RESEARCH ARTICLE

Effect of Amla (*Phyllanthus emblica*) Extract on Fresh and Cryopreserved Seminal Attributes of Jamnapari Buck

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Abstract

The aim of the present study was to determine the effect of an ethanolic extract of *Phyllanthus emblica* (Amla) fruit on the quality of goat semen after the freezing-thawing process. Twelve ejaculates collected from two Jamnapari bucks were evaluated. Semen samples were split-diluted with a Tris-based extender containing the additive ethanolic extract of *Phyllanthus emblica* fruit (0.6 and 0.9%) and an extender containing no additive (control); were filled in 0.25 mL French straws, and equilibrated for 4 h at 4° C and frozen in liquid nitrogen vapour. Frozen straws were thawed individually at 37° C for 30 s in a water bath. The samples were evaluated for sperm quality parameters at pre-freeze (on dilution) and post-thaw stages. The freezing extender supplemented with 0.6 and 0.9% ethanolic extract of *Phyllanthus emblica* fruit led to significantly higher percentages of post-thawed sperm motility, livability, and plasma membrane integrity with reduced percentage of sperm abnormalities and seminal plasma enzyme activities in comparison to the controls ($p < 0.001$). The study showed that adding 0.6% and 0.9% ethanolic extract of *Phyllanthus emblica* fruit to the extender (Tris-citric-acid-fructose-egg-yolk-glycerol) improved the quality of post-thawed Jamnapari buck semen. The highest preservation efficiency was observed in 0.6% ethanolic extract compared to other treatments.

Key Words: Buck semen, Cryopreservation, Enzyme leakage, *Phyllanthus emblica* (Amla) as extender additive, Post-thaw sperm quality

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Introduction

Cryopreservation can lead to the release of reactive oxygen species (ROS) and alterations in membrane potential due to changes in mitochondrial membrane fluidity (Said *et al.,* 2010). Hydrogen peroxide (H_2O_2), nitric oxide (NO), and superoxide anion (O_2–) exert beneficial effects on intracellular signaling, sperm capacitation, and acrosome reactions (Jin and Yang, 2017). High concentrations of these molecules are toxic and have a negative impact on sperm function, despite their significant role in sperm physiology, specifically in capacitation and acrosome reactions (Medeiros *et al.,* 2002). Sperm contains a high amount of polyunsaturated fatty acids (PUFA), which makes it susceptible to damage from ROS or lipid peroxides in the membrane (Silva, 2006). It is well recognized that the cryopreservation process decreases the viability and motility of spermatozoa in farm animals (Khan *et al.,* 2021). Antioxidants are compounds that prevent the generation of ROS and the oxidation of lipid peroxidation. Superoxide dismutase, glutathione peroxidase, and catalase are well-documented antioxidants that play a significant role in protecting sperm cells from oxidative stress (Sanocka and Kurpisz, 2004). Plant-derived extracts are sources of natural antioxidants with lower cytotoxicity as compared to artificial antioxidants.

Phyllanthus emblica, popularly known as Amla, is found in a wide range of tropical and subtropical countries, such as India, China, Indonesia, Burma, and the Malay Peninsula. *P. emblica* fruit is known to possess various bioactive constituents such as vitamin C, minerals, amino acids, tannins, phyllembelic acid, phyllemblin, rutin, curcuminoids, emblicol, and several phenolic compounds (Bhattacharya *et al.,* 2000). Previous studies have proven that *P. emblica* exhibits antimicrobial properties, acts as an antioxidant (Chatterjee *et al.,* 2011), and possesses anti-inflammatory effects (Golechha *et al.,* 2011). In addition, *P. emblica* contains ellagic acid, which is a potent antioxidant that can inhibit gene mutations and repair chromosomal abnormalities (Priya *et al.,* 2012). Moreover, Arun *et al.* (2018) reported that the administration of *P. emblica* leaf extract at a dosage of 50 mg/kg b.wt. effectively mitigates testicular damage in rats subjected to chronic stress. This protective effect is attributed to the

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prevention of low sperm quality and alterations in functional proteins, particularly tyrosine-phosphorylated proteins in the testes. To our knowledge, so far, no study has been conducted on the use of an ethanolic extract of *Phyllanthus emblica* as an antioxidant in goat sperm freezing extender. Therefore, the aim of the present study was to determine the effect of an ethanolic extract of *Phyllanthus emblica* fruit on the quality of goat semen after the freezing-thawing process.

Materials and Methods

The mature, healthy and fresh *Phyllanthus emblica* fruits were harvested from Experimental Farm of College of Horticulture, ANDUAT, Kumarganj, Ayodhya, UP (India). Fifty grams of dried *Phyllanthus emblica* fruits were grounded and extracted using a modified technique proposed by Khalaf *et al.* (2008).

Semen Collection

Semen samples from two mature Jamnapari bucks (2 to 3 years of age) were used in this study. The bucks, belonging to the Deep Frozen Semen Laboratory of the College, were maintained under uniform optimal nutritional conditions. Twelve ejaculates were collected twice a week from the bucks, with the aid of an artificial vagina. Immediately after collection, the ejaculates were incubated in a water bath at 34°C, until microscopic sperm quality assessments were performed.

