Effect of quercetin on lipid peroxidation, total antioxidant potential, mitochondrial activity and quality of frozen-thawed Indian red jungle fowl (*Gallus gallus murghi*) sperm

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Abstract

The study was designed to elucidate the effects of quercetin on lipid peroxidation, total antioxidant potential, mitochondrial activity and quality of frozen-thawed Indian red jungle fowl (*Gallus gallus murghi*) sperm. For this, semen was collected from seven adult Indian red jungle fowl through abdominal massage, and evaluated for volume, concentration and motility. The qualifying semen ejaculates having >80% motility were diluted in red fowl extender having quercetin 0 mM (control), 5 mM, 10 mM, 15 mM and 20 mM. Diluted semen was frozen following glycerol based protocol and semen microscopic (motility, plasma membrane integrity, viability, acrosome and chromatin damage) biochemical (mitochondrial activity and ferric reducing antioxidant power) and oxidative parameters (malondialdehyde) were determined after thawing at 37°C. Motility, acrosome and chromatin integrity were recorded highest with 15 mM and 20 mM quercetin compared to quercetin 5mM, 10mM, and control. Nevertheless, plasma membrane integrity, viability, mitochondrial activity and antioxidant potential were recorded highest with 15 mM quercetin compared to all experimental extenders. Malondialdehyde concentration in sperm and seminal plasma were recorded lowest (P<0.05) in extender having 15 mM quercetin. It is concluded that quercetin supplementation in red fowl extender improve motility, mitochondrial activity, plasma membrane, acrosme and chromatin integrity by elevating the total antioxidant potential and ameliorating the lipid peroxidation in frozen-thawed semen.

Material and Methods

2.1. Experimental birds

Seven Indian red jungle fowl cocks (*Gallus gallus murghi*) were kept at Avian Research Center, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi and exposed to natural photoperiod of 16 hours. Each bird was offered commercially available poultry feed (approximately 100g) on daily basis and freshwater *ad libitum*.

2.2. Semen collection, Initial evaluation and cryopreservation

Semen from mature males was collected through abdominal massage in graduated glass tube. Volume was measured with micropipette in microliters. Initial motility was evaluated by mixing 10 µL semen sample in 500 µL of phosphate buffer saline. For this purpose, a drop of diluted sample was placed on pre-warm glass slide and motility was observed with microscope. Sperm concentration was measured with Neubauer hemocytometer. Ejaculates having >60% motility were pooled, and diluted (1:1; V/V) in red fowl extender. Red fowl extender was prepared by mixing Fructose (1.15 g), Sodium glutamate (2.1 g), PVP (0.6 g), Glycine (0.2 g), Potassium acetate (0.5 g) to 100mL of double distilled water having pH (7.0) and Osmotic pressure (380 mOsmol/kg) (Rakha et al., 2016). The diluted samples was divided into five experimental extender and quercetin was added as 0.0 mM (control), 5mM, 10mM, 15mM, 20mM. The diluted semen was cooled to 4°C, 20% glycerol was added to chilled semen and filled in 0.5ml French straws (IMV, France) with suction pump and placed over liquid nitrogen vapors for 10 minutes, plunged into liquid nitrogen and stored. After 24 hours, the straws were removed from liquid nitrogen and thawed in a water bath at 37°C for semen quality evaluation.

2.3. Semen Quality Assay

2.3.1. Motility

Post-thaw sperm motility was assessed by placing a drop of sample on pre-warmed (37°C) glass slide, cover-slipped and observing under a phase contrast microscope (400x). percentage sperm motility was evaluated at a scale of 0 to 100% (Zemjanis, 1970).

2.3.2. Plasma membrane integrity

Sperm plasma membrane integrity was assessed through hypo-osmotic swelling (HOS) test (Santiago-moreno et al., 2009). The HOS solution was prepared by

Objectives

To identify the optimum concentration to reduce the oxidative stress and ameliorate the cryo-damages during cryopreservation of Indian red jungle fowl sperm.

