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Effects of *Mangifera indica* and *Punica granatum* extracts on semen after cryopreservation

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Abstract
Semen cryopreservation is used to sustain fertility in conditions like infertility and malignancy. It damages sperm organelles (mitochondria, DNA) due to augmented reactive oxygen species (ROS). In the current research, mango and pomegranate fruit extracts were evaluated for their effect on semen quality of Sahiwal bulls after cryopreservation and post thawing. The main object of the present study was to appraise the effects of herbal antioxidants on semen cryopreservation by assessment of sperm cell viability, motility as well as membrane stability by standard techniques. The addition of all treatments (MFE and PFE) to the semen extender increased the sperm viability, motility and membrane integrity at all evaluation times.

Keywords: Sahiwal bull, spermatozoa, cryopreservation, TEY, polyphenols, herbals

1. Introduction
Semen cryopreservation is used to preserve fertility in conditions like infertility and malignancy [1]. However, semen storage and cryopreservation are associated with cold shock that lead to higher production of reactive oxygen species (ROS) and an imbalance between free radicals and antioxidant system of extended semen [2, 3]. Cryopreservation involves various steps like temperature reduction, cellular dehydration, freezing, and thawing. Sperm are not adapted to endure low temperatures; so, cryopreservation of these cells happens at the expenses of cellular viability and normal function. Mammalian sperm are very sensitive to cooling from body temperature to near the freezing point of water. Damage to sperm, known as cold shock, is observed as an irreversible loss of motility upon rewarming [4].
ROS triggers the process of lipid peroxidation that results in irreversible arrest of sperm motility and sperm damage. Post-thaw sperm quality is adversely affected by ROS which include superoxide anion, hydrogen peroxide, nitric oxide and hydroxyl radicals [5]. During semen cryopreservation process, a large amount of the cytoplasmic component such as antioxidants will leak out to extracellular irreversibly. These events decrease enzymatic defensive system capacity of semen [6]. Semen cryopreservation damages sperm organelles, (mitochondria, DNA) due to increased reactive oxygen species (ROS). This diminishes motility, membrane integrity, amplifies metabolic alterations resulting in reduced sperm fertility [7].
Natural antioxidants protect from oxidative stress and associated diseases therefore play an important role in health care [8]. Plant foods are the primary source of natural antioxidants. Fruits and vegetables are important dietary sources of antioxidant polyphenols to humans [9]. Positive effects of antioxidants on oxidative stress are known. Present study was to appraise whether addition of selected fruit extract can raise spermatozoa resistance against oxidative stress.
Fruit is one of the major dietary sources of various antioxidant phytocompounds for humans. Mango (*Mangifera indica* L.) is one of the most important commercial crops worldwide in terms of production, marketing and consumption. Mango (*Mangifera indica* L.), as well as citrus fruits, can be considered a good source of dietary antioxidants, such as ascorbic acid, carotenoids and phenolic compounds. Mangoes can be considered a good source of dietary antioxidants, such as ascorbic acid, carotenoids and phenolic compounds. Polyphenols are natural substances in plants that are antioxidants with the potential to protect the body from disease. Early studies showed that the main phenolics found in mango are leucocyanidin, catechin, epicatechin, chlorogenic acid, quercitrin and quercetin. β-carotene is the most abundant carotenoidin several cultivars.

The nutritional value of mango as a source of vitamin C and provitamin A should also be emphasized [10, 11].

Pomegranate (*Punica granatum*), belonging to family Punicaceae [12], is native to the Iran plateau and the Himalayas in north Pakistan and Northern India. The edible portion (aril) of fruit is about 55–60% of the total fruit weight and consists of about 75–85% juice and 15–25% seeds [13]. Pomegranates contain fibre, pectin, sugar and several tannins, as well as flavonoids and anthocyanidins in their seed oil and juice [14]. Kim *et al*. [15] demonstrated that pomegranates possess chemopreventative and adjuvant therapeutic effects against human breast cancer cells. Due to such biological activities, the consumption of pomegranate-containing foods is increasing.

