphase apoptosis

Stage XIII in rat's seminiferous tubules is the stage during which diplotene spermatocytes divide (MI) to give 2^{ry} spermatocytes which in turn divide (MII) at stage XIV to give the early round spermatids that appear in stage I. To check checkpoint activation in the testes of treated rats (meta-phase I and II), we stained the testicular tissue sections of all groups for apoptosis using the TUNEL assay (Fig. 2 & 3). Staging of seminiferous epithelium cross section in rats has been done according to Russel et al., 1990. As illustrated in (Fig. 3E), the frequency of apoptotic cells in testes of UA treated rats in the stage XIII is significantly higher relative to the control and other treated groups (around 7 apoptotic nuclei per epithelial stage) probably indicating difficulties in segregation of homologous chromosomes during metaphase I (Fig. 3B). However, there were apoptotic spermatogonia (cells located at the boundaries of seminiferous epithelium) in UA, UA/TQ and UA/NAC treated groups (Fig 2F, arrows-head). Quantification of these cells in 45 tubules from 3 rats per group reveals no clear vacation between treated groups (Fig 3F).

Uranium exposure induces no obvious persistent damage related foci in Spermatocytes

To further investigate the reason of an activated spindle assembly check-point (SAC) during MI, we examined the presence of γ -H2AX foci at late spermatocytes in testicular sections stained for antibodies against the DSBs marker γ -H2AX. Data showed that some spermatocytes of UA treated group displayed γ -H2ax foci indicating persistent damage at only few spermatocytes (Fig 4B). Quantification of cells with foci have shown that there was insignificant increase in % of cells with clear foci (1-3) in UA groups (Fig 4E). This indicates that exposure to UA does not induce obvious persistent damage related foci at spermatocytes, however the pronounced increase in metaphase I apoptosis could be due to improper DSB repair during the meiotic recombination of UA treated group.

Double immunostaining of testicular sections with both another DSB marker, 53BP1 and TUNEL assay (Fig 4 C, D) revealed that apoptotic metaphase cells are negative for 53BP1. Then, the cytotoxic effect of UA might be a direct effect on metaphase check point or it is possible that the earlier improper repair has led to this partial metaphase arrest.

Discussion

In the current study, a single dose of UA caused testicular toxicity, this was evident by the reduction of the testicular GSH and NO levels and SOD activity, the activation of the metaphase check point at epithelial cycle stage XIII, and the appearance of degenerative and vascular pathological changes. On the other hand, TQ and NAC succeeded in attenuating the apoptotic and histopathological influences of UA on the testis without inducing any antioxidant effect.

GSH, the most abundant nonprotein thiol, is an essential players in establishment of redox balance and was reported to have radioprotection against DNA damage (Chatterjee, 2013). Therefore, depletion of testicular GSH induces oxidative stress, especially when taken into consideration the high content of polyunsaturated fatty acids in testis (Periyakaruppan et al., 2007). In the present study, SOD activity was significantly inhibited in the UA treated rats, which is consistent with the previously shown in erythrocytes (Bellés et al., 2007). SOD level in the testis is a relatively high compared to other organs and its inhibition resulted in impaired sperm motility, and hence infertility (Mruk et al., 2002; Garratt et al., 2013). This response has been attributed to down-regulation of SOD gene expression (Periyakaruppan et al., 2007). In the testis, NO displays a wide range of regulatory effects on blood flow, spermiogenesis, sperm viability, sperm motility, apoptosis, in addition to its cytoprotective functions (Kono et al., 2006). The significant reduction in the testicular NO level, observed in the present study, is in parallel with the decreased inducible NO gene expression following intratracheal instillation with DU particles in rats (Li et al., 2005). The non-significant changes in LPO levels relative to the control group in our study is consistent with that found in the renal cortex of UA exposed rat model (Kato et al., 1994). Selective differential sensitivity of enzymatic antioxidant to the inhibitory influence of DU on their activities could be a possible explanation (Lestaevel et al., 2015; Lestaevel et al., 2009; Linares et al., 2006).

