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Causes, Ameliorative Measures and Assays to Estimate Testicular Oxidative Stress in Bulls: A Review

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ABSTRACT

Effect of oxidative-stress (OS) on male fertility is much as it reduces fertility to a substantial low level. In bulls, a variety of testicular insults, such as exposure to toxins, X-ray and environmental chemicals, pollutants or specific physical ailments (varicocele) can result in OS which is then reflected in the spermatozoa health. The free-radicals induced damage can be either due to reactive oxygen or nitrogen species. The measures to alleviate such OS include either preventive measures to suppress the production of free-radicals or addition of supplements to counteract the negative effect *post-facto*. Since detrimental effect of free-radicals is well documented estimation of its presence in semen and effect on spermatozoa need to be carried out precisely to chalk out a strategy to counterbalance the OS. This review briefly touches upon aetiology, methods of combating OS and comparative analysis of various available determinative assays.

Key words: Bovine, Free radicals, Mitigation, Temperature.

Reproductive system in most eutherian species, especially the male gonads, is impacted with free radical-induced oxidative stress (OS). The reasons may be very high rate of cellular division and oxygen consumption by mitochondria, presence of increasing number of leucocytes (Kujur *et al.,* 2022) and rich polyunsaturated fatty acids (PUFA) in spermatozoa cellular compartments especially membranes which is the primary site of action for lipid peroxidation (LPO) (Aitken *et al*., 2006). The cytoplasm is limited to the mid-piece and contains insufficient antioxidant systems to provide a suitable protection against free- radical mediated cellular injury (boar, Gadea *et al*., 2004, bull, Biolodeau *et al*., 2000; Stradaioli *et al*., 2007). Moreover, reduction in superoxide dismutase (SOD) by 50% in bull semen stored for protracted period increased the probability of OS (Biolodeau *et al.,* 2000). The problem is further compounded by low oxygen pressure due to weakness of testicular artery increasing the competition for available oxygen. All such factors cause OS. Over 80 years before, MacLeod (1943) indicated oxidative stress as an important factor in disruption of sperm function in humans. His observations were later found to be true in the semen of livestock and laboratory animals (Tosic and Walton, 1950). Oxidative stress is caused by the increased presence of several reactive oxygen (ROS) or nitrogen (RNS) species consisting of peroxides, superoxide anions, nitric oxide, hydroxyl radicle and singlet oxygen, resulting from inability of the biological system to counterbalance it. Such highly reactive free radicals haveone or more unpaired electrons derived from oxygen metabolism (Halliwell, 2006). Though at physiological and controlled concentrations of free radicals play an important role in spermatozoa physiological processes such as capacitation, acrosome reactionand signalling processes to ensure fertilization (Bansal and Bilaspuri, 2011), the problem arises at the overproduction which can trigger pathological processes (Agarwal *et al.,* 2003). In more serious cases unchecked attack of free radicals of testicular cells tissues cause serious damage with consequent impaired spermatogenesis. It is, thus, obvious that cellular damage can only occur when balance between production of free radicals and its subsequent metabolism for appropriate gonadal function on one hand and inability of the biological system to detoxify excess of it on the other happens (Romeo *et al.,* 2004). Certain endogenous and exogenous conditions such as DNA damage, exposure to heat stress, senility, or radiation or other causes may alter the fine balance between free radical production, its physiological use and its counteraction (Srivastava *et al*., 2017). In such a scenario, antioxidants such as enzymes added in the semen can come handy by either decreasing the production of free radicals or counterbalancing it. Physiologically a mammalian body's defences against free radicals can be either the preventive antioxidant system or the scavenging antioxidant system or both. In the former, antioxidant enzymes such as Superoxide Dismutase (SOD), catalase and Glutathione Peroxidase (GPX) play a major role (Bahmani *et al.,* 2015),

