Ionizing Radiation Evokes Disturbed Redox-Active Metabolite Homeostasis and Antioxidant Status in Rat Testicular Tissue

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ABSTRACT: The present work aimed to determine the effect of different doses of ionizing radiation of x-rays on the level of some redox-active metabolites and gross antioxidant status in rats' testicular tissues that were previously exposed to different doses of ionizing radiation. Rats were exposed to different doses of x-rays ionizing radiation for 20 seconds/day for a period of 9 days. The level of reduced glutathione (GSH), vitamin C and level of malondialdehyde (MDA) were determined. In addition, the free radical scavenging property as well as the ability of the whole testicular tissue to reduce and chelate the redox-active transition metal iron was used as indices of the gross antioxidant status of the rats' testes. The results showed that while the ionizing radiation evoked a dose-dependent diminution on the level of GSH and vitamin C, it caused an increase in the level of MDA, an index of oxidative assault on cellular lipids. In addition, the ionizing radiation reduced the ability of the whole testicular tissue to scavenge radicals, reduced Fe³⁺ and chelate Fe²⁺. Consequently, it appears that while the ionizing radiation diminished the level of antioxidant biomolecules, it also exerted deleterious redox pressure on the gross antioxidant defense system of the testicular tissues.

Keywords: PLS PROVIDE KEY WORDS

INTRODUCTION

Ionizing radiation (IR) is a type of electromagnetic wave or particle that removes tightly bound electrons from an atom, causing it to become ionized (Pernot, et al., 2012). Consequently, after deposition of radiation energy in biological medium, several cascades of events ranging from physical, chemical, biochemical, and cellular responses are initiated, and this ultimately generates radicals in living matter with its attendant consequences (Panganiban et al., 2013). The physicochemical basis for ability of ionizing radiations to induce formation of radicals is integrally linked to the high energy associated with ionizing radiation which is significantly greater than the bond energies of many molecules and can cause homolytic bond scission and the generation of secondary electrons. The time scale of the initial steps of energy deposition and molecular bond scission is on the order of 10⁻¹³ s (Soule et al., 2007).

These radicals can either be created directly in critical biomolecules, or they can be formed in water and the ensuing radicals may directly attack other biomolecules of physiological importance (Kade et al., 2008). Of note is the fact that the overwhelming majority of the biological effect of ionizing radiation is thought to be mediated by free radicals in water. In fact, since water is the main constituent of cellular matter, it is primarily the ionization of water that results in the production of secondary species with high...
reactivity and short life times ($10^{-10} - 10^{-9}$ s) such as the OH radical, aquatic electrons, or hydrogen atoms which is the secondary species that mediate the chemical reactions that damage biologically important molecules that are possible critical targets in radiation-induced cytotoxicity (Pernot, et al., 2012). Although, it is a common knowledge that the main target of ionizing radiation damage is believed to be the DNA in the cell nucleus [Morgan, 2003], the validity of the current radiation damage models has been challenged [Pinto et al., 2002]. The discovery of non-targeted phenomena, such as radiation-induced genomic instability in the progeny of cells that survive after irradiation [Aypar, et al., 2011; Caputo et al., 2012] and bystander effects on cells that have not directly been exposed to radiation [Mancuso et al., 2012], call this central dogma of radiation biology further into question [Morgan, 2003]. There are reports of the effects of radiation on cell organelles other than the nucleus [Bloomet al., 1955; Deshpande et al., 1996; Hickman et al., 1994; Somosy, 2000]. It has been suggested that these extranuclear effects are not subsequent to nuclear responses to radiation but are instead due to the direct effect of radiation on other organelles [Deshpande et al., 1996; Hickman et al., 1994; Somosy, 2000].

Indeed, the fact that ionizing radiation-mediated free radical generation (or oxidative stress) can elicit deleterious effects on a wide variety of cellular processes ranging from disruption of cellular communication to cell death [Aypar et al., 2011], makes the elucidation of the precise mechanism(s) of the deleterious effect of ionizing radiation on biological processes an intriguing area of intensive research. This becomes imperative since many pathological situations such as neurodegenerative diseases, cardiovascular problems, infertility, cancer, and aging [Caputo et al., 2012] are thought to be mediated by radicals. Consequently, study on the effect of ionizing radiation on cellular processes must be all-inclusive.

More importantly, while several reports have clearly observed that there is a direct association between ionizing radiation and increased oxidative stress in living system, the oxidative stress parameters employed in such studies are generally not all-inclusive and thus still open. In most reports, the level of some redox-active tissue metabolites are used as measures of oxidative stress markers rather that the total antioxidant status of the whole tissues. In this regard, this study was intended to determine some key antioxidant parameters as indices of whole antioxidant status of testes of albino rats exposed to various doses of x-ray ionizing radiation.