In the present study aimed at evaluating the antioxidant effect of mango fruit extract (MFE) and pomegranate fruit extract (PFE) on semen quality after cryopreservation semen motility, viability and plasma membrane integrity, the hypo-osmotic swelling test at 0, 1 and 2 hours after freeze thawing.

2. Materials and Methods

2.1 Collection and Authentication of Plant Material

The fresh fruits of *Mangifera indica* (Family Anacardiaceae) and *Punica granatum* (Family Punicaceae) were purchased from local market (Amroha district), Uttar Pradesh, in April. 2017. Moreover, fruits were authenticated at Forest Research Institute, Dehradun (Voucher Number: Dis \657 \2017 \Syst. Bot.\Rev. Gen. \10-15, 16).

2.1.1 Preparation of Mango Extract

Fresh mango fruits were cleaned, edible portions were cut and homogenized with polytron homogenizer and extracted in 200 mL of ethanol: acetone (7:3, v/v) for 1 h at 37 ◦C. After 18 hours, the mixture was centrifuged at 6200 rpm for 20 minutes. The supernatant was filtered and pooled together. Extraction of the residue was repeated using the same conditions. Mango fruit supernatant was subjected solvent removal by rotary evaporator (< 45 °C) under vacuum to yield dark yellow colored viscous residue (Mango Fruit Extract) MFE which was kept at -20 °C until further use.

2.1.2 Preparation of Pomegranate Fruit Extract

Each pomegranate was washed in cold water and drained. Approximately two whole of pomegranate fruits were cut into two halves, to obtain aril juice and whole pomegranate juice from the same fruits. The arils were separated manually, and the inner white parts of the fruit were removed. All the edible portions of pomegranate were extracted with 50% of ethanol and homogenized with polytron homogenizer. The homogenized suspensions, obtained, were pooled and left at room temperature for 2 h with occasional vortexing. Then, it was centrifuged at 10,000g for 15 min at 10°C and the reddish brown supernatant (Pomegranate Fruit Extract) PFE stored at -20 °C until further use. Extraction and analysis of antioxidant activity and total phenolic content was done separately both the fruits and each sample was estimated in triplicate.

2.2 *In-Vitro* Antioxidant Activity conducted on Fruit Extracts

The total phenolic content of extracts was determined as per the method described by [16]. The antioxidant activity was evaluated using ABTS /TEAC {[2,2-Azinobis (3-ethylbenzothiazolin) 6-sulfonic acid]/ [Trolox equivalent antioxidant assay]} was done as per Re *et al*. [17], DPPH (2, 2-diphenyl-picryl-hydrazil) test as per Silva [18] and FRAP (Ferric reducing antioxidant power)according to Benzie, and Strain [19]. All the studies were carried out in triplicate.

2.3 Preparation of Semen Extender

Buffer for semen extender was prepared by mixing 1.34g citric acid (Merck, Germany), 2.42g N-Tris- (hydroxymethyl-aminomethane (MP Biomedical, France) in 73ml of distilled water. The pH of buffer was adjusted to 7.0 and supplemented with 1g Fructose (BDH, England), 20 ml Egg yolk, 7 ml Glycerol (BDH, England) and antibiotics (Penicillin, Streptomycin 50000 IU) [20].

2.3.1 Semen Collection and Evaluation

Present study was conducted on three mature normal Sahiwal bulls of the age group between 3 to 4 years and average weight was 450-500 kg, reared at the Chawdhary Farms, Mathura (UP). Bulls were maintained in standard conditions of feeding and housing for the entire study period (March, 2017 to May, 2017). Semen was collected with the help of artificial vagina (42˚C) at weekly interval for three weeks (3 replicates). Frequency of semen collection was two ejaculates weekly. The semen samples were Semen samples were evaluated; when achieving normal ranges (volume: 5-12 mL; sperm concentration :> 1 × 109 sperm mL^{-1}; motility: > 70%; total morphological abnormalities: ≤ 10%), bull semen was frozen. The ejaculates were then pooled, equalizing the sperm contribution of each male to eliminate individual differences [14].

