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The Effects of X-Ray Irradiation on Bovine Sperm Quality Indicators After Freezing and Thawing

Research Article

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ABSTRACT

This study was conducted to determine the suitable dose of x-ray irradiation on bovine sperm quality indicators after freezing and thawing. Sperm samples in liquid nitrogen were x-ray irradiated at doses of zero, 0.3, 0.6, 0.9, and 1.2 Gy. The results showed that irradiation influenced ($P<0.05$) motility and viability of sperms. The semen malondialdehyde concentration had no significant differences among treatments. Based on the comet assay result, DNA strand breaks parameters had no significant differences with the control group, except at a dose of 1.2 Gy. Based on flow cytometry results, sperm percentage with high activity of mitochondrial membrane was increased and those with low activity decreased ($P<0.05$). The HOST test showed that sperm percentage with plasma membrane integrity in irradiated samples at a dose of 0.3 Gy had no significant difference compared to the control group, but sperm percentage with intact plasma membrane was higher in the sample irradiated at doses of 0.6 and 0.9 Gy. Irradiation at a dose of 0.9 Gy increased sperm with the intact plasma membrane. Doses lower than 1.2 Gy did not affect acrosome integrity, but 1.2 Gy x-ray irradiation decreased sperm having intact acrosome ($P<0.05$). Based on these results, x-ray irradiation at a dose of 0.9 Gy could increase the mitochondrial membrane activity and enhance the motility and viability of sperms after thawing without a negative effect on semen and sperm quality while x-ray irradiation at a dose of 1.2 or more Gy deteriorates semen quality.

KEY WORDS DNA strand breaks, mitochondrial membrane activity, motility, viability, x-ray irradiation.

INTRODUCTION

Long-term storage of sperm by freezing causes sperm motility and viability damage after thawing (Holt, 2000). Although compounds such as glycerol, and some amino acids (glutamine, glycine, proline, alanine, and histidine) are used to induce sperm resistance at low temperatures and to protect against cold shock, a 25% decrease in motility of bovine sperm after freezing is still observed (Amirat-Briand *et al.* 2009). X-ray as ionizing radiation can cause changes in the quality of the sperm by creating free radicals (Shawrang and Sadeghi, 2013). Some reports on the impact of irradiation on sperm quality in laboratory animals (mouse, rat, and hamster) were published (Tateno *et al.* 1996). Researchers exposed laboratory animals to ionizing beams including gamma-ray and x-ray and studied its effects on the sperm chromosome of two different hamster breeds (Chinese and Golden). These authors stated that there was no great difference in the induction of structural chromosome aberrations between X-irradiated (0.91, 1.82, 3.63) and y-irradiated (1.10, 2.15, 2.95, 4.01) spermatozoa (Tateno *et al.* 1996). Lubart *et al.* (1997) studied the effects of laser irradiation on mitochondrial activity and the plasma membrane of mammalian sperm. These authors reported that

780 nm light inhibits Ca2+ uptake by sperm mitochondria and enhances Ca2+ binding to sperm plasma membranes. Parplys *et al.* (2012) studied the effects of x-rays on DNA damage. These authors reported that ionizing irradiation induces several types of DNA lesions, base damage, single-strand breaks, and double-strand breaks through the production of free radicals. Although there are different ways to evaluate the rate of DNA breaks, the Comet method has advantages over other methods. Among the benefits of this method is that it is possible to investigate damage to single cells, being able to detect minimal DNA damage (Olive and Banath, 2006). This method estimates DNA damage parameters including % DNA in tail and tail moment. Comet observations include the head + the tail (Frenzilli *et al.* 2014). Irradiation with laser red light has been described to improve the cryotolerance and longevity of frozen-thawed bovine and ram spermatozoa (Fernandes *et al.* 2015; Iaffaldano *et al.* 2016). Red-light stimulation, either with low-level lasers or light-emitting diodes (LED), has also been reported to increase the motility, ability to elicit *in vitro* capacitation, fertilizing ability, and lifespan of fresh and liquid-stored semen in fish (Zan-Bar *et al.* 2005), turkey (Iaffaldano *et al.* 2005), dogs (Corral-Baqués *et al.* 2005; Corral-Baqués *et al.* 2009), rabbit (Iaffaldano *et al.* 2010), buffalos (Abdel-Salam *et al.* 2011) humans (Salama and El-Sawy, 2015), boar (Yeste *et al.* 2016; Pezo *et al.* 2019; Prieto *et al.* 2019) and donkeys (Catalán *et al.* 2020). Similarly, Shawrang *et al.* (2020) studied the application of gamma irradiation to increase the motility and viability of bovine sperm after freezing and thawing. The results of that study demonstrated that gamma irradiation at a dose of 0.7 Gy could enhance the motility and viability of sperms after thawing without a negative effect on semen and sperm quality. The aim of this study was the determination of the suitable dose of x-ray irradiation for increasing sperm motility and viability after freezing and thawing and to assess its effects on malondialdehyde concentration, the rate of sperm DNA strand breaks and mitochondrial membrane activity, acrosome and plasma membrane integrity of bovine sperm.

