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Evaluation of sperm protein modifications in frozen-thawed rat spermatozoa

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ABSTRACT

Assessment of cryoinjury of rat spermatozoa is crucial to improving the probability of fertilization. The epididymal rat spermatozoa were subjected to 15, 30, 45 and 60 days freezing separately, and then determined the antioxidant and non anti oxidant enzymatic system were observed after thawing each sample. The results of thawed samples showed that the cryopreservation has significant effects of cryopreservation on rat epidydimal sperm lipid peroxidation, antioxidant and non enzymatic antioxidant system in different times of preservation ($p \leq 0.05$). The reduced activity of antioxidant enzymes in extreme cold is a common effect which accelerates the accumulation of ROS in higher amount. Our research findings have suggest that cryopreservation makes rat spermatozoa susceptible to external and internal damage, in particular during cooling process.

Keywords: Epididymis, Lipid Peroxidation, Antioxidant system.

Abbreviations:-

ROS: Reactive oxygen species

DNA: Deoxy ribonucleic acid

LPO: Lipid peroxidation

PBS: Phosphate buffered saline

HEPES: Hydroxy ethyl piperazine ethane sulfonic acid

NADPH: Nicotinamide adenine dinucleotide phosphate

INTRODUCTION:

Preservation of spermatozoa is an important tool to preserve genetic diversity and to assist in the reproduction of a species. Sperm cryopreservation allows the widespread dissemination of valuable genetic material, leading to an increased rate of genetic gain. However cervical AI, when using frozen semen, is limited by low fertility rates in rats. The exact mechanism of reduction of fertilizing ability of cryopreserved spermatozoa is not yet completely understood.

Moreover cryopreservation lead to significant reduction in the level of spermatozoa antioxidants and this generates reactive oxygen species such as superoxide radical, hydroperoxyl radical and hydroxyl radical (Silva, 2006) which can cause cell damage. Therefore, the amount of ROS should be limited to the minimum required to maintain cell function as sperm cell is easily damaged by lipid peroxidation, as its membrane has a high content of polyunsaturated fatty acids (Silva, 2006).

One of the major biological processes associated with ROS is lipid peroxidation. Lipid peroxidation triggers the loss of membrane integrity, causing increased cell permeability, enzyme inactivation, and structural damage to DNA, and cell death. At present, the most widely used assay for lipid peroxidation involves the measurement of malondialdehyde, a small molecular mass degradation product of peroxidative process that can be measured by virtue of its capacity to form adducts with thiobarbituric acid.

Antioxidants partially ameliorated the negative effects of reactive oxygen species produced during cryopreservation. The sperm lipid peroxidation in the presence of reactive oxygen species leading to decreased sperm quality (Bucak *et al.,* 2007). The anti-oxidant system comprising reduced glutathione, glutathione peroxidase, catalase, superoxide dismutase, Glutathione Reductase and various non-enzymatic molecules such as glutathione, vitamins (E and C) has been described as defense functioning mechanisms against the lipid peroxidation of semen and important in maintaining sperm motility and viability (Gadea *et al.,* 2004). This anti-oxidant capacity in sperm cells may however, be insufficient in preventing LPO during the freeze–thawing process. Thus mammalian spermatozoa lack a significant cytoplasmic component, which contains antioxidants that counteract the damaging effects of ROS and lipid peroxidation (Storey, 1997).

However, no previous study has examined the effect of cryopreservation on the induction of oxidative stress in the rat spermatozoa. Moreover, neither protection nor repair systems for sperm integrity are available and, therefore, addition of exogenous protectants is required to reduce this ROS-mediated damage (Vishwanath and Shannon, 1997). The present study was undertaken to evaluate the effect of cryopreservation on the antioxidant systems of rat epididymis.