Semen Extension, Freezing and Thawing

The volume of the ejaculates was measured in a conical tube, and the sperm concentration was determined using an Accucell photometer (IMV, France). Sperm motility was determined using phase-contrast microscopy (400× magnification) at 37°C. Only ejaculates with >75% progressive motility and sperm concentration of higher than 2.2×10^9 sperm/mL were selected. Selected ejaculates were diluted with Tris-citric-acid-fructose-egg-yolk-glycerol (TFYG) extender. Diluted semen was divided into three equal parts: the first part was taken as control (C), and the other two parts were supplemented with an ethanolic extract of *Phyllanthus emblica* extract at 0.6% and 0.9% (T2 and T3, respectively). The diluted semen samples were filled into 0.25 mL French straws, sealed by ISEVO (IMV, France), and equilibrated for 4 h at 4°C in cold cabinet (Minitube, Germany). After equilibration, the straws were frozen in liquid nitrogen vapour (4 cm above the liquid nitrogen) for 20 min and then plunged into liquid nitrogen for overnight storage. Next day, the frozen straws were thawed in a water bath individually at 37°C for 30 s for post-thaw evaluation.

The pre-freeze (on dilution) and post-thawed samples were assessed for percentage of progressive sperm motility, livability, abnormalities and plasma membrane integrity using standard procedures. The seminal plasma was separated from freshly diluted and frozen-thawed samples using centrifugation at 402 x g for 20 min. The levels of AST, ALT, ACP, and AKP in seminal plasma were determined using commercially available kits on the BEACONB Auto^{200} analyzer. All data were presented as mean ± standard error of the mean (SE) and analyzed using one-way analysis of variance (ANOVA) with Graph Pad Prism 5 software.

Results and Discussion

The impact of ethanolic extract of *Phyllanthus emblica* on the seminal attributes of buck semen on dilution and at post-thaw stage is depicted in Table 1. Semen samples processed with 0.6 % ethanolic extract of *Phyllanthus emblica* in TFYG extender had significantly ($p<0.05$) higher percent post-diluted and post-thawed sperm motility, viability and morphological normal sperm followed by 0.9 % ethanolic extract as compared to the control extender ($p < 0.05$). The mean post-thaw motility recorded was in agreement with the observation of Bucak *et al.* (2010), but higher than that reported by Aguiar *et al.* (2013) and lower than the finding of Archana *et al.* (2017). Our findings on sperm viability were in tune with Goswami *et al.* (2021), and lower than the findings of El-Battawy and El-Nattat (2018), but higher than those reported by Archana *et al.* (2017). The present findings on sperm quality parameters pre- and post-freezing of buck semen also concurred well with the observations of Alam *et al*. (2023) with inclusion of 1.0 mM Cysteine HCl in the cryo-extender for buffalo bull semen.

Table 1: Effect of ethanolic extract of *Phyllanthus emblica* (Amla, 0.6 % & 0.9 %) supplementation in Tris extender on sperm quality parameters and enzyme leakage during cryopreservation of Jamnapari buck semen

Stages	Additive concentration (%)	Progressive motility (%)	Sperm livability (%)	Sperm abnormality (%)	HOS reactive sperm (%)	AST (µmole/L)	ALT (µmole/L)	ACP (KAU/100 mL)	AKP (KAU/100 mL)
Post-dilution	Control (C)	78.67 ± 0.8^{a}	86.33 ± 0.6^{a}	6.83 ± 0.60^{b}	49.00 ± 1.0^{a}	60.33 ± 2.7^{c}	119.3 ± 3.20^{c}	27.17 ± 1.8^{c}	43.17 ± 2.6^{c}
	0.6 % (T_1)	81.17 ± 1.01^{b}	88.50 ± 0.76^{c}	5.50 ± 0.72^{a}	55.67 ± 1.80^{c}	57.33 ± 2.64^{a}	112.30 ± 2.67^{a}	24.50 ± 1.89^{a}	40.83 ± 2.44^{a}
	0.9 % (T_2)	79.67 ± 1.02^{a}	87.67 ± 0.67^{bc}	6.33 ± 0.71^{ab}	$52..00\pm2.22^{b}$	59.33 ± 2.87^{bc}	116.2 ± 2.87^{b}	26.17 ± 1.54^{bc}	$42..50\pm2.45^{b}$
Post- thaw	Control (C)	48.33 ± 0.67^{A}	75.00 ± 0.97^{A}	13.17 ± 1.4^{C}	39.33 ± 0.42^{A}	137.2 ± 4.73^{C}	263.5 ± 4.49^{C}	59.17 ± 1.35^{C}	151.80 ± 5.10^{C}
	0.6 % (T_1)	55.83 ± 0.87^{C}	79.67 ± 1.09^{C}	11.17 ± 0.8^{A}	44.67 ± 0.88^{C}	127.80 ± 3.29^{A}	249.20 ± 3.65^{A}	54.00 ± 0.89^{A}	147.30 ± 5.10^{A}
	0.9 % (T_2)	50.67 ± 0.67^{B}	76.33 ± 1.12^{B}	12.33 ± 1.3^{BC}	41.33 ± 0.42^{B}	133.00 ± 4.2^{B}	258.50 ± 4.22^{B}	57.33 ± 0.84^{B}	150.80 ± 4.99^{B}