MATERIALS AND METHODS

Sperm preparation and irradiation

25 sperm straws 0.5 mL containing frozen sperm samples were prepared at -196 ˚C liquid nitrogen from NDJ Company. Frozen sperm samples were x-ray irradiated by Nuclear Regulatory Authority. According to studies, irradiation at a dose of 1 Gy will cause DNA damage (Tateno *et al.* 1996); Therefore sperm samples in liquid nitrogen were x-ray irradiated in 5 replicates at doses of zero, 0.3, 0.6, 0.9, and 1.2 Gy using Balteau 235 Portable x-ray Generator (kV=120, mA=2.4) for 0, 3, 6, 9 and 12 minute respectively.

Sperm motility and concentration

Thawing of irradiated frozen sperm straws was done in 37 ˚C bain-marie for 45 seconds. The sperm quality and quantity parameters including concentration, motility, progressive motility, circle motility, fast motility, slow motility, local motility, and immotile sperm percentage were evaluated using the CASA system (12500/0000 AndroVision® Modul Concentration and Motility).

Sperm viability

Sperm viability analysis was performed using eosin/nigrosin staining. The staining was assessed on a microscope slide, with 20 µL of frozen-thawed sample to 100 µL of staining solution. A smear was made and allowed to dry on another slide. Unstained spermatozoa and spermatozoa with the damaged membrane (200 sperm) were counted under the microscope at 100X (Mohammadi *et al.* 2011).

Sperm morphology

Sperm morphology was performed using eosin/nigrosin staining, smears were made on slides, air-dried, and made permanent to calculate the abnormalities in spermatozoa. The slides were observed under the microscope using 100X and oil immersion objectives. Morphologically normal and abnormal spermatozoa were counted, and their percentage was calculated.

Determination the amount of malondialdehyde

Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a pink species absorbing at 535 nm (Zeb and Ullah, 2016). Solutions used in this method included: 0.67% solution of thiobarbituric acid (TBA), 10% solution of trichloroacetic acid (TCA), butylated hydroxytoluene (BHT, 0.2 g of BHT in 10 mL of ethanol), and ethylenediaminetetraacetic acid (EDTA, 0.37 g of EDTA in 10 mL of distilled water). Briefly, samples (0.5 mL of each sample) were homogenized with 0.5 mL EDTA, 0.5 mL BHT, and 1 mL TCA. The mixture was centrifuged at 936 × g for 15 min using a centrifuge (Hitachi, Tokyo, Japan). Then, 2 mL of TBA was added to 1 ml of supernatant. The mixture was heated in boiling water (95 ˚C) for 1 h, followed by cooling with running water. Absorbance was read at 532 nm using a spectrophotometer. Thiobarbituric acid reactive substances were calculated from the standard curve and expressed as nmol malondialdehyde (MDA)/mL of semen.

Detection of DNA damage by sperm comet assay

The assay was performed according to Frenzilli *et al.* (2014) with some modifications. The sperm sample was

centrifuged and diluted with 500 µL PBS [8 g NaCl, 2 g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 (pH 7.5)] after removal of the supernatant. Then, 30 µL of the sample was suspended in 150 µL of 0.7% (w/v) low melting point agarose (LMPA). From this suspension, 65 µL was applied to the surface of a microscope slide pre-coated with 0.8% normal melting point agarose (NMPA) to form a microgel and allowed to set at 4 ˚C for 10 min. Slides were dipped in cell lysis buffer [146.19 g NaCl, 37.24 g EDTA, 1.25 g Tris (pH 9.5) containing 1 mL Triton X-100 and 1 g sodium lauryl sarcosinate] for 24 h at 4 ˚C and protected from light. Following cell lysis, all slides were washed with deionized water to remove salt and detergent from the microgels. Slides were then coded and placed in a specifically designed horizontal electrophoresis tank containing electrophoresis buffer [24.6 g $NaCH_3COO$ and 12.1 g Tris-HCl (pH 8.3)]. Electrophoresis was conducted at 0.6 V/cm, 12 mA for 1 h. After electrophoresis, slides were neutralized in neutralization buffer [48.5 g Tris (pH 7.5)] and the DNA fluorochrome SYBR Green (CinnaGen DNA Safe Stain, Cat. No.: EP5082) was applied for 30 min. Slides were rinsed briefly with double-distilled water and the coverslips were placed before image analysis. The fluorescent-labeled DNA was visualized using a fluorescence microscope and the resulting images were captured on a computer and processed with Comet score software. The parameters for the DNA damage analysis include % tail DNA and tail moment. The Tail Moment value is calculated using the software and the following relationship (Frenzilli *et al.* 2014).

