Asian Journal of Biochemistry

ISSN 1815-9923

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Biochemistry 10 (3): 117-124, 2015
ISSN 1815-9923 / DOI: 10.3923/ajb.2015.117.124

Effect of Mn^{2+} on Glutathione-S-Transferase Activity of Human Ejaculated Spermatozoa

[1]Kuldeep Kaushik, [1]Pawan Kumar Mittal and [2]Natwar Raj Kalla
[1]Department of Zoology, Panjab University, Chandigarh, 160014, India
[2]Department of Biophysics, Panjab University, Chandigarh, 160014, India

Corresponding Author: Kuldeep Kaushik, Department of Zoology, Dev Samaj Post Graduate College for Women, Ferozepur City, Punjab, 152002, India

ABSTRACT

This study was undertaken to monitor the effect of 0.1 mM Mn^{2+} on the glutathione s-transferase activity (GST) in nicotine treated and untreated spermatozoa samples and to calculate the Michalis-Menten kinetic parameter of the reaction. Semen samples were collected from healthy volunteer donors after semen analysis, sample was subjected for preparation of 10,000 g supernatant. The enzymatic activity has been performed using 1-chloro, 2-4 dinitrobenzene (CDNB) as a substrate. The 0.1 mM Mn^{2+} supplementation was found to decrease the GST activities significantly ($p<0.001$). CDNB-GSH conjugate formation were almost significantly ($p<0.05$) lowered down with the addition of 0.1 mM Mn^{2+} to nicotine treated samples. The substrate affinity constant (k_m) of the reaction was found to be 44.44 μM. It remained unchanged throughout the V_{max} which was considerably lowered upon Mn^{2+} addition to the spermatozoal samples. The data was transformed to Lineweaver-Burk 1/v vs 1/[S] plot which indicate the behavior of this enzyme was non-competitive in nature.

Key words: Glutathione S-transferase, sperm, Mn^{2+}, V_{max}, k_m

INTRODUCTION

Cryopreservation of sperm is a common requirement and technique used in assisted reproductive technologies (ART), which has the potential to exacerbate sperm oxidative stress (Watson, 2000; Tremellen, 2008). It is observed that various sperm parameters like motility, membrane integrity as well as genetic makeup is impaired by oxidative stress implicated during assisted reproductive procedures. Sperm parameters are greatly influenced by oxidative stress and elicited the greatest curiosity to understand the underline mechanism(s) (Agarwal and Prabakaran, 2005). Micronutrients, vitamins and various enzymes are the integral part of human semen and prevent the damage of its genetic material, plasma membrane etc. caused by oxidative stress (El-Kannishy *et al*., 2011). During *in-vitro* fertilization and intra uterine insemination (IUI) treatment semen is centrifuged to separate sperm from seminal plasma. At the present moment commercial sperm preparation media do not contain any antioxidants aside from albumin and amino acids. Sperm preparation media may also be supplemented with a variety of antioxidants to guard against oxidative stress (Tremellen, 2008). It is reported that, among trace metal supplement, Mn^{+} is potent radical scavenger as compared Zn^{+}, Ni^{+} and Co^{+} (Coassin *et al*., 1992; Kaushik *et al*., 2015). Manganese is able to quench the peroxyl radical as, it is a potential chain breaking trace metal antioxidant (Coassin *et al*., 1992). It is also reported that lipid peroxidation (LPO) induced by free radical producing system is inhibited by Mn^{2+} (Cavallini *et al*., 1984).