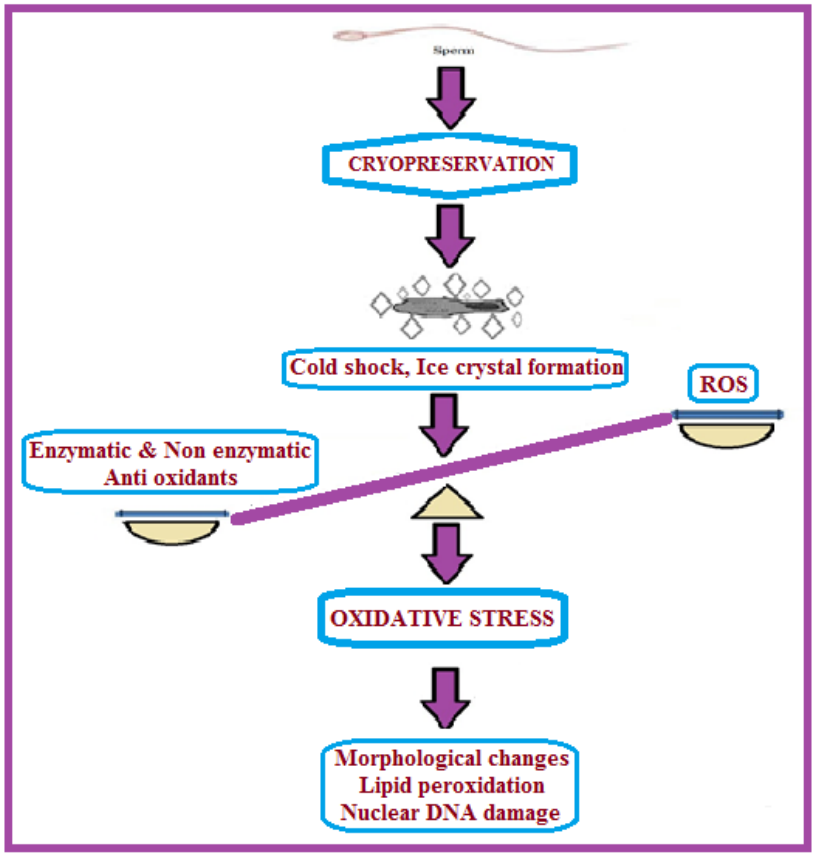

Figure 1: Schematic representation of the buildup of oxidative stress in the sperm. It explains the effect of cryopreservation and the imbalance caused by accumulating ROS and depleting antioxidant both enzymatic and non-enzymatic types due to cold shock and ice crystals formation, which brings about a state of oxidative stress.

MATERIALS AND METHODS:

Cryopreservation of sperm:

Male rats were euthanized via ethanol inhalation followed with induction of bilateral pneumothorax. Immediately following confirmation of sedation, the distal end of the scrotal sac was removed and dissection of the tunics allowed for exteriorization of the testis and *cauda epididymidis*. The *cauda epididymidis* without fat was removed from the testicle and was rssinsed in warm phosphate buffered saline (DPBS). Later using scissors it was cut into three slits. The epididymis was then placed in the selected cryopreservation medium (1.0:0.7), i.e. Sperm Freeze™, a 15% glycerol based cryoprotectant in HEPES buffer (Ferti Pro N.V., 8730 Beernem, Belgium), and was incubated for 10min to allow the sperm to swim out of the tissues. This sample was subjected to static vapour phase cooling for 15min before being plunged into liquid nitrogen. Samples were subsequently thawed at 37°C for 10min. Once totally thawed, the fresh and frozen-thawed semen samples were mixed with 50mM Tris-HCl buffer, pH 8.0, containing 0.25M sucrose and 1mM PMSF then subjected to the centrifugation at 3500xg for 10min. the resulting supernatant was used as source for various activities.

Assay of Enzymatic Antioxidants

Estimation of catalase activity

Catalase assay was carried out by the method of Harir and McHale (1987). The decomposition of H_2O_2 was followed directly by measuring the decrease in absorbance at 240 nm (ε=0.036mM^{-1}cm^{-1}). To 0.5 mL of the supernatant 1.5 mL of 100mM phosphate buffer (pH7.3) was added. Then one mL of 50mM H_2O_2 was added and the final volume was made upto 3ml using $H_2O_2$and change in absorbance was recorded after every 15 seconds up to one min. The activity of catalase was expressed as µmoles of H_2O_2 utilized/min/mg protein.

Estimation of superoxide dismutase activity

Superoxide dismutase was measured according to the method of Misra and Fridovich (1972) based on the oxidation of epinephrine transition by the enzyme. To 0.5ml of the supernatant 2ml of 0.05M carbonate buffer, pH 10.2, and 0.5mL of 0.6mM epinephrine was added to the formation of adrenochrome in the next 4 min, change in absorbance was recorded at 470 nm in spectrophotometer. SOD activity was expressed as the amount of enzyme required to cause 50% inhibition of epinephrine auto-oxidation).