Tail moment= tail length (px) × % DNA in tail.

In this relationship, the tail length is a parameter estimated by the software's light intensity (pixels).

Hypo-osmotic swelling test (HOST)

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on coiled and swollen tails. This was performed by incubating 250 µL of frozen-thawed sperm sample with 1 mL of a 100 mOsm hypo-osmotic solution (0.9 g of fructose+0.49 g of sodium citrate per 100 mL of distilled water) at 37 ˚C for 45 min. After incubation, 5 µL of the mixture was spread with a coverslip on a warm slide. Two hundred sperm were evaluated (magnification 400×) with bright-field microscopy. Sperm with coiled tails were recorded (Revell and Mrode, 1994).

Chlortetracycline (CTC) assay

Acrosomal and capacitation status were assessed using CTC staining as described previously (Perez *et al.* 1996) with a little modification. A CTC working solution (0.75 mM) was freshly prepared in a buffer containing Tris (20 mM), NaCl (130 mM), and D, L-cysteine (5 mM) at a pH of 7.4. Twenty microliters of sperm were mixed with 20 µL CTC working solution. The reaction was fixed by adding 5 µL of 1% (v/v) glutaraldehyde in 1 M Tris buffer (pH 7.4) and leaving 30 s at room temperature under dark conditions. Sperm were prepared on a clean microscope slide and examined under a fluorescent microscope at a magnification of ×400. All samples were processed in duplicates, and at least 200 spermatozoa per slide were scored. Two hundred sperm were categorized into three categories as follows: (1) uniform fluorescence head (un-capacitated spermatozoa); (2) post-acrosomal region without fluorescence (capacitated spermatozoa); and (3) fluorescent-free head or a thin fluorescent band on the equatorial segment (acrosome reacted spermatozoa).

Statistical analysis

Data were statistically analyzed by GLM procedure of SAS software in a completely randomized design (SAS, 2004). Significance test was performed using Duncan's multiple range tests at $P < 0.05$.

RESULTS AND DISCUSSION

The result of concentration and sperm motility percentage before and after x-ray irradiation is shown in Table 1. There was no significant difference in the concentration of sperm between the control and the irradiated groups. A significant increase in sperm motility percentage was observed after irradiation at a dose of 1.2 Gy compared to the control treatment ($P<0.05$). There was no significant difference between experimental treatments for other motion parameters such as the percentage of sperm with progressive and circle motility. The percentage of fast motility of sperm was affected by x-ray irradiation so that fast motility was reduced at doses 0.6 and 0.9 Gy ($P<0.05$). There was no significant difference in the fast motility of sperm between the control and other irradiated groups. X-ray radiation dose rate had a significant effect on the slow motility of sperm. Slow motility of sperm increased with increasing dose rate, with the lowest slow motility at a dose 0.3 Gy and the greatest slow motility was determined at a dose of 0.9 and 1.2 Gy ($P<0.05$). There was no significant difference in local motility of sperm between the control and the irradiated groups. The x-ray irradiation led to significant decreases in the percentage of immotile sperm ($P<0.05$). Sperm motility at a dose 1.2 Gy x-ray irradiation was higher ($P<0.05$) than in other treatments. Therefore, it was concluded that a dose of 1.2 Gy x-ray irradiation led to a 7% increase in sperm motility and 17% reduced in the immotility of sperm after freeze-thawing.

One theory is that increased sperm motility could be due to motility in immotile sperm.

Table 1 Concentration (10^9/mL) and sperm motility percentage before and after x-ray irradiation

Parameters	Control	0.3 Gy	0.6 Gy	0.9 Gy	1.2 Gy	SEM
Concentration	39.8	38.82	37.95	38.29	36.69	4.513
Motility	70.21^{b}	72.56^{ab}	71.59^{ab}	72.66^{ab}	75.43^{a}	5.147
Progressive motility	64.76	62.83	65.90	64.76	68.90	7.292
Circle motility	1.81	1.40	1.40	1.23	1.13	0.211
Fast motility	57.78^{a}	55.55^{a}	57.66^{bc}	53.83^{c}	58.00^{a}	7.986
Slow motility	5.14^{c}	6.88^{b}	6.80^{b}	10.70^{ab}	10.83^{a}	3.068
Local motility	5.45	9.76	4.16	7.86	6.50	4.735
Immotility	29.8^{a}	27.43^{ab}	29.00^{a}	27.33^{ab}	24.56^{c}	4.647

