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Nikhil Pal Bajia
Department of Veterinary Gynaecology and Obstetrics, PGIVER, Jaipur, RAJUVAS, Bikaner, Rajasthan, India

Sumit Prakash Yadav
Assistant Professor, Department of Veterinary Gynaecology and Obstetrics, PGIVER, Jaipur, RAJUVAS, Bikaner, Rajasthan, India

Anand Kumar
Department of Veterinary Gynaecology and Obstetrics, College of Veterinary and Animal Science, RAJUVAS, Bikaner, Rajasthan, India

Krishna Kumar Sharma
Department of Veterinary Gynaecology and Obstetrics, PGIVER, Jaipur, RAJUVAS, Bikaner, Rajasthan, India

Shobha Burdak
Assistant professor, Department of Veterinary Pathology, R.R. CVAS, Deoli, Tonk, Rajasthan, India

Pradeep Makawana
Veterinary Officer, Lumbia, Rajasthan, India

Rahul Kumar
Veterinary Officer, Deoria, Pali, Rajasthan, India

Corresponding Author:
Nikhil Pal Bajia
Department of Veterinary Gynaecology and Obstetrics, PGIVER, Jaipur, RAJUVAS, Bikaner, Rajasthan, India

Effect of Giloy (*Tinospora cordifolia*) ethanolic extract supplementation to ram semen extender on sperm abnormality in chilled semen

Nikhil Pal Bajia, Sumit Prakash Yadav, Anand Kumar, Krishna Kumar Sharma, Shobha Burdak, Pradeep Makawana and Rahul Kumar

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Abstract
Ethanolic extract of Giloy (*Tinospora cordifolia*) was supplemented at concentrations of 100 µg/ml, 300 µg/ml, 500 µg/ml, 700 µg/ml to ovine semen extender in order to study the effect of antioxidant potential on sperm abnormality parameter of Magra ram semen during cooled storage at 4 °C. The basic extender was Tris egg yolk citrate fructose extender (TEYCF) which is supplemented with different concentrations of Giloy extract. Results revealed that, the mean percentage of total sperm abnormality was significantly (p<0.05) lower in 300 µg/ml and 500 µg/ml Giloy extract supplemented group as compare to control after 72 hrs of preservation. After 96 hrs of preservation, the mean percentage of total sperm abnormality was significantly (p<0.05) lower in TC300 and TC100 groups as compared to control. In conclusion, this study provides a new data about positive effect on sperm abnormality parameter of Magra ram semen during cooled storage at 4 °C, after supplementation of the ethanolic extract of Giloy to the extender.

Keywords: *Tinospora cordifolia*, ram semen, sperm abnormality, antioxidant

1. Introduction
Ovine population in India is about 74.26 million and has risen by 14% over the previous census (20th Livestock Census, 2019) [23]. In rural areas, unorganized sheep sector play a significant role to meet demand of cash by selling of live animals, wool and manure. The reproductive management in intensive ovine breeding includes artificial insemination of a large number of sheep, which is associated with chilling or freezing of semen. The chilled semen was shown as alternative of the frozen semen by different authors (Abulizi *et al*., 2012; Fernandez-Abella *et al*., 2003; Salomon and Maxwell, 2000) [2, 12, 39]. Recently, different conception (Allai *et al*., 2018; Mehdipour *et al*., 2016) [3, 28] for a protective effect of plant derived antioxidants on the sperm cells has reported. Ovine spermatozoa are very sensitive to oxidative damage due to higher ratio of polyunsaturated fatty acids and lower molar ratio of cholesterol. Chilled ram semen are required to be used within 10-14 hrs for getting appreciable pregnancy rate. Improvement in ovine semen quality during cold storage still may appear to be a challenge. Preservation of ovine spermatozoa under chilling conditions further produce huge amount of reactive oxygen species (ROS) that negatively impacts sperm function that lead to loss of fertility (Bucak and Tekin, 2007) [5]. Poor quality semen represent over 80 percent failure of fertilization and embryogenesis, miscarriage and infertility in animals (Gadea and Matas, 2000; Rabbani *et al*., 2010; Enciso *et al*., 2011) [14, 35, 10] and this can be improved by incorporation of antioxidants in diluents (Perumal *et al*., 2011) [33]. A lot of studies have exhibited that exogenous antioxidant treatment enhances seminal quality in buck (Zaenuri *et al*., 2014) [55], bulls (Sariozkan *et al*., 2014) [41] and rams (Abadjieva *et al*., 2020) [1] during liquid storage. Thus, supplementation of a favorable antioxidant in semen extender could prove valuable in decreasing the oxidative stress and thereby conserving spermatozoa for longer time with improve semen quality. Recently, natural plants and extracts derived from

them have been used in semen extenders as antioxidants which are effective and have been confirmed to be used on a large scale. Flavonoids, hydrolysable tannins, phenolic compounds, and terpenes are the most valuable constituents responsible for antioxidative properties of plants (Gupta and Sharma, 2006; Ogunlesi *et al*., 2009; Carlsen *et al*., 2010) [17, 32, 6]. *Tinospora cordifolia* (Desai *et al*., 2002) [7], *Thymus vulgaris* (Miura *et al*., 2002) [30], *Moringa oleifera* (Sreelatha and Padma, 2009), *Opuntia ficus-indica* (Meamar *et al*., 2012) [27], Clove bud (*Syzygium aromaticum*; Guan *et al*., 2007) [16] these are the some known examples of natural plants that show antioxidant properties.