Glutathione-s-transferases (GST) are a family of enzymes that catalyze a number of glutathione dependent reactions and have been primarily described as a cytosolic or micrsomal detoxification enzyme (Hayes and Pulford, 1995). Glutathione-s-transferases activities form a part of adaptive response of germ cells to oxidative stress and are important constituents in detoxifying the product of LPO (Rao and Shaha, 2000). Glutathione-s-transferases enzymes associated with spermatozoal plasma membrane show considerably higher activity as compare to the plasma membrane of brain cell (Hemachand *et al*., 2002). It catalyzes the formation of thioether conjugates from glutathione (GSH) and broad range of electrophilic compound and thereby, converts into more hydrophilic product and enhances its excretion (Jakoby and Habig, 1980). Glutathione-s-transferases catalyze the conjugation reactions in part, by stabilizing the thiolate anion GS-at the active site (Graminski *et al*., 1989). The catalytic advantage is clear, as GS is a superior nucleophile compared to GSH. Glutathione-s-transferases activity directly takes part in the elimination of product of lipid peroxidation and there is enhancement GST activity in spermatogenic cells after H_2O_2 exposure (Rao and Shaha, 2000). Being a house keeper, GST actively take the responsibility to detoxify endobiotics and xenobiotics substances (Laborde, 2010). Kumar *et al*. (2014) have been reported that GST plays an important role in sperm capacitation, acrosome reaction and fertilization. Mn^+ as well as GST plays a pivotal role in living cells under oxidative stress. The k_m value for GSTs for electrophiles is typically in the micromolar range which suggest that if the concentration of reactive lipid species is the nanomolar range, then its metabolism through GSH-GSTs pathway is minimal (Sheehan *et al*., 2001; Higdon *et al*., 2012). Keeping with the view of antioxidant potential of Mn^{2+}, the present work was undertaken to investigate the effect of trace metal ion, manganese (Mn^{2+}) on GST activity of human ejaculated spermatozoa and to calculate various Michalis-Menten kinetic parameter to understand the nature of Mn^{2+} supplemented enzymatic reaction.

MATERIALS AND METHODS

Chemicals: Nicotine, 1-chloro, 2-4 dinitrobenzene (CDNB), reduced glutathione (GSH), bovine serum albumin (BSA) Cohn fraction V and sodium dodecyl sulphate (SDS) were purchased from Sigma. Folin phenol reagent and $MnCl_2$ were procured from Himedia Laboratories (India) and other chemical used in the present investigation were purchased from SRL Private Limited, India.

Nicotine stock solution was prepared by dissolving in 0.2 M phosphate buffer saline (PBS) solutions. The 5% SDS solution was prepared in 0.5 N NaOH.

Collection of semen samples: After ethical approval, semen samples were collected from eighteen healthy nonsmoker volunteer donors through the courtesy of the General Hospital, Sector 16, Chandigarh between age group (20-25 years), accordance with the donor's consent. Semen analyses were performed after complete liquefaction and sperm concentration motility forward progression were evaluated subjectively at room temperature. Sample having final concentration $70\text{-}80\times10^6$ sperm cells mL^{-1} and with more than 70% motile sperm were selected. Seminal plasma discarded by centrifugation at 300×g for 10 min. The pellet, so obtained was suspended in equal volume of 0.2 M PBS.

Preparation of 10,000×g supernatant: The spermatozoal samples were suspended in 0.2 M PBS and gradually cooled to 4°C. They were homogenized in an ice bath using Teflon fitted homogenizer. The homogenate was centrifuged first at 1000×g and finally at 10,000×g for 30 min at 4°C. The supernatant was filtered through a plug of glass wool to remove floating lipids. The resultant solution was labeled as 10,000×g supernatant and was used for biochemical assays.

Measurement of GST activities: The activity of GST was determined by using method of Habig *et al*. (1974). The 0.1 mL of 10,000×g supernatant was treated or not with 0.5 mM nicotine and 0.1 mM Mn^{2+} was supplemented or not to these samples. Suitable control and blank were run simultaneously. The pH of the reaction mixture was maintained at 6.6 with 0.2 M phosphate buffer. The 0.1 mL of 30 mM CDNB was added and the reaction mixture was incubated at 37°C for 5 min, to initiate the reaction 0.1 mL of 30 mM glutathione (GSH) was added. The reaction was allowed to proceed for 5 min at 340 nm. The increase in absorbance was recorded after every minute. The average absorbance per minute was calculated thereafter. The substrate and reagent blanks were run simultaneously. The activity of GST was expressed as the amount of CDNB-GSH conjugate formed mg $prot^{-1}$ min^{-1}. The molar extinction coefficient (ε) of GSH at 340 nm is 9600 M^{-1} cm^{-1}. Substrate kinetics was performed by using GSH at final concentrations ranging from 10-50 mM.