**MATERIALS AND METHODS**

**Animals**

Male albino Wistar rats weighing 200-250 g were kept in plastic cages and were allowed free access to water and normal pellet diet and were maintained under controlled conditions of humidity, temperature and a diurnal environment. Animals were acclimatized to laboratory before starting the experiment and during the irradiation period. All animal procedures were carried out in accordance with standard practice of the use of experimental animals.

**Exposure specification**

- **Radiation dose 1:** Voltage, $V = 40$ kV; Electric current, $I = 80$ mA; Time of exposure per day, $t = 20$ sec; Power, $P = 40$ kV $\times 80$ mA $= 3200$ W, Energy, $E = 4.0 \times 10^3$ eV $= 64$ kJ
- **Radiation dose 2:** Voltage $V = 60$ kV; Electric current $I = 120$ mA; Time of exposure per day, $t = 20$ sec; Power, $P = 60 \times 120$ mA $= 7200$ W; Energy $E = 120$ mA $\times 60$ kV $\times 20$ sec $= 144$ kJ
- **Radiation dose 3:** Voltage, $V = 90$ kV; Electric current, $I = 160$ mA; Time of exposure per day, $t = 20$ sec; Power, $P = 160$ mA $\times 90$ kV $= 14.4$ kW.
Energy, \( E = 160 \text{ mA} \times 90 \text{ kV} \times 20 = 1.8 \times 10^4 = 288 \text{ kJ/eV} \).

The radiation dose absorbed by the animals is estimated using equations (1) and (2). [Andrew. 1978]

\[
Dose = \left( \frac{\mu_{en} \cdot N \cdot E \cdot \rho \cdot A}{A \cdot \rho \cdot x} \right) = \mu_{en} \cdot N \cdot E \quad (1)
\]

where energy absorbed/mass, \((\mu_{en}/\rho) = \text{mass energy absorption coefficient (cm}^2/\text{g})\), \(N = \text{photon fluence (photons}/\text{cm}^2)\), \(E = \text{energy per photon}, \rho = \text{density}, x = \text{thickness}, A = \text{area}\). On the other hand, the equivalent ionizing radiation dose absorbed by the tissue is obtained from the relation:

\[
H_T = \sum R W_R \cdot D_{T,R} \quad (2)
\]

where \(D_{T,R} = \text{the absorbed dose in tissue } T\) by radiation type \(R\) (X-ray); \(S W_R\) = weighting factor.

**Irradiation procedure**

Rats were kept in plastic cages (18 cm length by 9 cm width) approximately at 20 cm from the x-ray source. Whole body irradiation of animals was performed using different doses of x-ray for nine days with each rat exposed to the radiation for 20 seconds per day. The animals were divided into four groups. Group 1, control animals received no radiation; Groups 2, 3 and 4 were exposed to different energy doses of ionizing radiation of 64 kJ, 144 kJ and 288 kJ respectively. The animals in the control group, though not irradiated, were transported to the irradiation facility to ensure environmental homogeneity. At the end of the experimental period, all rats and the corresponding control animals were anesthetized with ether and euthanized by decapitation. Rats were fasted 12 h prior to euthanasia.

**Preparation of Tissue Homogenates**

Rat testes were quickly removed, placed on ice and homogenized in cold 50 mM Tris–HCl pH 7.4. The homogenates were centrifuged at 4,000 x g for 10 min to yield the low-speed supernatant (S1) fraction that was used for lipid peroxidation assay. For all other antioxidant assays, protein in S1 were precipitated in 10 volumes of cold 4% trichloroacetic acid (TCA) solution to give supernatant S2 which was used for other antioxidant assays.

**Antioxidant Defense Systems**

**DPPH Free Radical Scavenging Activity**

The free radical scavenging ability of the testicular tissue against DPPH (1, 1-diphenyl-2 picrylhydrazyl) free radicals was evaluated as described earlier in our laboratory with slight modifications (Omololu et al., 2011). Briefly, 600 µL of properly diluted S2 were mixed with 600 µL, 0.3 mM methanolic solution containing DPPH radicals, the mixture was left in the dark for 30 min and the absorbance of the resulting golden yellow products was measured at 518 nm.