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whereas in the later prevention of lipid per oxidation of sperm membranes and other testicular cells which are rich in poly unsaturated fatty acids (PUFA) takes place. The prevention of oxidation by the enzyme-system includes decreasing the chain formation by finding primer free radicals or by stabilizing metal radicals (copper and iron). In scavenging antioxidant system vitamins such as C and E neutralize free radicals and obliterate them from damaging the spermatozoa or other gonadal cells. In the situations when body's cellular mechanisms is not able to cope with the disturbances in the anti-oxidant mechanism the use of antioxidant supplements such as enzymes, vitamins, hormones (melatonin, Kumar *et al.,* 2016) or amino acids (Cysteine, Arunpandian *et al.,* 2022) may be a practical solution. It is to be noted that seminal OS status is emerging as a significant diagnostic and prognostic tool in assisted reproductive technology. Therefore, precise determination of OS may help to develop strategies to reduce OS during extension and cryopreservation of bovine semen. In this brief review, an attempt has been made to delineate the causes of OS on testicular and spermatozoa function, its ameliorative measures and significance of various procedure of measuring OS in semen samples and their comparative analyses is provided.

Free radicals inducedmorphological alterations in Spermatozoa

Recent studies have shown an imbalance in either the production or degradation of ROS may have serious adverse effects on sperm. Free-radical induced damage to spermatozoa includes mitochondrial impairment (Baumber *et al.,* 2000), nucleic acid fragmentation (Morte *et al*., 2008) and oxidative breakdown of fatty acids and amino acids (Aitken *et al.,* 1989; Mammato *et al.,* 1996), all of which individually or collectively are associated with loss of spermatozoa motility. Furthermore there may be decreased capacity for sperm-oocyte fusion (Guthrie and Welch, 2006; Ahmad *et al*., 2015), resulting in poor fertilization rates and alterations during embryogenesis (Lewis and Aitken, 2005; Simoes *et al.,* 2013). Various authors have opined that free radicals attack the integrity of DNA in the spermatozoa nucleus by causing base modifications, DNA strand breaksand chromatin cross-linking (Said *et al.,* 2005). *In vivo*, such damage may not be of much importance as damaged spermatozoa may be incapable to participate in the fertilization process (Cocuzza *et al*., 2007). However, during sperm storage by either cooling or cryopreservation such safeguards are hacked off, particularly in situations where much of seminal plasma isseparated from ejaculates *e.g*. spermatozoa washing or at semen extension, for, much of the antioxidant capacity in semen resides with seminal plasma. One must also keep in mind the particular morphology of spermatozoa where in tail or acrosome does not contain much of cytoplasm, thus resulting in failure of endogenous antioxidants in cells.

Lipid per oxidation in the spermatozoa membrane by free radicals can disrupt fluidity and permeability of cell membranes, exposing exposure of the entire cell to risk. Malondialdehyde (MDA) molecules produced due to the degradation of the peroxides of unsaturated fatty acids penetrate cell membrane structures causing asymmetric distribution of lipid membrane components. This results in reducing sperm-oocyte interaction, particularly zona binding of spermatozoa. Thus, in recent times, the per oxidation induced damage to testicular function is most commonly investigated area (Peltola *et al.,* 1994), with MDA used as a marker (biomarker) to determine the rate of oxidative damage to spermatozoa lipids.

Aetiology of testicular oxidative stress

Though endogenous antioxidants play a vital role in protecting gonadal function during spermatogenesis and thereafter, a plethora of internal and external factors can alter the fine balance between free radicals and antioxidants defence mechanism resulting in oxidative stress (Fig 1).

High testicular temperature

Any factor that cause abnormal increase in testicular temperature can lead to testicular OS as they decrease SOD and catalase function (Ahotupa *et al*., 1992). This may arise due to increases in the H_2O_2 level and increased rate of apoptosis. The temperature- humidity index is a common measure of summer stress in animals to measure the output of animals raised in subtropical and tropical climates. Environmental factors such as extreme heat waves have a negative impact on animal reproductive efficiency (Arunpandian *et al.,* 2022).