AST-ALT=Asparate and Alanine amino-transferases, ACP-AKP= Alkaline and Acid Phosphatases, Mean ± SE values bearing different superscript within the stage (a, b, c / A, B, C) in a column differ significantly ($p<0.05$).

Ice crystallization and lipid peroxidation during cryopreservation result in oxidative stress, leading to the generation of reactive oxygen species (ROS). Excessive production of ROS can lead to increased sensitivity in sperm, causing detrimental effects on their morphology, ultra-structures, and functions. This is often accompanied by reduced sperm motility, viability, and fertilizing capacity (Bilodeau *et al.,* 2001). The antioxidant capacity of sperm cells may not be enough to prevent lipid peroxidation (LPO) during the freeze-thaw process (Qamar *et al.,* 2023). *Phyllanthus emblica* is a plant that contains high levels of vitamin C and low molecular weight hydrolyzable tannins (Priya *et al.,* 2012). It also has significant amounts of phenolic compounds and exhibits antioxidant properties (Iamsaard *et al.,* 2014). *Phyllanthus emblica* extract has been found to possess lipid peroxidation inhibition and anticancer properties (Krishnami and Mirunalini, 2012). Generally, dietary supplementation with *Phyllanthus emblica* leaf extract has been used to increase sperm quality in rats (Arun *et al.,* 2018). Moreover, Rajak *et al.* (2004) found that amla at doses of 250, 500, and 750 mg/kg for 30 days against ischemic reperfusion (IR) injury in rats prevented oxidative stress and myocyte injury by inhibiting lipid peroxidation and increasing levels of myocardial antioxidants like catalase (CAT) and glutathione peroxidase (GPx), reduced glutathione (GSH), and superoxide dismutase (SOD).

The highest mean percentages of HOS-positive sperm were observed both on dilution and at post-thaw stage in buck semen extended with Tris extender containing 0.6% ethanolic extract of *Phyllanthus emblica*, followed by 0.9% ethanolic extract and the control group (Table 1). These findings concurred well with the observations of Shokry *et al.* (2020), but were lower than those reported by Goswami *et al.* (2021) and higher than those observed by Kumar (2022). Plasma membrane is essential for the survival of sperm cells (Makarevich *et al.,* 2010). It is used to determine the fertilizing capacity of spermatozoa in animals (Brito *et al.,* 2003). Additionally, it is involved in various essential processes such as sperm metabolism, capacitation, the acrosome reaction, and sperm-oocyte fusion (Forouzanfar *et al.,* 2013).

In the present study the extracellular ALT, AST, ACP and AKP activities in frozen thawed seminal plasma were found to be significantly ($p<0.05$) lower in extender containing 0.6 % ethanolic extract of *Phyllanthus emblica* followed by 0.9 % ethanolic extract and the control extender ($p < 0.01$). The variations in leakage of ALT and AST could be attributed to individual variations and age of bucks (Tiwari, 2000), rate of dilution as well as composition of diluents, glycerol levels, equilibration periods, cooling rates and freezing rates (El-Khawagah *et al.,* 2020). *Phyllanthus emblica* extracts containing phenolic compounds exhibit antioxidant properties in the carotene bleaching method. These extracts inhibit auto-oxidation by scavenging free radicals, quenching singlet oxygen, and donating hydrogen (Mayachiew and Devahastin, 2008). The present findings on enzyme activity of buck semen pre- and post-freezing concurred well with the observations of Alam *et al.* (2023) with inclusion of 1.0 mM Cysteine HCl as antioxidant in the cryo-extender for buffalo bull semen. Purohit *et al.* (2010) found that amla juice (0.01 mL/day) reduced brain cell damage in radiation- and cadmium-induced rats by decreasing lipid and protein peroxidation, polyphenol-mediated free radical scavenging, increasing the levels of GSH, and reducing the activities of acid and alkaline phosphatases. In the absence of reports of *P. emblica's* use as a semen additive, our observations could not be compared and discussed properly, but its beneficial effect as a semen additive in post-thawed semen is noticeable, and further studies should be done with other possible concentrations and complete semen analysis.

Conclusions

The findings of this study indicated that adding 0.6% and 0.9% ethanolic extract of *Phyllanthus emblica* (Amla) fruit to the extender (Tris-citric-acid-fructose-egg-yolk-glycerol) improved the quality of post-thawed Jamnapari buck semen, the highest preservation efficiency was observed with 0.6% ethanolic extract compared to higher level of ethanolic extract (0.9%) and control extender.

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