**ABTS**

\( \text{ABTS}^{+} \) free radical-scavenging activity

\( \text{ABTS}^{+} \) free radical-scavenging activity was determined as previously described by Re et al. (1999) with some slight modifications. Briefly, equal volume of 7 mM 2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and 2.45 mM potassium persulfate solutions were mixed and left overnight in the dark to generate \( \text{ABTS}^{+} \) radical cation. Later, \( \text{ABTS}^{+} \) solution was diluted with ethanol to an absorbance of 0.7 ± 0.05 at 734 nm. Properly diluted S2 (200 µL) was mixed with 1800 µL of diluted \( \text{ABTS}^{+} \) solution. The absorbance was measured 30 min after the initial mixing at 700 nm.

**GSH Level**

The GSH level in the testicular homogenates was determined as non-thiol protein and was estimated using Ellman’s reagent in S2 Ellman
The deproteinized testicular homogenates was also used for the assessment of antioxidant capacity of the testes. In all cases, the yellow color formed in the reaction systems were measured at 412 nm. The content of GSH is related to the weight of the tissue (µmol GSH/g wet tissue).

**Vitamin C content**

Vitamin C level in the testicular homogenate was determined colorimetrically as described by Jacques-Silva et al. (2001). An aliquot (1 ml) of S2 was incubated for 3 h at 37°C then 1 ml H₂SO₄ 65 % (v/v) was added. The reaction product was determined using a color reagent containing 4.5 mg/ml dinitrophenylhydrazine and CuSO₄ (0.075 mg/ml), and the absorbance of the colored product was measured at 520 nm using UNICO visible Spectrophotometer (USA). The content of ascorbic acid is related to tissue amount (µmol ascorbic acid/g wet tissue).

**Reducing Property**

The reducing property of homogenates was determined by assessing their ability to reduce FeCl₃ solution as described by Pulido et al. (2000). Briefly appropriated dilution of S2 were mixed with 250 µL, 200 mM sodium phosphate buffer (pH 6.6) and 250 µL of 1 % potassium ferrocyanide, the mixture was incubated at 50°C for 20 min, thereafter 250 µL, 10 % TCA was added, and subsequently centrifuged at 650 rpm for 10 min, 250 µL of S2 was mixed with equal volume of water and 100 µL of 0.1 g/100 ml ferric chloride, the absorbance of the resulting deep green solution was later measured at 700 nm, a higher absorbance indicates a higher reducing power.

**Chelating Assay**

The Fe²⁺ chelating ability of S2 was determined using a modified method described by Puntel et al. (2005). Freshly prepared 500 µmol/L FeSO₄ (150 µL) was added to a reaction mixture containing 168 µL of 0.1 mol/L Tris–HCl (pH 7.4), 218 µL saline or testicular tissue homogenate. The reaction mixture was incubated for 5 min, before the addition of 13 µL of 0.25 %, 10-phenanthroline (w/v). The absorbance of the orange color formed was subsequently measured at 510 nm. The Fe(II) chelating ability was subsequently calculated with respect to the reference (which contains all the reagents without the testicular tissue).

**TBARS Assay**

Thiobarbituric acid reactive substances (TBARS) in homogenates (S1) were determined as described by Ohkawa et al. (1979) except that the buffer for the color reaction was pH 3.4. The color reaction was developed by adding 300 µl 8.1 % SDS to S1, followed by sequential addition of 500 µl acetic acid/HCl (pH 3.4) and 500 µl 0.8 % TBA. This mixture was incubated at 95°C for 1 h forming a purple color product which was measured at 532 nm and the absorbance was compared to that of a standard curve obtained using malondialdehyde (MDA).

**Data analysis**

Data were grouped by treatment and are presented as mean ± S.E.M. Potential differences in bar plots were determined by one-way ANOVA followed by Duncan’s test. Differences were considered statistically significant when \( p < 0.05 \).

**RESULTS**

**Effect of x-ray radiation on radical scavenging ability of tissue**

The ability of the tissues to scavenge free radicals is presented in Figure 1 (panel A and B). In this Figure, it is apparent that the ionizing radiation markedly diminished the free radical scavenging activity of the testicular tissues in a dose dependent manner (\( p < 0.05 \)). More interestingly, the adverse effect of the ionizing radiation to be multi-factorial as the testicular
tissue lost its ability to scavenge the different radicals, either the DPPH radicals (panel A) or the protonated radicals (ABTS) (panel B).

**Effect of x-ray radiation antioxidant metabolites of tissue**

The levels of the antioxidant metabolites (GSH and vitamin C) are used as common indices to assess the antioxidant status of tissues under conditions of oxidative stress. As presented in Figure 2 (panel A), it is apparent that the ionizing radiation evoked a significant diminution in the level of the redox-active metabolite (GSH) (panel A), and vitamin C (panel B) in the testicular homogenate. Equally, as indicated in the Figure, the effect of the radiation on the amount of the testicular antioxidant biomolecules evaluated is dependent on the absorbed radiation dose.