Infection/injury

Any inflammatory condition of the testicles causes a significant increase in leucocytes, increased expression of inflammatory genes (Allen *et al.,* 2004), decrease in testosterone production, lipid per-oxidation in Leydig cell membrane and considerable increase in steroid making and beta-hydroxyl dehydrogenase activity leading to disturbed spermatogenesis (Reddy *et al.,* 2006).

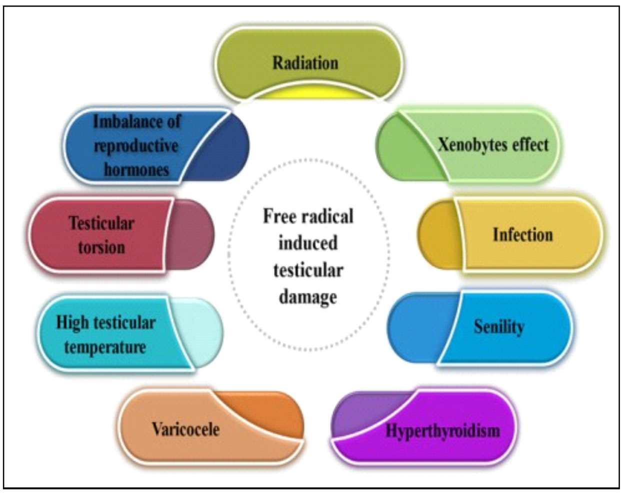

Fig 1: Causes leading to testicular oxidative stress.

Moreover, Cholesterol transport activity required for maintaining membrane fluidity is also inhibited (Husain and Somani, 1998).

Reproductive hormones disharmony

Exposure of the male gonads to harmful chemicals may negatively affect antioxidant activity *e.g.* administration of dimethane sulfanate can cause inhibition of expression of antioxidant enzymes such as glutathione peroxidase, SOD and catalase and decrease in testosterone concentration, disturbance in spermatogenesis and increase in cell apoptosis in testicles (Zini and Sclegel, 2003). Similarly, chronic administration of human chorionic gonadotropin may cause excessive production of free-radicals in male gonads and subsequent increase in lipid per-oxidation, induction of apoptosis of germinal cells and disturbances in spermatogenesis (Gautam *et al*., 2007). In concurrence, Gupta *et al*. (2022) concluded that OS and hormonal imbalance might resultin high number of dead and defective spermatozoa, with resultant poor quality semen ejaculates.

Xenobytes effect

It refers to the effect induced by the chronic accumulation of residues of antibiotics, other drugs and chemicals in the body. Ozyurt *et al.* (2006) demonstrated a wide spectrum of xenobytes causing testicular OS alongside suppressing antioxidant mechanism.

Exposure to toxins

Exposure of foraging animals to a variety of accumulated toxins is foregone conclusion, more so because of their dependence on fodder, such as pesticides, industrial waste, or wastage from heavy machinery (brake oils). Accumulation of such toxins in the body can cause testicular OS and consequently disturbance in spermatogenesis. For example mice treated with certain pesticides such as hexachlorocyclohexane (Samanta and Chainy, 1997) and industrial pollutants such as 3,1-dinitrobenzene or nonylphenol (Han *et al.,* 2004) exhibited a significant increase in testicular OS, injury to germ cells and apoptosis. Methoxyethanol, a glycol etherand its main metabolite, methoxyacid, used in brake oilsand colours can increase in testicular OS and atrophy (McClusky *et al.,* 2007). Studies have shown that prolonged exposure to high concentration of particular metals such as iron and cadmium can cause OS (Koizumi and Li, 1992). Also, prolonged exposure to lead causes decline in testicular sperm output, increase in production of sperm free radicals, decrease in epididymal sperm motility and increase in lipid per-oxidation (Hsu *et al*., 1997; Marchlewicz *et al.,* 2007).