Glutathione Peroxidase

Glutathione peroxidase assay was carried out by monitoring the oxidation of NADPH in a recycling assay as described by Wendel, 1981. Total GPx activity was determined using cumene hydro peroxide CHP (ROOH) as substrate. Selenium dependent GPx activity was measured using H_2O_2 as substrate. Non Se-GPx activity of GST was measured using CHP as substrate.

Estimation of glutathione reductase activity

Glutathione reductase activity was determined by a slightly modified method of Carlberg and Mannervik (1985) at room temperature. The reaction mixture contained 50µl of 3mM NADPH in 0.1% $NaHCO_3$, 50µl of 20mM GSSG in phosphate buffer, 800µl of phosphate buffer and 100µl of enzyme source as prepared above. The reaction was monitored at 340nm for 3min. The molar extinction coefficient of 6.22 x 10^3 M cm^{-1} was used to determine the GR activity. One unit of enzyme activity is equal to micromoles of NADPH oxidized/mg protein/min.

Estimation of lipid peroxidation

Lipid peroxidation was carried out by the method of Okhawa *et al.,* (1979). The 10% semen samples were prepared by diluting with 1.15% of KCl for lipid peroxidation. To the 0.1ml of the sample, added 0.2 ml of 8.1% sodium dodecyl sulphate (SDS) and 1.5 ml of 0.8% TBA. The mixture was finally made up to 4 mL with distilled water and boiled at 95^{o}C for 1 hr. After cooling, 1 mL of distilled water and 5 mL of n-butanol: pyridine mixture (15:1 v/v) were added and shaken vigorously and then centrifuged at 4000 rpm for 10 min. Then the absorbance of the organic layer was measured at

532 nm. Amount of lipid peroxidation was expressed as nmoles of MDA produced/mg protein using a standard graph of 1, 1, 3, 3-tetraethoxy propane as standard.

Estimation of Non-enzymatic Antioxidants

Estimation of Reduced Glutathione

The estimation of total reduced glutathione (GSH) was carried out by the method of Ellman *et al.*, (1972). To 0.1ml of tissue enzyme source added 1.9ml of 5% TCA and the proteins precipitated were centrifuged at 3000rpm for 15min and the precipitate was discarded. To 1ml of the supernatant 2ml of 0.6mM DTNB in 0.2M phosphate buffer, p^H 8.0, was added and the colour developed was read at 412nm against a blank containing TCA instead of sample. The amount of GSH was expressed as μmoles of GSH/mg protein.

Estimation of ascorbic acid Ascorbic acid was estimated by the method of Omaye *et al* (1979). 0.5 ml of sample was mixed thoroughly with 1.5 ml of 6% TCA and centrifuged for 20 min at 3500xg. To 0.5 ml of the supernatant, 0.5 ml of 2, 4 DNPH reagent was added and mixed well. The tubes were allowed to stand at room temperature for 3 hrs. and placed in ice-cold water to cool to 4^{o}Cand added 2.5 ml of 85% sulphuric acid and then allowed to stand for 30 min. A set of standards containing 10-50 μg of ascorbic acid were taken and processed similarly along with a blank, containing 0.5 ml of 4% TCA. The color developed was read at 530 nm.

Estimation of α-Tocopherol

α-tocopherol was estimated by the method of Baker *et al* (1951). To 0.1 ml of sample, 1.5 ml of ethanol and 2.0 ml of petroleum ether were added, mixed and centrifuged. The supernatant was evaporated to dryness at 80^{0}C. To this was added 0.2 ml of 2, 2'dipyridyl solution and 0.2 ml of ferric chloride solution. Mixed well and kept in dark for 5 min and added 2.0 ml of butanol. The intense red color developed was read at 520 nm. Standard tocopherol in the range of 10-100 μg were taken and treated similarly along with blank containing only the reagent.