Protein estimation: For the calculation of specific activity of GST, protein concentrations of various samples were estimated by SDS-Lowry method as described by Lees and Paxman (1972), using BSA (Cohn fraction V) as standard. The 0.9 mL of 5% SDS in NaOH was added to 0.1 mL of samples in test tubes. Tubes were allowed to remain at room temperature for at least 2 h and were agitated 2-3 times vortex. To make sure that the samples were dissolved thoroughly to this 2.5 mL of copper carbonate solution were added and tubes were allowed to stand for 20 min. Then 0.25 mL of 1 N folin phenol reagent was added, sample were mixed immediately and allowed to stand for 45 min. The intensity of color developed was read at 740 nm. Bovine serum albumin (BSA) standard (20-100 μM mL^{-1}) was also run simultaneously.

Statistical analysis: Statistical analysis was carried out employing SPSS-12 software. Data was expressed as Mean±SD for observation in each group. The statistical significance of inter group difference of various parameters was determined by unpaired student's t-test. Comparison was made between untreated (without nicotine) supplemented with Mn^{2+} and nicotine treated supplemented or not with Mn^{2+} samples.

RESULTS

The activity of GST was measured in the Mn^{2+} supplemented and unsupplemented (Control) spermatozoal samples using reduced glutathione as reaction substrate, the amount of CDNB-GSH conjugate formed as an end product of the reaction has been expressed in Table 1. The 0.1 mM Mn^{2+} supplemented was found to decrease the GST activities significantly ($p<0.001$). The reaction was also carried out in the presence of nicotine. The 0.5 mM nicotine added was observed to increase the activity of this enzyme. However, the addition of 0.1 mM Mn^{2+} to nicotine treated samples could lower the amount of CDNB-GSH conjugate formation almost significantly ($p<0.05$).

Table 1: Activity of glutathione S-transferase in human spermatozoa samples

Parameters	Values
Without nicotine	
Without Mn^{2+} (Control)	0.4000±0.015
With Mn^{2+}	0.2898±0.0098***
With 0.5 mM nicotine	
Without Mn^{2+}	0.4651±0.031
With Mn^{2+}	0.4122±0.037*

Each datum represent Mean±SD of six independent observations each made in triplicate, Mean values are CDNB-GSH conjugate mg $prot^{-1}$ min^{-1}, *$p<0.05$ as compared to nicotine treated unsupplemented samples, ***$p<0.001$ as compared to unsupplemented untreated samples