**Figure 1:** Radical scavenging activity of whole testicular homogenates exposed to different doses of ionizing radiation. Evaluation was done using DPPH (panel A) and ABTS (panel B) radicals. Data are expressed as means±SD (n=7). *indicates a significant difference from control at p<0.05.

**Figure 2:** Levels of GSH (panel A) and Vitamin C (panel B) in whole testicular homogenates exposed to different doses of ionizing radiation. Data are expressed as means±SD (n=7). *indicates a significant difference from control at p<0.05.
Effect of x-ray radiation on ability of the tissue to reduce Fe$^{3+}$ or chelate Fe$^{2+}$

Figure 3 (panel A) shows the ability of the tissue to reduce Fe$^{3+}$. Herein, it is apparent that the ionizing radiation markedly abolished the ferric reducing antioxidant potency of the tissue in a dose dependent fashion. Similarly, as presented in Figure 3 (panel B), the ionizing radiation also exerted a deleterious effect on the Fe$^{3+}$-chelating potential of the tissue in a manner similar to what is observed in Figure 3 (panel A).

Effect of x-ray radiation on malondialdehyde level of the tissue

The level of malondialdehyde often expressed as a measure of thiobarbituric acid reactive substances has often been employed to measure the extent of lipid damage in cells and tissues. In Figure 4, it is apparent that the ionizing radiation exerted a profound dose-dependent increase in the level of malondialdehyde in tissue homogenates pre-exposed to the radiation.

Figure 3: Ferric reducing antioxidant power (panel A) and Fe$^{2+}$-chelating ability (panel B) of whole testicular homogenates exposed to different doses of ionizing radiation. Data are expressed as means±SD (n=7). *indicates a significant difference from control at p<0.05.

Figure 4: Level of MDA in whole testicular homogenates exposed to different doses of ionizing radiation. Data are expressed as means±SD (n=7). *indicates a significant difference from control at p<0.05.
DISCUSSION

Several reports have described the relationship between ionizing radiation and generation of free radicals in biological systems (Azzam et al., 2011; Smith et al., 2012). In fact, it is now a common knowledge in the field of radiation research that there is a strong correlation between the role of ionizing radiation in generation of reactive oxygen species and the extent of genetic damage and oxidative damage consequent from such radiation assault (Costantini, 2014; Galván et al., 2014). Indeed, oxidative damage has been proposed as a possible fundamental mechanism of the diverse detrimental effects of radiation which is often characterized by increased concentrations of ROS, decreased activity of antioxidant enzymes, and genetic damage (Spitz et al., 2004), which ultimately leads to severe medical conditions mainly radical mediated degenerative pathologies (Halliwell and Gutteridge, 2007). Elegant and intriguing as these reports may be, the measures employed to assess antioxidant status of animals exposed to ionizing radiation is not all inclusive. In most cases, researchers have quantified the amount of redox-active metabolites, expression and activities of antioxidant enzymes, yet the antioxidant status of the gross structure of the assaulted organs are often not evaluated. Hence, this study was intended to evaluate the gross antioxidant status of the testicular organ of rats exposed to intermittent doses of ionizing radiation. Particularly, the radical scavenging capacity and ability of the whole testicular tissue to counteract the deleterious effect of transition metals in the generation of oxidative stress will be given attention.

In general routine evaluation of antioxidant potency of any substance, the ability of the antioxidant substances to scavenge DPPH radicals have always been employed. DPPH radicals are unstable free radicals that attains stable configuration on interaction with any proton-rich species (Omololu et al, 2011). Similarly, the ability of antioxidants to scavenge the protonated radicals, ABTS, has also been employed as an index of antioxidant potency of any substance. Plausible as these approaches have been in the evaluation of the antioxidant properties of natural and synthetic compounds, this approach has not been frequently employed as a critical parameter to determine the radical scavenging ability of the whole tissues in mammals (Kade and Rocha, 2013). However, it appears that in Figure 1, the ionizing radiation significantly diminished the gross DPPH (Figure 1 panel a) and ABTS (Figure 1 panel b) radicals scavenging activity of the testicular tissues. A possible reason for this observation may be directly related to depleted levels of the possible antioxidant biomolecules that possess potent radical scavenging activity. In this regard, the observed reduction in the radical scavenging potency of the renal tissue appear to positively correlate with the reduction in the amount of the radical scavenging biomolecules, GSH (Figure 2 panel a) and vitamin C (Figure 2 panel b). The observed depletion of the antioxidant biomolecules mediated by the ionizing radiation may be difficult to explain within the limit of the current data. However, in line with basic physics and chemistry of ionizing radiation, there is the possibility that the ionizing radiation may directly modify these antioxidant biomolecules thus leading to the consequent loss of their radicals scavenging functions. Conversely, their depletion may be related to their likely utilization by antioxidant enzyme defense systems such as glutathione peroxidase that depends on glutathione for its redox catalytic cycle to reduce potentially hazardous hydroperoxides (Kade et al., 2008, 2009).