RESULTS AND DISCUSSION:

Effect of cryopreservation on rat epididymal spermatozoa

There were significant effects of cryopreservation on rat epidydimal sperm lipid peroxidation, antioxidant and non enzymatic antioxidant system in different times of preservation ($p \leq 0.05$).The reduced activity of antioxidant enzymes in extreme cold is a common effect which accelerates the accumulation of ROS in higher amount. Yang *et al.* (2011) have found that chilling stress could reduce the activities of antioxidant enzyme.

Lipid peroxidation in fresh and frozen thawed rat spermatozoa

There was 24.65% increase in lipid peroxidation observed in the cryopreserved samples by freezing and thawing. The cryopreservation has elevated the levels of MDA compared to the control group. The concentration of MDA was 42.51±0.82 (n-6)µmoles for fresh semen, was increased to 58.42±0.72**,** 59.02±0.81, 61.06±0.89 and 62.91±0.80 (n-6) in 15days, 30 days, 45 days and 60 days frozen-thawed samples, respectively **(Figure. 2)**. It indicates significant ($p \leq 0.05$) effect of cryopreservation on lipid peroxidation of spermatozoa.

In the present study, significant increase in the epididymal spermatozoa LPO levels were observed as compared to the fresh, possibly due to increase in the generation of free radicals by freezing in the spermatozoa which may causing cell membrane damage and enzyme system repression. Freezing-thawing process decreases semen quality (Oettle 1986, Wang *et al.* 1997). An analysis of human sperm cells cryopreserved with the use of the same method produced similar results (Wang *et al.* 1997). Neagu *et al.* (2011) demonstrated a decrease in the peroxidation of membrane lipids in thawed samples of canine semen.

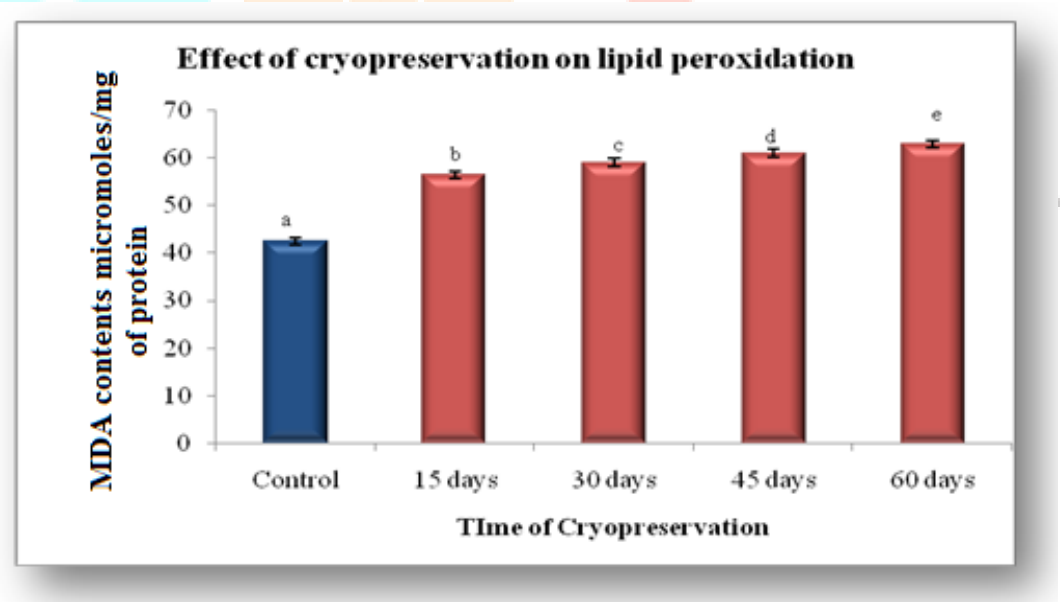

Figure 2: Data were expressed as mean SD (n=6). Units: Lipid peroxidation: micromoles of malondialdehyde/mg of protein. Values marked as a, b, c, d and e differ significantly from fresh value (P≤0.05).

Malonaldehyde produced from the breakdown of polyunsaturated fatty acids serves as a reliable index for determining the extent of the peroxidation reaction and lipid damage. (Buege and Aust, 1978). Lipid peroxidation appears to be the major process of membrane damage as evident from increased MDA concentration in the frozen-spermatozoa. The results of this study shows that cryopreservation of rat semen decreased the percentage of spermatozoa with the simultaneous significant increase in the concentration of final product of lipid peroxidation.