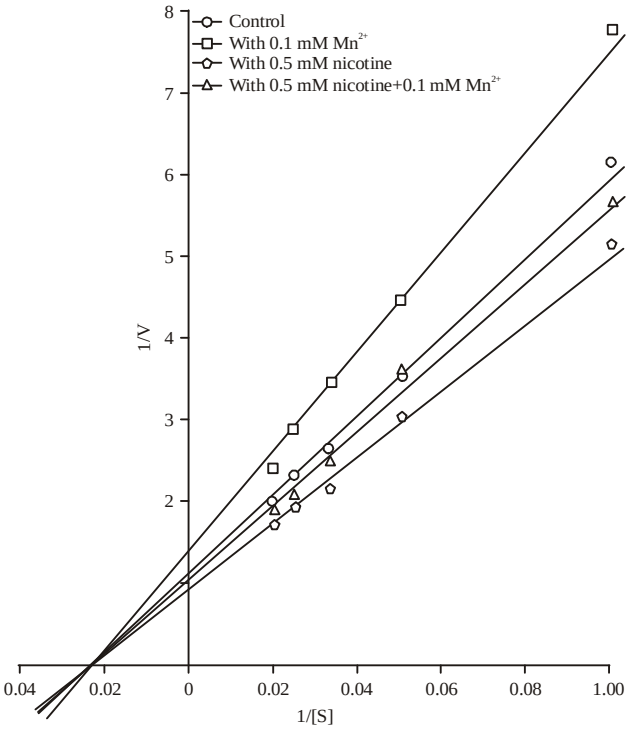

Fig. 1: Activity of glutathione S-transferase in the human spermatozoal samples, treated or not with 0.5 mM nicotine, in the presence or absence of 0.1 mM Mn^{2+} supplementation using different substrate concentrations, v: CDNB-GSH conjugates mg $prot^{-1}$ min^{-1} and [S]: GSH concentration

Table 2: Activity of glutathione S-transferase in the human spermatozoal samples, treated or not with 0.5 mM nicotine, in the presence or absence of 0.1 mM Mn^{2+} supplementation using different substrate concentrations

Substrate concentrations	Without nicotine		With nicotine	
GSH (mM)	Without Mn^{2+}	With Mn^{2+}	Without Mn^{2+}	With Mn^{2+}
10	0.1618±0.021	0.1281±0.027	0.1942±0.013	0.1751±0.017
20	0.2801±0.0175	0.2228±0.012**	0.3405±0.016	0.3250±0.009
30	0.4000±0.015	0.2898±0.0098***	0.4651±0.031	0.4000±0.037$^{\$}$
40	0.4347±0.028	0.3545±0.012**	0.5128±0.029	0.4812±0.024$^{\$}$
50	0.5128±0.013	0.4187±0.023**	0.5882±0.048	0.4061±0.4061

Each datum represent Mean±SD of six independent observations each made in triplicate, Mean value are CDNB-GSH conjugate mg $prot^{-1}$ min^{-1}, **p<0.01, ***p<0.001 as compared to unsupplemented-untreated samples, $^{\$}$p<0.05, $^{\$\$}$p<0.01 as compared to nicotine treated unsupplemented samples

In order to calculate the kinetic parameters of the enzyme, various concentrations ranging from 10-50 mM of GSH were used and the amount of CDNB-GSH conjugate so formed were measured (Table 2). After regression analyses of mean values, the data was transformed to Lineweaver-Burk 1/v vs 1/[S] plot. Figure 1 shows the behavior of this enzyme in the presence or absence of nicotine and with or without Mn^{2+}. Table 3 shows the calculated kinetic parameters. The substrate affinity constant (k_m) of the reaction was found to be 44.44 μM. It remained unchanged throughout the V_{max} was considerably lowered upon Mn^{2+} addition to the spermatozoal samples. Maximum GST activities recorded in nicotine treated samples, upon Mn^{2+} supplementations the V_{max} values were decreased by 12.38%. The enzymatic reaction therefore, was non-competitive in nature.

Table 3: Kinetic parameters of the glutathione S-transferase in the 0.5 mM nicotine treated or untreated spermatozoal samples, supplemented or not with 0.1 mM Mn^{2+}

Sample and supplementation	k_m (μM)	V_{max} (CDNB-GSH conjugate mg $prot^{-1}$ min^{-1})
Untreated		
Without Mn^{2+}	44.44	0.9090
With Mn^{2+}	44.44	0.7246
Treated		
Without Mn^{2+}	44.44	1.0869
With Mn^{2+}	44.44	0.9523