Varicocele

Varicocele found less commonly in bulls, is the dilation of spermatic vein in the testicle thereby increasing testicular blood flow and subsequently testicular temperature. It affects spermatogenesis process by creating OS, greater DNA damage and loss of seminal plasma antioxidants (Asmis *et al*., 2006).

Radiation

Exposure of testicular tissueand spermatozoa to the ionizing radiation can be associated with OS (Manda *et al*., 2007), which may induce permanent damage. On the other hand, Sertoli and Leydig cells seem to be relatively resistant to X-ray, which following exposure can be restored to health by use of antioxidants (Lee *et al.,* 2002; Ahotupa and Huhtaniemi, 1992).

Senility

As far as breeding bulls are concerned senilityand its associated problems are not much to bother about, for, a strict breeding and culling policy is in place. However, in certain exceptional cases when elite bulls with a very high genetic merit are used well beyond its production age certain age related problem may arise. In agreement, a well documented study in rats by Syntin *et al*. (2001) showed with increasing age the expression of enzymatic and non-enzymatic antioxidants decreasesand also the level of glutathione, an antioxidant decreases. Different authors have opined that degenerative changes such as appearance of vacuoles in germinal cells in testicular tissue and decline in sperm quality in rats are associated with advancing age. This effect is further exaggerated by decline in number of germinal cells with advancing age (Luo *et al*., 2006).

Testicular torsion

Testicular torsion is a condition not so often encountered in bulls. However, its occurrence coincides with disturbed testicular blood flowwith increase in levels of OS in the testicles of the same side, increase in production of NO and H_2O_2, formation of lipids peroxidation, accumulation of isoprostane, decrease in antioxidant enzymes and increase in apoptosis rate (Anderson and Williamson, 1990). Corrections of testicular torsion requires corrective measures within 3-4 hours to avoid permanent testicle shrinkage. The ill effects of ischaemic condition can be minimized by providing supplements such as resveratrol, L-carnitine, selenium, caffeic acid phenethyl ester and *Allium sativum* extract (Sarica *et al*., 1997).

Hyperthyroidism

This is another condition not frequently encountered in the bulls. However, when it occurs it is associated with OS, an increase in lipid per-oxidation and GSH level, arising due to increased mitochondrial activity and concurrent release of electrons from mitochondrial electron transport chain due to increased production of thyroxine (Sahoo *et al.,* 2007). Administration of melatoninand antioxidant may be used as a therapeutic measure.

Minimization of the ill effects of free radicals on spermatozoa and testicular function

Seminal plasma of bulls contains antioxidant compounds that protect spermatozoa against OS and in the process offset deficiency of sperm cytoplasmic enzymes. A group of enzymatic antioxidants present in seminal plasma of bulls include superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione reductase (GR) (Bansal and Bilaspuri, 2011, Srivastava *et al.,* 2016). It also contains certain non-enzymatic antioxidants such as taurine, pyruvate, ureate, ascorbate and α-tocopherol.

To counteract and minimize the oxidative damage to live spermatozoa several investigators have used different techniques with varying success rate (Fig 2). The addition of antioxidants in the semen extender is a well-known method of the minimization of OSon the spermatozoa during storage. Antioxidants, in general, are the compounds which dispose, scavengeand suppress the formation of ROS, or oppose their actions.

Addition of anti-oxidants in the semen

The imbalance of the free-radicals is corrected by supplementation of the semen extenders with either enzymatic or non-enzymatic anti-oxidants in liquid stored semen (Agarwal *et al.,* 2014) or frozen thawed semen (Bathgate, 2011) which have proven to be effective in relieving the OS.