Antioxidant enzymatic activity in fresh and frozen thawed rat spermatozoa

Cryopreservation is a technique commonly used for gene banking purposes and is routinely employed in artificial reproductive technologies. Moreover as mentioned earlier cryopreservation lead to significant reduction in the level of spermatozoa antioxidants and this generates superoxide radical, hydroperoxyl radical and hydroxyl radical (Silva, 2006) which are responsible for cell damage. In this study the effect of cryopreservation on rat epididymal sperm anti oxidant enzymatic system were observed at different intervals of time, represented in **Table.1**

		Cryopreservation			
Activities	**Control**	**15 days**	**30 days**	**45 days**	**60 days**
CAT	0.367±0.03	0.226±0.072	0.186±0.06	0.177±0.07	0.163±0.02
SOD	3.442±0.08	1.677±0.03	1.409±0.04	1.212±0.02	1.094±0.03
GPx	0.774±0.04	0.474±0.03	0.419±0.01	0.394±0.11	0.373±0.05
GST	0.975±0.02	0.3543±0.03	0.340±0.01	0.323±0.05	0.303±0.01
GR	1.875±0.008	0.949±0.02	0.865±0.187	0.814±0.164	0.780±0.17

Table 1: Data were expressed as mean ± SD (n=6). Note: Catalase: The enzyme activity expressed as micromoles of H_2O_2 consumed /min/mg of protein, Superoxide dismutase: The enzyme activity expressed as micromoles of epinephrine utilized/mg of protein, Glutathione peroxidase: The enzyme activity expressed as nanomoles of NADPH oxidized/min/mg of protein. GST: µmoles of CDNB-GS conjugate formed per minute per mg protein., GR: nanomoles of NADPH oxidised /minute/ milligram protein.

Effect of cryopreservation on catalase activity in fresh and frozen thawed rat spermatozoa

In the present study the activity of catalase in fresh spermatozoa was 0.367±0.32, which was declined to 0.226±0.72 in the 15 days frozen-thawed samples. Significant decrease in CAT activity was observed continuously like0.186±0.06, 0.177±0.07, 0.163±0.02 in 30, 45, 60 days cryopreservation. In this study, CAT activity decreased in the spermatozoa after cryopreservation, but activity variations in the group with time difference were much more evident. The result was in agreement with Sreejith *et al.* (2007), who reported that CAT activity declined in spermatozoa seminal plasma of bull semen after cryopreservation.CAT activity in rat sperm cells showed a strong reduction after cryopreservation (38%), when compared with control samples. The significant increase of CAT activity in cryopreserved sperm cells could be used as evidence for ROS formation and development of oxidative stress. In the scientific literature there are many controversial publications about the effects of low temperature on CAT activity.