DISCUSSION

It is well recognized that oxidative stress is one of the major causes of *in-vitro* impairment of motility, sperm DNA integrity and sperm-oocyte fusion capacity which is accompanied by decrease in antioxidant capacity. Reactive oxygen species (ROS) are generated at low level during normal oxidative processes in all aerobic organisms (Mittler, 2002) and these low levels of free radicals are generally not harmful to the cell (Martindale and Holbrook, 2002). At moderate concentrations, ROS are necessary for a number of protective reactions, as it can act as molecular signals that trigger endogenous defence mechanisms and recently associated with increased resistance and longevity (Ristow and Schmeisser, 2011). Souza *et al*. (2012) reported that how short-term ultra violet radiation exposure at similar to natural, sub-lethal levels simultaneously triggers responses in two different enzyme systems involved in key cellular processes: GST (antioxidant defence), Casp-3 (related to apoptosis) and how this short exposure may affect physiological endpoints of cell. Some previous findings also suggest that GST is a suitable signal of antioxidant responses in some zooplankton (Martindale and Holbrook, 2002; Souza *et al*., 2007; Souza *et al*., 2010). Activity of GST is directly associated with oxidative stress in germ cells. Increased GST activity also observed in increased oxidative stress in testis (Kaur and Bansal, 2004). Gopalakrishnan and Shaha (1998) reported the location of GST in the sperm head, which makes it more likely for the cell to use the catalytic function of this molecule.

In the present investigation the activity of GST was found to be elevated significantly ($p<0.01$) in nicotine treated spermatozoal samples. Hayes and Pulford (1995) have suggested that increased oxidative stress due to ROS, induce GST activity. The increased GST activity in the present case clearly indicates the effective participation of GST or glutathione dependent defense mechanism against oxidative stress induced by compound like nicotine. The plausible reason for this may be explained as follows: under increased oxidative stress, the Thiobarbituric acid reactive substance (TBARS) production increase. This stimulates the sperm cells glutathione to reduce the ROS, including H_2O_2 to H_2O and itself get oxidized to oxidized glutathione (GSSG). In case of nicotine treated samples, nicotine might be getting conjugated to GSH and thus ultimately get stabilized and/or excreted in less toxic form: leaving the cell free from its deleterious impacts. Conjugation of glutathione with nicotine has also been suggested by Anand *et al*. (2000).

The kinetic analysis further reveals that Mn^{2+} is non-competitive inhibitor of GST. Nicotine on the other hand, registered highest value of the V_{max}. The V_{max} was considerably lowered upon Mn^{2+} addition to the spermatozoal samples. The substrate affinity constant (k_m) of the reaction remained unchanged throughout and was calculated to be 44.44 μM.

GST plays multiple roles in spermatozoa apart from the ROS detoxification, it also plays a significant role in oocyte binding proteins. Hemachand and Shaha (2003) demonstrates that the sperm surface GSTs are able to use extracellular reduced glutathione to inhibit the loss of functional competence of goat spermatozoa; however, in the presence of GST inhibitors, they are unable to do so. The capability of the spermatozoon to utilize extracellular GSH gives a survival

advantage to it, as sperms have very meager cytoplasm ((Hemachand and Shaha, 2003). Glutathione-dependent reduction of hydroperoxide by glutathione peroxidases, a selenoprotein, is believed to be a major defense mechanism of aerobic organisms against ROS, which not only catalyze the reduction of ROS like H_2O_2 but also of organic and fatty acid hydroperoxides (Awasthi *et al*., 1975). It appears that spermatozoal GST also possesses glutathione peroxidase like activity. Since, the present work has been conducted independently of selenium, therefore, not only relive the cell via conjugation reaction but it also helps in quenching the ROS attack by catalyzing the reduction of these reactive oxygen species (particularly H_2O_2) and consequently oxidizing GSH to GSSG. Biochemical levels of response represent early warning signals and may provide useful information on how organisms respond to environmental stresses. Moreover, that several enzymes involved suggest that neither of them is sufficient but the combination of different enzyme systems would be necessary to reduce the stress experienced by the cell (Souza *et al*., 2012).