Enzymatic anti-oxidants

Enzymatic anti-oxidants are the natural anti-oxidants which neutralize the excess ROS and prevent further damage to the cellular structures. The enzymatic anti-oxidants consists of SOD, catalase, glutathione peroxidase (GPx) and glutathione reductase (GR) (Bansal and Bilaspuri, 2011) that prevent damage to cell structure and causes the reduction of hydrogen peroxide into water and alcohol. High concentration of glutathione is found in genital tract and epididymal fluids of bull (Agrawal and Vanha Perttula, 1988). Furthermore, this enzyme protects spermatozoa against O_2 toxicity and lipid membrane against per-oxidation. Catalase decomposes H_2O_2 into O_2 and H_2O and separates the superoxide anion produced by NADPH oxidase from neutrophils and protects spermatozoids against OS. Supplementation of bull semen with GSH improved the seminal characteristics and in vitrofertilization rate (Gadea *et al*., 2008) but there was not much beneficial effect in field AI trials (Tuncer *et al.,* 2010). Meanwhile, SOD supplemented with a comparably higher amount than other antioxidant enzymes leads to conversion of superoxide (NO_2) anion into O_2 and H_2O_2.

Ngou *et al.,* (2020) explored the applicability of Oxyrase as an oxygen scavenging agent and showed it can preserve the post-thaw quality of Sahiwal bull spermatozoa. Oxyrase is an enzyme derived from *E. coli* cell membrane electron transport system, which in the presence of hydrogen donor substrate like lactate, decreases oxygen of solutions to lower levels (Kressin *et al.,* 1997).

Non-enzymatic antioxidants

In addition to containing enzymatic antioxidants, semen has a number of non-enzymatic antioxidants such as vitamins-A, -C and -E ascorbate, pyruvate, ubiquinole, glutathione, albumin, ureate, taurineand hypotaurine, each of which plays a vital part in fighting OS (Sheweitta *et al.,* 2005). Moreover, a small quantity of glutathione found in a number of cellsreacts directly with ROS. Glutathione is a cofactor for GPX and protects mammalian cells against OS while reducing H_2O_2 and other peroxides. Certain study results show that glutathione exerts protective effects on sperm against freezing and is associated with enhanced spermmotility after freezing. Furthermore, this compound increases enzymatic activity in sheep semen (Saleh and Agarwal, 2002). Various non-enzymatic or chemical antioxidant supplementations have been attempted by many workers with varying results. Resveratol, a flavonoid inhibited the lipid peroxidation of the ram spermatozoa when incubated at low concentrations (Sarlos *et al.,* 2002). Quercetin, an another flavonoid having more antioxidant