2.3.3. Viability

Sperm viability was assessed by Lake's glutamate solution (Bakst & Cecil, 1997); prepared by adding potassium citrate (0.00128g) sodium glutamate (0.01735) sodium acetate (0.0085g) in 100 ml distilled water. Water soluble Eosin-blue (1g) and nigrosine (5g) were added in Lake's glutamate solution. A smear was prepared by mixing 12 drops of Lake's glutamate solution with one drop of semen sample on glass slide, air dried and observed under a phase contrast microscope (1000x) with oil immersion. Unstained sperm appeared as shiny white and recorded as live sperm while stained sperm appeared as pink and read as dead. A total of 200 spermatozoa were counted in five different fields of glass slide from each experimental extender.

2.3.4. Sperm acrosome integrity

Sperm acrosomal integrity was evaluated by giemsa stain (Jianzhong & Zhang, 2006). The stain was prepared by adding giemsa (3 g), in and PBS in 35ml water. A drop of semen sample was taken on a glass slide, smear was made, air dried and fixed in neutral formal saline solution for 30 minutes. After fixing, the slides were washed with distilled water and kept in giemsa stain for 1.5 hours. Slides were again washed two times with distilled water to remove extra stain, air dried and observed under a phase contrast microscope (1000× with oil immersion). Live sperm with intact acrosome were stained and appear as blue, dead sperm with damaged acrosome were unstained and appeared as white or partially stained. A total of 200 spermatozoa were counted in five different fields of glass slide from each experimental extender.

2.3.5. Sperm chromatin damage

The chromatin integrity was assessed by aniline blue stain (Auger et al., 1990). Aniline blue solution was prepared through mixing of aniline blue (5g) with 100ml of PBS, filtered and pH was adjusted to 3.5 by adding of 2% glacial acetic acid. Smear was made with 10µL of semen on clean glass slide, air dried and kept in aniline blue stain for 5min, washed with distilled water and air dried. Prepared slides were observed under a phase contrast microscope (1000x with oil immersion). Spermatozoa having damaged chromatin material appeared as dark blue. A total of 200 spermatozoa were counted in five different fields of glass slide from each experimental extender.

2.4. Sperm biochemical Assays

2.4.1. Lipid peroxidation assay

The Lipid peroxidation assay was assessed by measuring malondialdehyde (MDA) concentration in semen samples. Briefly, 200µL of seminal plasma was mixed with 10 µL of probucol, 640µL of NMPI (Total Lipid Liquid, Fardiag, Italy) and 150 µL of HCL, vortexed and incubated at 45°C for 1 hour. Clear seminal plasma was obtained by 10 minutes of centrifugation at 10,000rmp. The level of MDA was evaluated by measuring absorbance at 586 nm and expressed its concentration as µM/ml in seminal plasma and µM/10^9 in sperm.

2.3.7. Mitochondrial activity

MTT reduction assay was used to measure the mitochondrial activity of sperm cells. In this assay, NADPH-dependent cellular oxireductase enzyme presents the viable cells in the sample by reducing MTT to purple color Formazan. For this purpose, MTT stock solution was prepared by adding 5mg of MTT (Sigma-Aldrich, USA) in per ml of PBS. 10µL of stock solution was added to 100µL of semen sample. The absorbance was measured at 550nm and samples were then incubated for 1 hour at 37°C. After incubation, absorbance was measured again. The rate of MTT reduction was calculated by recording the difference between initial and final reading of absorbance (Hazary and Wishart, 2001).

Results

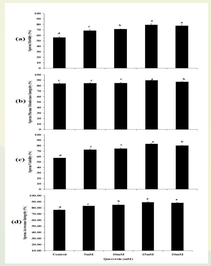

Figure 1: Effect of addition of different concentrations of Quercetin (mM) (0mM, 5mM, 10mM, 15mM and 20mM) on post-thaw percentage sperm motility (a), plasma membrane integrity (b), viability (c) and acrosome integrity (d). Bars with different letters showed significant differences (P<0.05) among different concentrations.

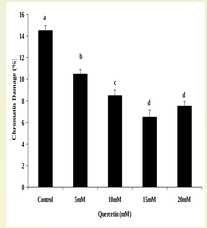

Figure 2: Effect of addition of different concentrations of Quercetin (mM) (0mM, 5mM, 10mM, 15mM and 20mM) on sperm chromatin damage after post-thawing. Bars with different letters showed significant differences (P<0.05) among different concentrations.