Lapointe *et al*. (1996) reported that Mn^{2+} and Mg^{2+} were potent stimulators of bovine spermatozoa motility probably by stimulating the adenylate cyclase activity. Anand and Kanwer (2001) also observed that 0.1 mM Mn^{2+} supplementation to human ejaculated spermatozoa and suspension stimulated sperm motility. They also reported that 0.1 mM Mn^{2+} supplementation resulted in a slight increase in Ca^{2+} and Mg^{2+} ATPase in the human ejaculated spermatozoal samples. Mn^{2+} supplementation improves certain sperm parameter like sperm motility and viability under *in-vitro* as well as under induced oxidative stress condition (Bansal and Bilaspuri, 2008). In continuity of the present study, authors have been calculated the C_{max} and k_i values of Mn^{2+} in LPO reaction under same circumstances. On the basis of these parametric values it has been suggested that Mn^{2+} is the most potent trace metal ion inhibitor of LPO in the human ejaculated spermatozoal suspension (Kaushik *et al*., 2015).

CONCLUSION

It can be safely concluded that Mn^{2+} is non-competitive inhibitor of GST and it can be used as an *in-vitro* antioxidant additive for the spermatozoal samples and/or sperm preparation media.

ACKNOWLEDGMENTS

Authors are thankful to Dr. Ravinderjit Kaur Anand Ivell, Lecturer, Faculty of Life Science, University of Nottingham, UK, for her valuable suggestion and constructive criticism during the course of research work.

REFERENCES

Agarwal, A. and S.A. Prabakaran, 2005. Mechanism, measurement and prevention of oxidative stress in male reproductive physiology. Indian J. Exp. Biol., 43: 963-974.

Anand, R.J.K., M. Arabi, K.S. Rana and U. Kanwar, 2000. Role of vitamin C and E with GSH in checking the peroxidative damage to human ejaculated spermatozoa. Int. J. Urol., 7: S74-S74.

Anand, R.K. and U. Kanwar, 2001. Role of some trace metal ions in placental membrane lipid peroxidation. Biol. Trace Elem. Res., 82: 61-75.

Awasthi, Y.C., E. Beutler and S.K. Srivastava, 1975. Purification and properties of human erythrocyte glutathione peroxidase. J. Biol. Chem., 250: 5144-5149.

Bansal, A.K. and G.S. Bilaspuri, 2008. Effect of manganese on bovine sperm motility, viability and lipid peroxidation *in vitro*. Anim. Reprod., 5: 90-96.

Cavallini, L., M. Valente and A. Bindoli, 1984. On the mechanism of inhibition of lipid peroxidation by manganese. Inorganica Chimica Acta, 91: 117-120.

Coassin, M., F. Ursini and A. Bindoli, 1992. Antioxidant effect of manganese. Arch. Biochem. Biophys., 299: 330-333.

El-Kannishy, S., S.S.A. El Gawad, I. Abdelaal, A.A. Metwaly, S.A. Hassan and T.A. Shokeir, 2011. Seminal plasma oxidant-antioxidant imbalance is a dominant feature of primary idiopathic infertile male smokers. Report Opinion, 3: 59-65.

Gopalakrishnan, B. and C. Shaha, 1998. Inhibition of sperm glutathione *S*-transferase leads to functional impairment due to membrane damage. FEBS Lett., 422: 296-300.

Graminski, G.F., Y. Kubo and R.N. Armstrong, 1989. Spectroscopic and kinetic evidence for the thiolate anion of glutathione at the active site of glutathione S-transferase. Biochemistry, 28: 3562-3568.

Habig, W.H., M.J. Pabst and W.B. Jakoby, 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem., 249: 7130-7139.

Hayes, J.D. and D.J. Pulford, 1995. The glutathione S-transferase supergene family: Regulation of gst and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. Crit. Rev. Biochem. Mol. Biol., 30: 445-600.

Hemachand, T. and C. Shaha, 2003. Functional role of sperm surface Glutathione S-transferases and extracellular glutathione in the Haploid spermatozoa under oxidative stress. FEBS Lett., 538: 14-18.