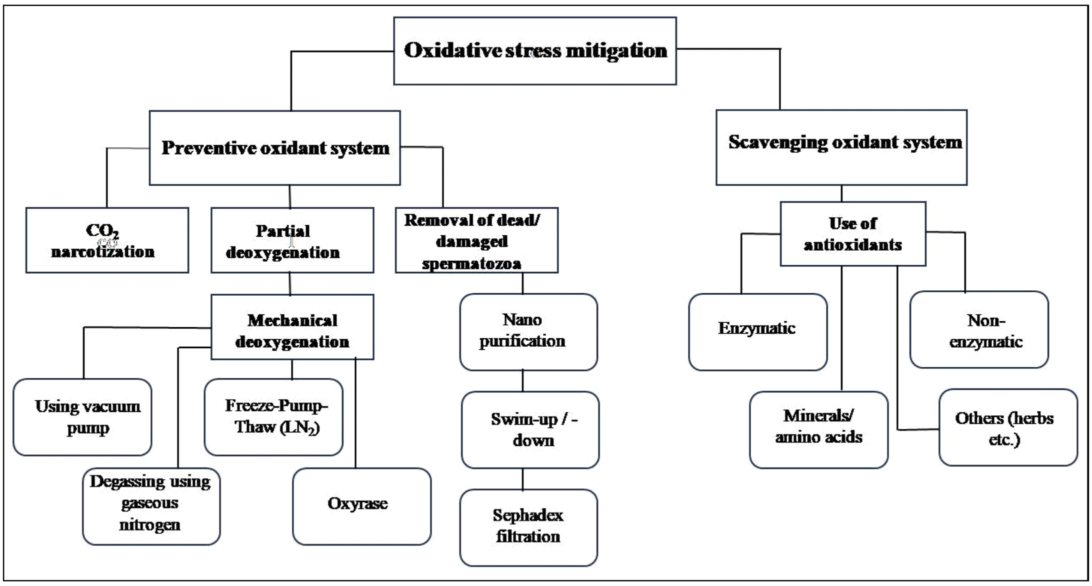

Fig 2: Various protocols to alleviate oxidative stress to bull spermatozoa.

capacity than vitamin C and E improved stallion semen motility, acrosome integrity, zona binding ability and reduced DNA fragmentation in cryopreserved spermatozoa (Gibb *et al*., 2013).

Minerals (Zinc and Selenium)

Zinc and the selenium arepotent antioxidant agent. Zinc is a catalyst and a main component of free radical-inhibiting free enzymes such as SOD (Asadi *et al.,* 2017). It can prevent lipid peroxidation through relocating or transferring metals including iron and copper (Bray and Bettger, 1990). In concurrence, Gupta *et al.* (2020) reported positive relationship between semen quality of bulls with that of seminal zinc concentration. Selenium protects important antioxidants in the body including vitamins C and E and decreases free radical-induced damage. Moreover, selenium plays a part in producing thyroid hormones and contributes to the function of this important gland. Use of vitamin E supplement with selenium considerably reduced Malondialdehyde (MDA), improved sperm motility and viability and protects spermatozoa DNA (Takasaki *et al.,* 1987).

Vitamins C and E

Vitamin E (α-tocopherol) and Vitamin C (ascorbic acid), potent antioxidant, are the major chain-breaking antioxidant found in the spermatozoa membrane (Hull *et al.,* 2000). Addition of vitamin E to the semen extender increased the spermatozoa resistance to the lipid per-oxidation (LPO) (Cerolini *et al*., 2000). It has been observed that supplementation of vitamin E had the positive effect on the spermatozoa quality, membrane integrity and mitochondrial membrane potential during cryopreservation (Cerolini *et al.,* 2000; Pena *et al.,* 2004). Vitamin E, besides being present in the spermatozoa membrane, contributes greatly to the activity of Sertoli cell lines and spermatocytes (Johnson, 1979). Moreover, use of vitamins E and C is highly effective to fight testicular OS following exposure to oxidants such as arsenic, endosulfan, cadmiumand alcohol. In agreement, investigation showed vitamin E was effective on testicular function through suppressing LPO in testicular and mitochondrial microsomes and fighting adverse effects of OSdue to exposure to certain agents such as ozone gas, iron overload, intense exercise, aflatoxin, cyclophosplamide and formaldehyde (Verma and Nair, 2001). Moreover, vitamin E activity is complementary to GPX activity, which facilitates the peroxides reduction in seminal cytoplasm. Vitamin C, a water soluble chain breaking antioxidant has ability of scavenging and neutralizing the hydroxyl, superoxide and hydrogen peroxide free radicals and prevents the spermatozoa agglutination (Agarwal *et al*., 2004). The incorporation of both vitamin E and C into the semen extenders improved the motility and livability of boar and bull spermatozoa (Pena *et al.,* 2004; Jeong *et al.,* 2009).

Melatonin

Since melatonin is fat- and water-soluble it can easily pass through testicular blood barrier to protect germinal epithelium thus playing an important physiological role as an antioxidants. Moreover, it shares one electron, rather than two electrons, in oxidation reaction. Therefore, free radicals are more likely to target melatonin, increasing its efficacy as antioxidants. In agreement, Perumal *et al.,* (2018) demonstrated that administration of slow release exogenous melatonin modulates semen quality and OS profile and *in vitro* fertilizing ability of cryopreserved mithun (*Bos frontalis*) spermatozoa. In humans, intraperitoneally administered melatonin caused reduction in testicular OS after experimental induction of varicocele of the left side (Awad *et al.,* 2006).