2.3.2 Semen processing

Each part of the semen was extended upto 80 million spermatozoa/ ml. The semen was given 10 minutes holding time at 37°C in water bath before dilution. During experiment 3 aliquots of semen for mango fruit extract (MFE) and (Pomegranate Fruit Extract) PFE (0.0%, 1.00%, 1.50%) extracts were diluted in single step at 37°C.The extended semen was further incubated for 5 minutes to permit uptake of MFE and PFE by spermatozoa. Semen samples were cooled from 37°C to 4°C in 2 hours and equilibrated at this temperature for 4 hours.

Extended semen was filled into 0.5ml straws in a biological cell freezer (IMV Technology, France) as per the standard protocol (fall of temperature from 4 °C to 10 °C @5 °C/min, -10°C to -100 °C@40 °C/min, -100 to -140 °C @20 °C / min) as developed in the S B Laboratory, Duvasu, Mathura, India. Semen straws were then frozen at -196°C by dipping in liquid nitrogen

2.3.3 Post-thaw sperm functional assays

One straw of semen from each treatment was thawed at 37°C for 30seconds and the semen quality parameters like Sperm Counting, Spermatozoa motility, Spermatozoa viability and Plasma membrane integrity were evaluated.

2.3.4 Sperm Counting

The diluted sperm suspension (10 µL) was loaded into each chamber of the Neubauer hemocytometer (LABART, Darmstadt, Germany) and the total sperm numbers were counted per 250 small squares as per Asadi *et al*. [21]

2.3.5 Sperm Motility

The percentage of sperm motility was determined randomly from 4 different fields for each sample and the numbers of

motile and non-motile sperm were 200 spermatozoa per slide [22].

2.3.6 Sperm Viability

The viability of sperm was evaluated by eosin Y staining. Accordingly, the sperm samples were transferred to the Neubauer slide, mixed with 10 µL of eosin, and assessed under a light microscopy. After that, according to the staining or non-staining, spermatozoa were classified into dead spermatozoa with pink or red coloration and live without any coloration. Randomly, at least 200 sperms were counted for each sample [23]. Finally, the obtained result of viability was presented as a percentage of live sperms.

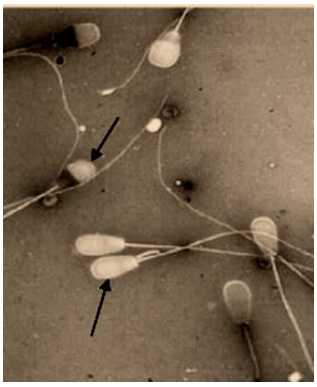

Fig 1: Photograph showing live and dead spermatozoa

2.3.7 Assessment of Functional Integrity for Membrane

The hypoosmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane after thawing, based on curled and swollen tails. This was performed by incubating 30 mL of semen with 300 mL of a 100 mOsM hypoosmotic solution (9 g fructose + 4.9 g sodium citrate per liter of distilled water) at 37 °C for 60 min. The osmotic pressure of solution was controlled and calibrated via an Osmometer (Labx, Mark III, USA). Following incubation, 0.2 mL of the mixture was spread with a cover slip on a slide. Four hundred sperm were examined with 400X magnification with bright-field microscopy. Sperm with swollen or coiled tails were recorded [24].

2.4 Statistical Analysis

Analysis of variance was carried out and significance was tested at 5% level. The data obtained were presented as Mean ± SE. Mean values were compared by using 2-way ANOVA with Post Hoc Tukey Test. Tukey *post hoc* test was applied for comparisons of means by using SPSS 16.1 version, USA, $p < 0.05$ was considered significant.