Other antioxidant indices measured in this study are the ability of whole renal tissue to reduce Fe$^{3+}$ or chelate Fe$^{2+}$. Iron as a proxidant have been reported to be involved in the generation of hydroxyl radicals via Fenton reaction (Graf et
al., 1984), which ultimately leads to the formation of other reactive oxygen species (ROS) (Klebanoff et al., 1992). Moreover, antioxidants could chelate transition metals, specifically iron, preventing it from being oxidized since it is physiologically useful in the Fe$^{2+}$ state. Hence, the antioxidant activity of compounds can be measured by assessing their ability to chelate transition metals of physiological relevance (Omololu et al., 2011, Kade and Rocha, 2013). Equally since oxygen transport by hemoglobin in the blood is mediated by Fe$^{2+}$, it therefore becomes highly imperative that any available Fe$^{3+}$ is reduced to the physiologically relevant oxidation state of Fe$^{2+}$ (Ogunmoyole et al., 2011). In fact, as indicated in Figure 3, the ionizing radiation markedly diminished the effectiveness of the whole renal tissue to either reduce Fe$^{3+}$ or chelate Fe$^{2+}$.

In line with the foregoing, it may be helpful to state that the reducing power as a defense mechanism is related to the ability of the antioxidant agents to transfer electron or hydrogen atom to oxidants or free radicals (Ogunmoyole et al., 2009; 2011). Consequently, in the whole testicular tissue homogenate, the observed diminution in the ability of the whole testicular tissue to reduce Fe$^{3+}$ with increasing dose of ionizing radiation may be associated to the depleted antioxidant biomolecules. Interestingly, GSH and vitamin C have been reported as potent reductant (Barbosa et al., 2006).

However, there are reports indicating that most biologically active Fe$^{2+}$ chelators such as polyphenols possess multiple hydrophilic groups that are efficient scavengers because phenolic groups inhibit iron-mediated oxyradical formation like other iron chelators, such as desferrioxamine, 1,10-phenanthroline, and pyridoxal isonicotinoyl hydrazone (Omololu et al., 2011). Chelation of transition metals often leads to their deactivation and prevents such metals from participating in the initiation of lipid peroxidation, protein carbonylation, DNA assault and oxidative stress through metal-catalyzed reactions and this is considered an antioxidant mechanism. (Kade et al., 2008). Consequently, the fact that the iron chelating ability of the whole testicular tissue diminished with increasing doses indicates that these polyphenolic compounds possibly derived from the diet of the rats may be implicated. These polyphenols may be depleted arising from antioxidant defense pressure possibly arising from the oxidant stress mediated by the radicals. An important biochemical and molecular implication arising from the disturbed iron homeostasis is the generation of hydroxyl radicals in the presence of endogenous hydroperoxides. An increased generation of these radicals leads to increased reactive oxygen species (Ogunmoyole et al., 2009). However, overproduction of reactive oxygen species attack membrane lipids abstracting protons thereby causing oxidation of lipid otherwise called lipid peroxidation. In fact, several reports have associated increased lipid peroxidation measured as malondialdehyde equivalent as a first line marker of the deleterious effect of ionizing radiation (Pernot et al., 2012). Consequently, the result presented in the study (Figure 4) is only in part confirmatory and is in agreement with earlier reports (Pernot et al., 2012). Apparently, the increasing doses of the ionizing radiation evoked a dose-related increase in the level of malondialdehyde generation (Figure 4).

Summarily, the uses of pharmacological and pathological parameters have been suggested as an alternative way of biodosimetry. When physical or chemical indicators of exposure to ionizing radiation, such as ionization chambers or film dosimeters, are not available, an estimate of the dose can be obtained by determining certain biological parameters [Pinto et al., 2010; Vinnikov et al., 2010]. Herein, free radical scavenging, ability to reduce and chelate transition metals such as iron, amount of
antioxidant biomolecules and the extent of oxidative damage to cellular lipids measured as malondialdehyde in the whole tissue are possible candidates for biodosimetric parameters at moderate doses.

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