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Generally, it has been reported that the elevation in Ca2+ uptake by sperm plasma membrane vesicles was a consequence of Ca2+ binding to the membrane after irradiation (Lubart *et al.* 1997). Sperm basal motility depends on three crucial factors: the presence of ATP, concentration of intracellular calcium, and normal to alkaline intracellular pH (Lishko *et al.* 2016). The cation channel of sperm is a sperm-specific, weakly voltage-dependent, Ca2+ selective, pH-sensitive ion channel that controls the entry of positively charged calcium ions into sperm cells, which is essential for sperm motility and fertility (Singh and Rajender, 2014).

The viability and morphological parameters of the frozen-thawed sperm before and after x-ray irradiation are presented in Table 2. The x-ray irradiation did not affect any of the sperm morphological parameters. The percentage of sperm viability was affected by x-ray irradiation after freezing and thawing. Therefore, sperm viability increased with increasing dose rate up to 0.9 Gy. The greatest viability level was found at a dose 0.9 Gy (P<0.05). The lowest sperm viability was observed at a dose 1.2 Gy.

The non-significant changes that occur in the percentage of normal sperm in irradiated samples can be due to the effect of radiation on the creation resistance of sperm to morphological changes. Beneficial effects on body systems by some external agent, which with large doses exhibits a damaging effect, are called hormesis (Obodovskiy, 2019). Thus, hormesis is a process in which exposure to low-dose radiation that is damaging at higher doses induces an adaptive beneficial effect on the cell (Mattson, 2008; Kudryasheva and Rozhko, 2015).

Tateno *et al.* (1996) studied the cytogenetic effects of x- and ¥-rays on the spermatozoa of the Chinese hamster and the Syrian hamster. These authors stated that there was no great difference in the induction of structural chromosome aberrations between X-irradiated (0.91, 1.82, 3.63) and y-irradiated (1.10, 2.15, 2.95, 4.01) spermatozoa.

The amount of malondialdehyde (MDA) of the frozen-thawed sperm before and after x-ray irradiation is presented in Table 3.

There was no significant difference in the amount of malondialdehyde in semen between the control and the irradiated groups. Furthermore, the increase in peroxide production rates was positively related to irradiation dose. Thus, irradiated treatment by low doses had a better preservation effect. However, Tateno *et al.* (1996) reported that doses of 0.91, 1.82, and 3.63 Gy of x-rays and 1.10, 2.15, 2.95, and 4.01 Gy of ¥-rays did not change the number of peroxides in sperm samples of two hamster species. Similarly, Hawas (2013) reported that low-dose gamma rays did not change the amount of malondialdehyde in the testis tissue of rats.

DNA strand breaks parameters before and after x-ray irradiation are demonstrated in Table 3. The radiation dose rate had a significant effect on DNA damage in spermatozoa. DNA damage of sperm (observed comet, % tail DNA, Tail Moment) only showed a significant increase after the highest dose (1.2 Gy, 11.37, 21.72, 20.28) of x-ray irradiation was used (P<0.05). Therefore, it is concluded that a dose of 1.2 Gy x-ray irradiation led to an 18% increase in % tail DNA.

There was no significant difference in DNA damage of sperm (observed comet, % tail DNA, Tail Moment) between the control and other irradiated groups.

The obtained results were in-line with estimates of Parplys *et al.* (2012) and Han *et al.* (2014) who reported that ionizing irradiation induces several types of DNA lesions, base damage, single-strand breaks, double-strand breaks through the production of free radicals.

The percentage of plasma membrane integrity of sperm before and after x-ray irradiation is shown in Table 4. Plasma membrane integrity increased with increasing dose rate up to 0.9 Gy. The lowest plasma membrane integrity was determined at a dose of 1.2 Gy and the highest plasma membrane integrity was determined at a dose of 0.9 Gy (P<0.05).

The HOST test assessed the resistance of the sperm plasma membrane to damage induced by the loss in permeability under the stress of swelling driven by the hypo-osmotic treatment (Sarıozkan *et al.* 2015).