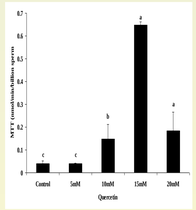

Figure 3: Effect of addition of different concentrations of Quercetin (mM) (0mM, 5mM, 10mM, 15mM and 20mM) on mitochondrial activity (nmol/min/billion sperm) of frozen-thawed semen sample. Bars with different letters showed significant differences (P<0.05) among different concentrations.

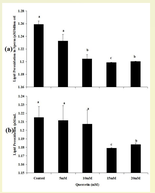

Figure 4: Effect of addition of different concentrations of Quercetin (mM) (0mM, 5mM, 10mM, 15mM and 20mM) on lipid peroxidation in sperm (a) and seminal plasma (b) in frozen-thawed semen sample. Bars with different letters showed significant differences (P<0.05) among different concentrations.

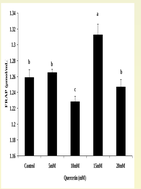

Figure 5: Effect of addition of different concentrations of Quercetin (mM) (0mM, 5mM, 10mM, 15mM and 20mM) on total antioxidant potential (µmol/ML) of Quercetin in frozen-thawed semen sample. Bars with different letters showed significant differences (P<0.05) among different concentrations.

Conclusions

- Study evaluated the effect of quercetin in extender on sperm quality, antioxidant potential, lipid peroxidation and mitochondrial activity of frozen-thawed Indian red jungle fowl sperm
- Motility, acrosome Plasma membrane integrity, viability, antioxidant potential and mitochondrial activity were recorded highest with 15 mM quercetin
- Chromatin damage and Malondialdehyde concentration in sperm and seminal plasma were recorded lowest with 15 mM quercetin

References

Partyka, A., Niżański, W., Ochota, M., 2012. Methods of assessment of cryopreserved semen. In Current Frontiers in Cryobiology. IntechOpen

Peterson, A. T., Brisbin, I.L., 1999. Genetic endangerment of wild red Jungle fowl *Gallus gallus*. Bird Conservation International 8, 387-394.

Peterson, A.T., Brisbin, I.L., 1998. Genetic endangerment of wild Red Junglefowl Gallus gallus?. Bird Conservation International 8(4), 387-394.

Purdy, P. H., 2006. A review on goat sperm cryopreservation. Small Ruminant Research 63, 215-225.

Rakha, B.A., Ansari, M.S., Akhter, S., Hussain, I., Blesbois, E., 2016. Cryopreservation of Indian red jungle fowl (*Gallus gallus murghi*) semen. Animal Reproduction Science 174, 45-55.

Rakha, B.A., Ansari, M.S., Akhter, S., Zafar, Z., Hussain, I., Santiago-Moreno, J., Blesbois, E., 2017. Cryopreservation of Indian red jungle fowl (*Gallus gallus murghi*) semen with polyvinylpyrrolidone. Cryobiology 78, 27-33.

Rakha, B.A., Ansari, M.S., Hussain, I., Malik, M.F., Akhter, S., Blesbois, E., 2015a. Semen characteristics of the Indian red jungle fowl (*Gallus gallus murghi*). Euopean Journal of Wildlife Research 61,379-386.

Rakha, B.A., Hussain, I., Malik, M.F., Akhter, S., Ansari, M.S., 2015b. Impact of ejaculate frequencies on the quality of Red Jungle Fowl (*Gallus gallusmurghi*) semen. Avian Biology Research 8, 109-112.

Rakha, B.A., Ansari, M.S., Akhter, S., Santiago-Moreno, J., Blesbois, E., 2018. Cryoprotectant effects of egg yolk on Indian red jungle fowl (*Gallus gallus murghi*) sperm. Theriogenology 119, 150-155.

Rakha, B.A., Hussain, I., Akhter, S., Ullah, N., Andrabi, S.M.H., Ansari, M.S., 2013. Evaluation of Tris-citric acid, skim milk and sodium citrate extenders for liquid storage of Punjab Urial (*Ovis vignei punjabiensis*) spermatozoa. Reproduction Biology 13, 238-242.

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