Hemachand, T., B. Gopalakrishnan, D.M. Salunke, S.M. Totey and C. Shaha, 2002. Sperm plasmamembrane-associated glutathione S-transferases as gamete recognition molecules. J. Cell Sci., 115: 2053-2065.

Higdon, A., A.R. Diers, J.Y. Oh, A. Landar and V.M. Darley-Usmar, 2012. Cell signalling by reactive lipid species: New concepts and molecular mechanisms. Biochem. J., 442: 453-464.

Jakoby, W.B. and W.H. Habig, 1980. The Glutathione Transferase. In: Enzymatic Basis of Detoxication, Jakoby, W.B. (Ed.). Academic Press, New York, USA., ISBN-13: 978-0123800015, pp: 63-94.

Kaur, P. and M.P. Bansal, 2004. Influence of selenium induced oxidative stress on spermatogenesis and lactate dehydrogenase-X in mice testis. Asian J. Androl., 6: 227-232.

Kaushik, K., P.K. Mittal and N.R. Kalla, 2015. Antioxidant potential of Mn^{2+} in the human ejaculated spermatozoa under oxidative stress. Int. J. Pharm. Sci. Res., 6: 2153-2162.

Kumar, R., V.K. Singh and S.K. Atreja, 2014. Glutathione-S-transferase: Role in buffalo (*Bubalus bubalis*) sperm capacitation and cryopreservation. Theriogenology, 81: 587-598.

Laborde, E., 2010. Glutathione transferases as mediators of signaling pathways involved in cell proliferation and cell death. Cell Death Differ., 17: 1373-1380.

Lapointe, S., I. Ahmad, M.M. Buhr and M.A. Sirard, 1996. Modulation of postthaw motility, survival, calcium uptake and fertility of bovine sperm by magnesium and manganese. J. Diary Sci., 79: 2163-2169.

Lees, M.B. and S. Paxman, 1972. Modification of the lowry procedure for the analysis of proteolipid protein. Anal. Biochem., 47: 184-192.

Martindale, J.L. and N.J. Holbrook, 2002. Cellular response to oxidative stress: Signaling for suicide and survival. J. Cell. Physiol., 192: 1-15.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci., 7: 405-410.

Rao, A.V.S.K. and C. Shaha, 2000. Role of glutathione S-transferases in oxidative stress-induced male germ cell apoptosis. Free Radical Biol. Med., 29: 1015-1027.

Ristow, M. and S. Schmeisser, 2011. Extending life span by increasing oxidative stress. Free Radical Biol. Med., 51: 327-336.

Sheehan, D., G. Meade, V.M. Foley and C.A. Dowd, 2001. Structure, function and evolution of glutathione transferases: Implications for classification of non-mammalian members of an ancient enzyme superfamily. Biochem. J., 360: 1-16.

Souza, M.S., B.E. Modenutti and E.G. Balseiro, 2007. Antioxidant defences in planktonic crustaceans exposed to different underwater light irradiances in Andean lakes. Water Air Soil Pollut., 183: 49-57.

Souza, M.S., B.E. Modenutti, P. Carrillo, M. Villar-Argaiz, J.M. Medina-Sanchez, F. Bullejos and E.G. Balseiro, 2010. Stoichiometric dietary constraints influence the response of copepods to ultraviolet radiation-induced oxidative stress. Limnol. Oceanogr., 55: 1024-1032.

Souza, M.S., L.A. Hansson, S. Hylander, B. Modenutti and E. Balseiro, 2012. Rapid enzymatic response to compensate UV radiation in copepods. Plos One, Vol. 7. 10.1371/journal.pone. 0032046

Tremellen, K., 2008. Oxidative stress and male infertility-a clinical perspective. Hum. Reprod. Update, 14: 243-258.

Watson, P.F., 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci., 60: 481-492.