In the current model, no obvious persistent damage related γ -H2ax foci was found in spermatocytes, 25 days post exposure to a single dose of UA. However, a pronounced increase in metaphase I apoptosis was observed. In rats, 52 days are required for spermatogonia stem cells to be differentiated into mature spermatozoa, and since the cycle duration of rats is 12.9 day (Clermont and Harvey 1965). In the current model, 25 days are enough for the early spermatocytes to do the meiotic recombination and reach the 1st meiotic division. Although some spermatocytes of the UA treated group displayed γ -H2ax foci, that was insignificant and is not enough to propose that UA induced damage-related foci in spermatocytes persist for 25 days post exposure.

Previously, we and others have indicated that a single dose of ionizing radiation (doses of 0.5-1 Gy, X- or gamma-irradiation) induced persistent damage related foci for several hours/few days post exposure (Ahmed et al. 2007; Ahmed et al. 2010; Paris et al. 2011; Ahmed et al. 2015). Considering that UA exposure has dual effects, cytotoxic and radioactive, it seems that UA induces genotoxic damage in germ cells during meiotic recombination, after spermatogonia stage, may persist but not for weeks or is improperly repaired and thus activates the metaphase checkpoint when reaching metaphase I. Even at very low doses of IR, spermatogenesis are being arrested, primarily by inducing apoptosis in spermatogonia, with Spermatogonia stem cells revealed elevated levels of DSBs for weeks after radiation (Grewenig et al. 2015). Here, there was insignificant increase in number of apoptotic spermatogonia in UA treated group, probably for UA genotoxicity, 25 days is a longer period to find an obvious effect on spermatogonia.

Failure of either TQ or NAC to enhance the redox sensitive balance in this study represents a major surprise owing to their well-known antioxidant activities. Nevertheless, evidences had been amassed from the literature denoting the diversity in the response patterns to TQ and NAC supplementation in relation to the differences in the studied doses and experimental models. TQ maintains its antioxidant property when utilized in low concentration and switches to be oxidative stress inducer when high concentrations were utilized, or even low concentrations in presence of transition metal ions as copper (Zubair et al. 2013). The antineoplastic property of TQ has been related to its ability to produce reactive oxidants and reduce GSH levels behaving as a pro-oxidant (Koka et al. 2010; Ashour et al. 2016; Taha et al. 2016). The same mechanistic factors were also involved in its antifungal effect on *candida glabrata* planktonic cells and biofilms (Almshawit and Macreadie 2017).

In the present study, TQ exhibited a marked cytoprotection in parallel with the findings in previous gamma irradiated models (Ahlatci et al. 2014; Guida et al. 2016). Also, TQ improved the testicular architecture against cadmium and titanium dioxide nanoparticles induced toxicity in rats (Sayed et al., 2014; Hassanein and El-Amir, 2017). According to our experimental design and measured parameters we are unable to suppose the antioxidant role of TQ in this outcome, however

its anti-inflammatory and anti-apoptotic properties may be the main causative factors in the testicular cytoprotection by reducing the pro-inflammatory cytokines levels, and down-regulating PD-1, Bax and caspase-3 expression and up-regulating Bcl-2 expression (Guida et al. 2016). Nevertheless, the partial alleviation in the testicular histopathological deteriorations and the reduction of apoptotic cells numbers following NAC supplementation indicates improvement at least on the cytological level which is corresponding to previous studies on gamma irradiated liver and intestine (Sridharan and Shyamaladevi 2002; Mansour et al. 2008). This has been explained by inhibition of DNA fragmentation, down-regulation of caspase-3 expression and stimulation of radioprotective IL- 1α and β and IL-2 production (Mansour et al. 2008; Li et al. 2015).