Amino acids and lipids

Arunpandian *et al.* (2023) evaluated impact of cysteine as a semen additive on mitigating the decline in sperm quality due to summer stress in riverine buffaloes (bubalus bubalis) and observed that the cysteine molecules at 4 mmol concentration produced more pronounced improvement in the freezability potential by countering free-radicals.Albumin is also known to improve the spermatozoa motility, plasma membrane integrity and acrosome intactness during freeze-thaw of the ram semen (Uysal and Bucak, 2007). Albumin also improves the fertility as well as catalase antioxidant activity in the bull semen following freeze-thaw (Schäfer and Holzmann, 2000). Katiyar *et al.* (2022) showed supplementation of Humanin, a mitochondrial derived peptide originally isolated from brain of a patient affected with Alzheimer's disease, improved the freezability of buffalo spermatozoa and increased the per cent recovery of freezable ejaculates by neutralizing free radicals. Perumal *et al*. (2016, 2017) investigated effect of low density lipoproteins as additive in fresh ejaculates and found improvement in quality parameters and biomarkers of OS following cryopreservation of mithun (Bos frontalis) spermatozoa. Cytochrome C, a small protein, is another antioxidant that can effectively reduce testicular H_2O_2. The cytochrome C isoform is considered a potent apoptosis activator and plays a part in increasing the protective capacity of testicular tissue through eliminating damaged germ cells (Liu *et al*., 2006).

Mechanical methods of reducing oxygen tension

Pande *et al.,* (2015) investigated the effects of degasification using nitrogen gas on semen quality and OSparameters in frozen-thawed cross bred bull semen and found significant improvement in semen quality. The rationale behind this may be thatliquid N_2 flushingmay have reduced the oxygen tension as well as substantially reduced the metabolic activity of spermatozoa. In agreement, to reduce oxygen tension in the ejaculates of crossbred bull Bhutia *et al.* (2021) and Kumar *et al.* (2018) attempted partial deoxygenation of semen extender to minimize post-thaw damage of spermatozoaand concluded that reducing the dissolved oxygen level to 4 ppm before cryopreservation improved the freezability by reducing OS and apoptotic changes. Omerdin *et al*. (2018) and Amarjeet *et al*. (2020c) following their investigation on spermatozoa swim-up

Table 1: Comparative merit and demerit of protocols for estimating oxidative stress.

Asaay	Principle	Advantage (s)	Disadvantage (s)
Chemiluminescence	Free radicals combine with luminol to produce a light signal that is then converted to an electrical signal (photon) by the instrument called luminometer. The number of free radicals produced are then expressed as relative light units.	Reacts with a variety of ROS and allows. both intracellular and extracellular ROS to be measured (1). It can measure various oxidants like H_2O_2, O_2^-. and OH^-. levels. It is easy to use and can measure the global level of ROS under physiological conditions (2). More sensitive test.	Fresh semen samples with high sperm count needed. Presence of leukocytes confounds the result (3). It cannot distinguish oxidants from one another
Nitroblue tetrazolium test	Nitroblue tetrazolium (NBT) is an electron acceptor that becomes reduced in the presence of free oxygen radicals to form a blue-black compound, formazan.	Easily performed, inexpensive and sensitive test (4).	May require more studies and validation in bovine semen.
Flow cytometry (FC)		Better speed, accuracyand reproducibility as compared to chemiluminescence method. It can detect even minute intracellular ROS. Can also detect ROS level in oligozoospermic semen samples (5).	FC sperm sorting itself may lead to generation of lipid pre-oxidation thus enhancing OS (6) Expensive and requires technical expertise
Myeloperoxidase or the Endtz test		ROS generated by the polymorphonu clear granulocytes can be effectively indicated (6).	It cannot be used to detect ROS generation by spermatozoa (4)
Electron-spin resonance spectroscopy or electron paramagnetic resonance	ESR uses the magnetic properties of unpaired electrons to detect free radicals directly	Permits the direct detection of free radicals and reports on the magnetic properties of unpaired electrons (1, 6).	Expensive technology so used only for research purposes. Requires fresh sample and great technical expertise. Short life span of ROS makes the application of this technique difficult (7).
ROS-TAC Score		This score minimizes the variability of the individual parameters (ROS or TAC) of OS hence considered superior (7).	More time taking and laborious.
Xylenol orange based assay		Claimed to be rapid, sensitive fully automated, easy and reliable (8).	Comparatively newer test so more validation is required (9).