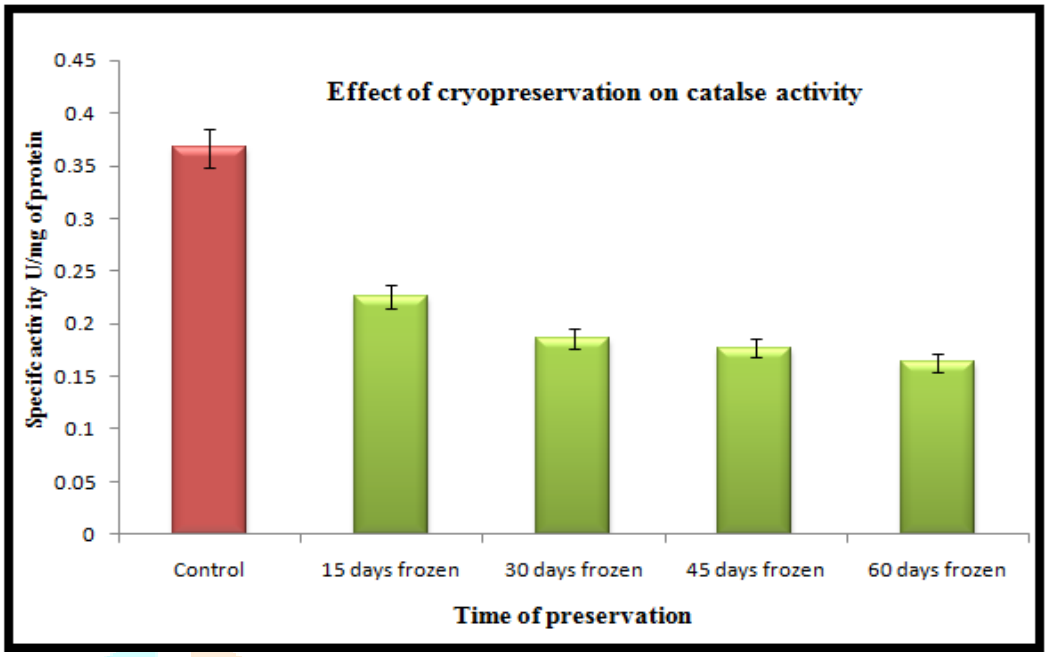

Figure.3: The activities of catalase in control and cryopreserved semen samples in rat.

Effect of cryopreservation on SOD activity in fresh and frozen thawed rat spermatozoa

SOD is considered the first line of defense against deleterious effects of ROS in cell by catalyzing dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen. In the same way to catalase, there was a decrease in the activity of SOD from 3.442±0.08 in fresh semen to 1.677±0.03, 1.409±0.04, 1.212±0.02, 1.094±0.03 in frozen-thawed semen, for respective storage days. Marti *et al.* (2008) indicated that decrease in SOD activity of spermatozoa after freezing-thawing the impairment of the antioxidant defense system of mammalian cryopreserved semen might be due to removal rate dilution of plasma during the freezing- thawing process. Moreover, the decrease of SOD activity after freezing-thawing was found also in the bull semen (Bilodeau *et al*., 2000) and in the human sperm (Lasso *et al.*, 1994).

The present study has observed that cryopreservation induced adecrease in SOD in all frozen-thawed samples. According to this data, the activity of SOD in rat spermatozoa was highly decreased51% above the fresh variant. That decrease in the activity of SODcould be considered as a consequence of the cryopreservation.

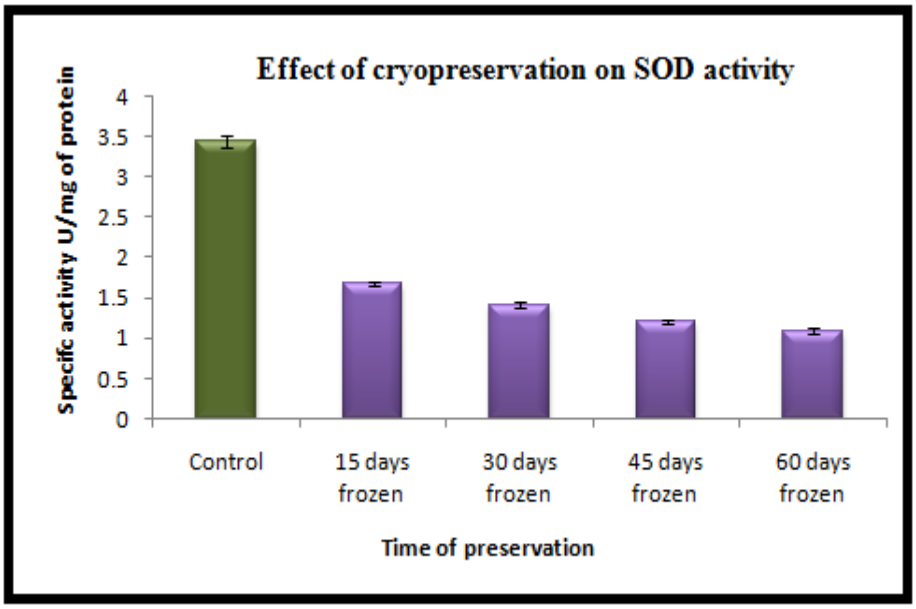

Figure.4: The activities of SOD in control and cryopreserved semen samples in rat.

Effect of cryopreservation on GPx activity in fresh and frozen thawed rat spermatozoa

Lower levels of GPx activity were reported in cryopreserved sperm cells in comparison with fresh semen (Fig 7). A dramatic drop in GPx activity was noted on cryopreservation ($p<0.05$). 15 days frozen-thawed samples of semen were characterized by lower levels of activity GPx. The range of changes in cryopreserved semen varies across different preservation intervals like 38.78% in 15 days frozen-thawed samples and in 30, 45 and 60 days frozen samples 45.86%, 49.09% and 51.80% activity were observed. All these results are in agreement with Frozen-thawed samples of bull semen showed 50% drop in SOD activity (Bilodeau *et al.* 2000).