3. Results

Earlier studies from literature indicate that consumption of fruits and vegetables prevent degenerative diseases caused by oxidative stress. Since DPPH, ABTS and FRAP radical scavenge assays are widely used due to their simplicity, stability, accuracy and reproducibility [17]. Extracts of fruits of *M. indica* and *P. granatum* confirm excellent antioxidant activity in all tests performed. The antioxidant capacities of foods are summarized in Table 1. The highest antioxidant capacities were detected for MFE by DPPH assay (47.1 ± 2.89 mg TE/100 g) and for PFE by ABTS assay (30.9 ± 1.25mg TE /100 g)

Table 1: Evaluation of fruit extracts for the *in vitro* free radical assay(s) and total phenolic content

Extract	Total polyphenols	ABTS /TEAC [b]	DPPH [b]	FRAP [b]
MFE	65.45 ± 1.91	38.0 ± 1.90	47.1 ± 2.89	19.1 ± 0.15
PFE	70.41± 2.14	30.9 ± 1.25	15.4 ± 1.93	11.1 ± 0.23

a Total polyphenols (mg gallic acid/g FW)
b Trolox (mg Trolox /g FW)

All semen samples were examined by light microscopy and classified as normal according to WHO guidelines. The aliquots of all experimental groups were incubated for 30 min with 0, 1.0, and 1.50 % w/v of the MFE and PFE. Data regarding the effect of natural antioxidants on the sperm motility, viability and plasma membrane integrity is given in Table 2 and Table 3 respectively. Figure 1 point out unstained live sperm cell also partially or completely stained dead sperm cells. The addition of MFE and PFE improved the post thawed semen parameters only to limited extent. The plasma membrane integrity did not remarkably improve by its addition. All the parameters deteriorated aggressively by its addition at 1.5%. This needs further studies to understand the impact at lower concentrations.

Table 2: Effect of fruit extracts MFE on post thawed Sahiwal bull semen quality cryopreserved in TEY

Extract	Motility (Incubation Time)			Viability	HOS Positive
	0 min	60 min	120		
0%	45.66±1.85 [a]	29.66±1.85[a]	24.66±1.75[a]	37.89±2.53[a]	34.31±2.09[a]
1.00%	90.25 ± 3.4 [a] 5	81.4± 1.14 [a]	73 ± 0.83 [a]	60.15±3.35[b]	43.33±2.40[b]
1.50%	78.71 ± 1.01 [a]	63.5 ± 2.99[a]	57.1 ± 2.9 [a]	47.60±3.62[b]	45.13±3.27[b]

Data shown are mean ± SEM (n=15), a,b,c denote differences (P<0.05) in the columns

Table 3: Effect of fruit extracts PFE on post thawed Sahiwal bull semen quality cryopreserved in TEY

Extract	Motility (Incubation Time)			Viability	HOS Positive
	0 min	60 min	120		
0%	45.66±1.85 [a]	29.66±1.85[a]	24.66±1.75[a]	37.89±2.53[a]	34.31±2.09[a]
1.00%	67.41 ± 2.21 [a]	50.6 ± 1.67 [a]	45.3 ± 1.39[b]	46.40±2.10[b]	40.66±2.16[a]
1.50%	76.41 ± 1.46[a]	65.8 ± 1.38 [a]	61.4 ± 1.13 [c]	29.60±2.71[c]	24.73±2.96[b]

4. Conclusions

Supplementation of TEY with fruit extracts (MFE and PFE) particularly 1.00% and 1.50% concentration in the freezing medium can contain the undesirable effects on the motility, viability and plasma membrane integrity in bull spermatozoa during freeze–thaw process. Present study it could be concluded that MFE/PFE significantly decreased LPO. Further studies required to investigate and purify the active ingredients present in these natural compounds.

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