Table 2 Sperm viability, normal and abnormal sperm percentage before and after x-ray irradiation (Gy)

Parameters	Control	0.3 Gy	0.6 Gy	0.9 Gy	1.2 Gy	SEM
Normal	84.8	85.7	82.6	83.7	83.4	4.37
Detached head	1.6	1.0	0.5	1.0	1.5	1.12
Abnormal head	1.0	1.0	2.0	0.3	1.2	1.59
Abnormal midpiece	5.4	6.2	5.1	8.3	4.0	4.85
Abnormal tail	4.6	4.2	3.6	5.2	3.7	2.81
Cytoplasmicdroplet proximal	2.0	1.3	2.0	1.2	2.4	1.60
Cytoplasmic droplet distal	0.6	1.0	0.8	1.2	1.7	1.24
Viability	81.2^{b}	82.5^{ab}	84.1^{a}	85.7^{a}	80.4^{b}	3.41

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 3 Malondialdehyde (MDA) and DNA strand breaks parameters before and after x-ray irradiation (Gy)

Parameters	Control	0.3 Gy	0.6 Gy	0.9 Gy	1.2 Gy	SEM
MDA	0.004	0.005	0.005	0.003	0.004	0.0027
Observed comet%	2.86^{b}	3.62^{b}	5.81^{b}	7.59^{b}	11.37^{a}	5.428
% Tail DNA	3.11^{b}	9.24^{b}	11.88^{b}	13.01^{b}	21.72^{a}	8.531
Tail moment	2.14^{b}	7.38^{b}	10.43^{b}	11.67^{b}	20.28^{a}	9.182

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 4 Acrosome and plasma membrane integrity of sperm before and after x-ray irradiation (Gy)

Parameters	Control	0.3 Gy	0.6 Gy	0.9 Gy	1.2 Gy	SEM
Plasma membrane integrity %	82.3^{b}	82.9^{b}	87.1^{a}	88.2^{a}	81.9^{b}	5.31
Acrosome integrity %	90.2^{a}	87.2^{a}	86.5^{ab}	83.3^{ab}	82.4^{b}	6.12

The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

In this study, the percentage of plasma membrane integrity (HOST test) was increased up to 4% with the greatest plasma membrane integrity observed at a dose of 0.9 Gy x-ray irradiation. Moreover, an increase in the percentage of sperm membrane integrity in irradiated samples compared with non-irradiated samples may be due to the phenomenon of Hormesis.

Holt (2000) and Portas *et al.* (2009) obtained some evidence that phase transitions might be involved in the manifestation of cryoinjury during the rewarming of cells after thawing. Accordingly, spermatozoa undergo these lipid phase transitions typically within the temperature of 37 ˚C.

One interpretation of this data is that as the thawing temperature increases to 37 ˚C (40% of sperm is alive), the plasma membrane is subjected to structural rearrangements involving lipids and proteins, the extent and nature of which are governed by interactions of temperature and solute effects during the freezing process. Therefore, these results (plasma membrane integrity) could be attributed to the positive effects of radiation (in some cases) on improved sperm quality.

The percentage of acrosome integrity of sperm before and after x-ray irradiation is shown in Table 4. A significant reduction in acrosome integrity percentage was observed after irradiation at a dose of 1.2 Gy compared to the control treatment (P<0.05).

There was no significant difference in acrosome integrity of sperm between the control and other irradiated groups except 1.2 Gy.

Lubart *et al.* (1997) and Singh and Rajender (2014) reported that the elevation in Ca2+ uptake by sperm plasma membrane vesicles was a consequence of Ca2+ binding to the membrane after irradiation. The cation channel of sperm is a sperm-specific, weakly voltage-dependent, Ca2+ selective, pH-sensitive ion channel that controls the entry of positively charged calcium ions into sperm cells, which is essential for sperm motility and fertility. Intracellular calcium plays a vital role in cell proliferation, and in mammalian spermatozoa, it has a pivotal role in the control of sperm motility and acrosome reaction. Moreover, the researchers found an elevation in the concentration of cytoplasmic calcium ions in spermatozoa after irradiation (Tateno *et al.* 1996).

According to sperm evaluation standards, sperm quality is desirable with a thawing temperature of 37 ˚C and acrosome integrity of more than 60% (Portas *et al.* 2009; Holt, 2000). Thus in the present study, although the acrosome integrity of sperm at a dose 1.2 Gy x-ray irradiation was lower than the control treatment, its amount was reported as more than 60% (sperm evaluation standard).

CONCLUSION

The results of this study demonstrated that x-ray irradiation at a dose of 0.9 Gy could increase the mitochondrial membrane activity and the motility and viability of sperms after thawing without negative effect on malondialdehyde concentration of semen and DNA strand breaks parameters, plasma membrane integrity, and acrosome integrity of sperm while x-ray irradiation at a dose of 1.2 or more Gy deteriorates semen quality.

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