Glutathione peroxidase plays a major role in maintaining the balance of pro and antioxidation, because it removes lipid peroxides from the cells, by converting them into non-reactive products (Fraczek and Kurpisz, 2005).The above could suggest that in rat semen GPx is more susceptible to cryogenic changes. The mechanism responsible for the loss of antioxidant enzyme activity in cryopreserved semen remains unexplained.

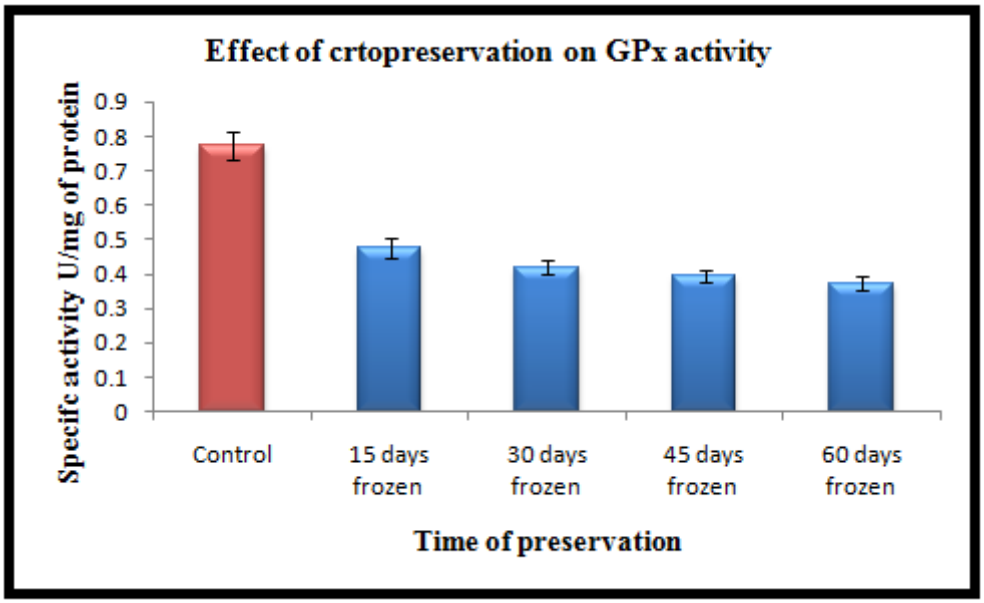

Figure.5: The activities of glutathione peroxidase in control and cryopreserved semen samples in rat.

Effect of cryopreservation on GR activity in fresh and frozen thawed rat spermatozoa

My study as mentioned earlier, the activity of the antioxidant enzymes differed greatly in both fresh and frozen samples. Another enzyme that was studied was Glutathione reductase of rat spermatozoa activity was reduced in spermatozoa compared fresh in frozen samples **(Figure 6)**.The GR activity was significantly (P≤0.05) decreased in frozen-thawed semen samples. The activity of GR in fresh spermatozoa was 1.875±0.008which was declined to 0.949±0.02in the 15 days frozen-thawed semen samples, this decrease in GPx activity was continued in 30, 45 and 60 days respectively by 0.865±0.187, 0.814±0.164,0.780±0.17. There was significant decrease in frozen thawed semen samples.

According to Park *et al.*(2003) the increased production of ROS in the process of freezing thawing of semen was observed. An aerobic cell contains substrates and enzymes to prevent the formation and propagation of ROS, but the antioxidant defenses of spermatozoa are relatively weak and these germ cells are very susceptible to oxidative stress (Jones and Mann, 1977). Some authors reported that ROS accumulation could be an important factor leading to further damage in post-thaw sperm (Guthrie *et al.*, 2008). These studies suggested that partial loss of antioxidant enzymes in cells with damaged sperm caused them more susceptible to peroxidative damage after thawing.