Conclusion

An obvious disturbance in the testis redox homeostasis and histopathological and cytological alterations in the testicular tissues following 25 days of exposure to a single dose of UA had been observed. That was associated with a partial arrest of metaphase cells (MI-MII) which may explain why less spermatids and low fertility have been reported earlier after oral exposure to doses of UA (10-40 mg/kg/BW) for two weeks (Linares et al., 2005). However, TQ and NAC attenuated the adverse effects of UA on the testicular microenvironement through anti-apoptotic and cytoprotective but not through antioxidant effects. Further studies are highly recommended to investigate UA toxicity within several hours and few days post-exposure, in addition to the protective effects of other natural compounds.

Disclosure statement. The authors declare that there are no conflicting interests.

Author's contribution: H Waly, S MM Ragab, N S Abou Khalil and E A Ahmed, designed the experiment, performed the practical work and wrote the paper, KA Hassanein analyzed the H&E stained slides.

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Figures legends

Figure 1. (A) Testis of control rats showing normal histological architecture. B) Testis of UA treated group showing interstitial edema (arrow), C) Congestion (arrow), D) Necrosis of seminiferous tubules (arrows). E) Testis of TQ treated group demonstrating normal histology. F) Testis of NAC treated group demonstrating normal histology as well as edema in the interstitial tissue (arrow).

Figure 2. UA exposure increases spermatocytes metaphase (stage XIII) apoptosis. Control testes stages XI-XII (A) and stage XIII (B), arrow shows normal metaphase cells, arrowhead shows 2ry spermatocytes. High frequency of apoptotic cells in UA treated rats (stage XIII, C & D), arrows show apoptotic dividing MI cells, Stage XIII from TQ (E) and NAC (F) treated rats with no apoptotic metaphase cells, (sp; spermatogonia, eSC; early spermatocytes, ISC; late spermatocytes. magnification bar = 10 um.

Figure 3. Difficulties in segregation of homologous chromosomes seen only in UA treated rats (B), but not in other treated groups, WT (A), TQ (C) and NAC (D. (E) a significant increase in Metaphase apoptosis after UA exposure. (F) an insignificant variation in number apoptotic spermatogonia from control and treated groups. magnification bar = 10 um.^{abc} Different letters indicate significant difference at *P*<.05 (one-way ANOVA followed by Duncan posthoc test). Fig. 4. Persistent damage related foci in spermatocytes following UA exposure induction (A & B). Double immunostaining of 53BP1 and TUNEL assay (stage XIII) from WT (C) and UA group (D). (E) Insignificant increase in % of cells with foci in UA group relative to other treated groups. magnification bar = 10 um. **Table 1.** Effects of TQ and NAC on the oxidant/antioxidant markers in the testicular homogenate of UA intoxicated rats. UA, urenyl acetate; TQ, thymoquinone; NAC, N-acetyl cysteine; LPO, lipid peroxidation; NO, nitric oxide; SOD, superoxide dismutase; GSH, reduced glutathione. Results are represented as means \pm SEM (n = 10 rats/group). ^{ab} Different letters indicate significant difference at *P*<.05 (one-way ANOVA followed by Duncan posthoc test).

Group				
	Control	UA	UA/TQ	UA/NAC
Parameter				
LPO (nmol/mg protein)	1.00±0.265 ^a	0.796±0.206 ^a	0.586±0.098ª	0.928±0.154 ^a
NO (nmol/mg protein)	0.035±0.005 ^a	0.019±0.002 ^b	0.015±0.001 ^b	0.025±0.003 ^{ab}
SOD (U/mg protein)	7.815±0.666 ^a	4.679±0.648 ^b	4.665±0.318 ^b	4.959±0.269 ^b
GSH (nmol/mg	177.57±48.341 ^a	64.846±6.984 ^b	64.191±10.853 ^b	59.917±2.654 ^b
protein)				

Table 2. Incidence of the histopathological lesions in testes of the experimental groups, - nulllesions; + presence lesion in (1-3 rats); ++ presence lesion in (4-6 rats); +++ presence lesion in (7-10 rats).

Group	Control	UA	UA/TQ	UA/NAC
Lesion				
Congestion	-	+++	-	+
Interstitial edema	-	+++	-	+
Testicular degeneration	-	++	-	-
Necrosis	-	++	-	-





Fig 4.





Fig 2.