(1) Cocuzza *et al.*, 2007; (2) Agarwal *et al.*, 2004; (3) Esfandiari *et al.*, 2003; (4) Mahfouz *et al.*, 2009; (5) Shekarriz *et al.*, 1995; (6) Weber 1990; (7) Buettner 1987; (8) Sharma *et al.*, 1999; (9) Erel 2005; (10) Table, courtesy Pande and Srivastava 2017.

technique concluded that enriched spermatozoa had reduced effect of free-radicals in buffalo bull. Since dead and damaged spermatozoa are considered primary source of detrimental free radicals Amarjeet *et al.* (2020a,b) and Rautela *et al.* (2022) attempted to purify fresh semen by depleting the source of ROS itself from fresh ejaculates using nano-technological approach. Their seminal work reported 15-18% improvement in post-thaw semen quality and concluded that nano-technology could be an easy to use approach to improve post-thaw semen quality by minimizing the damaging effect of free radicals in bulls.

Miscellaneous antioxidants

Supplementation of Mito TEMPO, a mitochondria-targeted superoxide dismutase mimetic with effective superoxide scavenging properties and Acetovanillone, a potent NADPH oxidase inhibitor, individually and in combination improved the freezability of buffalo spermatozoa (Kumar *et al*., 2021a, Kumar *et al.,* 2021b). Mito-Tempo has effective superoxide scavenging properties by converting toxic superoxide molecules into hydrogen peroxide or oxygen and subsequently detoxified to oxygen and water by catalase or glutathione peroxidase (Liang *et al*., 2010, Srivastava and Pande, 2016; Kumar *et al.,* 2022). Acetovanillone (Apocynin) is effective in preventing the production of the cellular superoxide without affecting their functional properties (Madureira *et al*., 2020). Curcumin, a lipophilic polyphenol (Ruby *et al*., 1995) when supplemented with the bull semen, increased the GSH content of the spermatozoa post-thawing (Bucak *et al.,* 2012).

Comparative analysis of assays to determine oxidative stress in spermatozoa

Determination of OS in semen is important diagnostic and prognostic tool in infertility/assisted reproductive technology. Thus, precise OS measurement may help to develop strategies to reduce OS during extension and cryopreservation of bovine semen. The measurement of OS/ROS is dependent on the analytic target along with the ROS in question. Also there are numerous methods for measurement of ROS. It can be measured both in neat as well as washed semen samples

Therefore, all assays employed to assess OS of spermatozoa have their own fundamental advantages and disadvantages. The investigator is advised to choose the assay after considering all the information in entirety. Table 1 summarises the pros and cons of various assays employed in measuring OS.

CONCLUSION

It is obvious that free-radicals can arise beyond physiological limits from a variety of aetiology. Such unwarranted increase in ROS concentration can severely hamper bull health and quality semen production. It is therefore, imperative that immediate corrective measures are undertaken by choosing appropriate antioxidant mechanism/agent such as cysteine. Moreover, accurate determination of free-radical level in seminal plasma or spermatozoa is essential before one embarks on therapeutic measures to enhance fertility.

Conflict of interest

All authors declare that they have conflict of interest.

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