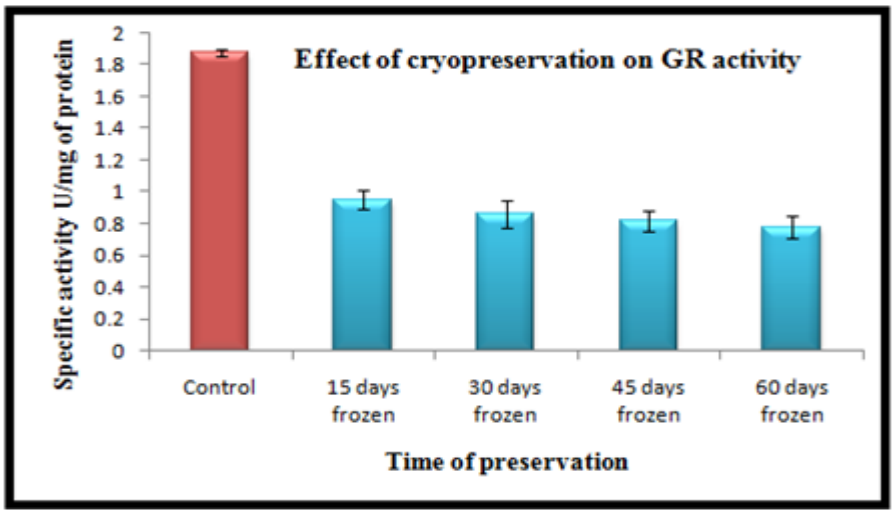

Figure.7: The Glutathione reductase activities of control and cryopreserved semen samples in rat.

Non enzymatic antioxidant activity in fresh and frozen thawed human semen

The results on nonenzymatic antioxidant activities in cryopreserved rat spermatozoa were shown in **Table 3**. Reduced GSH content was decreased significantly in frozen-thawed samples compared to the fresh semen samples. Spermatozoa Vit. E and Vit. C levels were also declined gradually in cryopreserved 15, 30, 45 and 60 days samples, compared to fresh samples. In all the frozen samples the values for GSH was lower than fresh semen, however, there was great variation between samples in the reduction of GSH (**Table.3**). GSH content in fresh semen was 11.67± 2.58. After freezing, there was a significant reduction in GSH content to 5.33±0.516 ($p < 0.05$), representing a 54% reduction in 15 days, 4.83±0.408 in 30 days, 4.16±0.408 and 3.66±0.258 in 60 days of cryostorage GSH content was found. The GSH content was reduced to 41%, 36% and 31% depending on cryostorage. Hence cryostorage of semen was found to deplete the GSH levels.

From the **Table 2** it was known that GSH, Vit.E and C contents decrease in frozen and thawed semen samples from 15 to 60.Reactive oxygen species (ROS), is a highly reactive oxidizing agent belonging to the class of frees radicals, there is production of ROS during cryopreservation and frozen spermatozoa are highly sensitive to lipid peroxidation (LPO), significant reduction in the level of spermatozoa antioxidants. Thuwanuta *et al.* (2011) found an improvement of ram sperm survival after the addition of antioxidants (vitamins C and E) to semen diluents, other lipid-derived radicals (Silva, 2006), vitamin E was more efficacious in improving post-thaw motility of human spermatozoa (Askasi *et al.*,1994). Sinha *et al.*, 1996 demonstrated marked increase in post thaw motility of buck spermatozoa with reduced glutathione to the freezing extender.

In this study the percentage decrease in GSH content of human spermatozoa was 64%, while in boar the decrease was 32%(Gadea *et al*., 2004)and in bull it was 58% or 78%(Bilodeau *et al*., 2000). My data confirm previous studies that were reported on damage of components of the antioxidant system during cryopreservation (Lasso *et al*., 1994).A decrease in intracellular GSH and an increase in ROS production during cryopreservation could be one of the concomitant causes of low viability in thawed spermatozoa.

Antioxidant	Control mg/gm	15 days	30 days	45 days	60 days
Glutathione	11.67± 2.58	5.33±0.516	4.83±0.408	4.16±0.408	3.66±0.258
Vit. E	4.28±0.469	2.908±0.242	2.561±0.222	1.943±0.379	1.641±0.083
Vit. C	1.205±0.062	0.768±0.057	0.682±0.052	0.554±0.052	0.469±0.033

Table 2: The levels of GSH, Vit. E and Vit. C in control and frozen semen samples.

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