Giloy (*Tinospora cordifolia*) is a large glabrous deciduous climbing shrub that commonly grows in the tropical and subtropical regions of India. *Tinospora cordifolia* is commonly referred to as the Rasayan-plant because it contains more than 100 constituents in their structure, responsible for its common tonic, antioxidant, antibacterial, immunomodulator and hepatoprotective properties. This herb is well known for their therapeutic value in Veterinary folk, Ayurveda and other systems of medicine (Krishna *et al*., 2009) [21]. According to Desai *et al.* (2002) [7] *Tinospora cordifolia* (Giloy) has a large range of antioxidant properties which minimizes reactive oxygen species and reactive nitrogen species. Antioxidant activity of *Tinospora cordifolia* is due to an arabinogalactan polysaccharide and phenolic compound (epicatechin; Subramanian *et al*., 2002; Pushp *et al*., 2013) [49, 34]. According to Upadhyay *et al.* (2014) [51] ethanolic extract of *Tinospora cordifolia* stem bark showed the highest antioxidant activity as compared to methanolic extract of *Tinospora cordifolia* stem bark. Kumar *et al.* (2018) [22] analyze the hydro-ethanolic extract of *Tinospora cordifolia* and stated that the major compounds present in the extract are tinocordioside, cordifolide A, palmatine, quercetin, β-sitosterol, heptacosanol, syringing and noticed excellent antioxidant activities for various antioxidant assays. *Tinospora cordifolia* reduces the effects of oxidative stress mediated cell injury and brings in to play antioxidant effects on gene expression as well as in the cytosol (Rawal *et al*., 2004) [37]. Ethanolic extract of this plant is capable of restoring a normal level of lipid peroxidation, enzymatic and nonenzymatic antioxidants in the case of N-nitrosodiethylamine induced liver cancer in rats (Jayaprakash *et al*., 2015) [20]. Dietary supplementation of *Tinospora cordifolia* improved antioxidant enzyme levels in ram semen. This plant has a protective effect on spermatozoa during cryopreservation due to increased levels of cholesterol, SOD and catalase in seminal plasma (Jayaganthan *et al*., 2013) [19]. Vitamin E and *Tinospora cordifolia* improve fertility in Bisphenol-A exposed goat testis and exhibits protective effect against Bisphenol-A induced detrimental effects in testis *in vitro* (Sharma and Gandhi, 2017) [44]. Various studies have been carried out by utilizing *Tinospora cordifolia* as dietary supplementation, but the effect of *Tinospora cordifolia* stem (ethanolic extract) on sperm abnormality during cooled storage at 4 °C is still limited. Keeping the aforesaid facts in view the major aim of the present study was be to evaluate effectiveness of different concentrations (100 µg/ml, 300 µg/ml, 500 µg/ml, 700 µg/ml) of *Tinospora cordifolia* stem (ethanolic extract) on sperm abnormality of Magra rams.

2. Materials and Methods

The present study was carried out at Indian Council of Agricultural Research-Central Sheep & Wool Research Institute, Arid Region Camps, Bikaner, Rajasthan (India), during February-March of year 2021. Bikaner is located at 73˚18'E longitude and 28˚1'N latitude at an altitude of 230 m above mean sea level. The area has an arid environment with yearly rainfall ranging from 200 to 300 mm with an inconsistent distribution throughout the year. The annual minimum and maximum ambient temperature ranges from 4 °C to 49 °C.

2.1 Chemicals

All the chemicals used in this study were procured from Sigma Aldrich (St. Louis, MO, USA), unless otherwise indicated.

2.2 Animals, semen collection and preliminary evaluation

Healthy breeding Magra rams (n = 8), with an average age of 1.5–3 years, body weight 38 ± 5 kg with good libido were used as semen donors using artificial vagina technique. Rams were permitted to graze in free land pasture for at least 7 hrs per day and provided dry roughage and concentrate in pellets form according to the requirement given by Indian Council of Agricultural Research. All the rams were housed under the same managerial conditions. Semen donor rams were used twice per week for three consecutive weeks (6 ejaculates/ram, total 48 ejaculates). Soon after collection, semen collection cups were labelled and transferred to water bath at 37 °C and screened for volume, colour, consistency, mass activity, sperm concentration, viability (live sperm percentage), sperm abnormality and pH. The semen samples were further subjected for evaluation after fulfilling the standard criteria (concentration >2.5 × 109 spermatozoa/ml; mass motility >3+; individual motility >70% and total abnormality <10% (Gil *et al*., 2003) [15].

2.3 Preparation of ethanolic extract of *Tinospora cordifolia* and extender

Stem of *Tinospora cordifolia* was purchased from herbal medicine provider of the local market and identification was confirmed by a botanical expert. The stem was washed and then dried in a moisture extraction oven at 40 °C for four days. The dried stem was then grounded to a coarse powder. The ethanolic extraction of dried powder of *Tinospora cordifolia* stem was done by Soxhlet apparatus (continuous hot extraction) method as described by Redfern *et al.* (2014) [38]. In current investigation, Tris-egg yolk citrate fructose extender (TEYC) (Tris 3.63 g, egg yolk 14% (v/v), citric acid 1.99 g, fructose 0.5 g, 100,000 i.u. penicillin, 100 mg streptomycin and distilled water up to 100 ml) was prepared and used for all the ejaculates (Salamon and Maxwell, 2000) [11]. Further, different concentrations of ethanolic extract of *Tinospora cordifolia* were supplemented to the extender.

2.4 Semen processing and cooling

For reducing individual variation, all fresh semen samples were pooled. The pooled semen w***as*** split into five equal aliquots and taken in 5 separate clean and sterilized small test tubes. These aliquots were extended (1:10) with pre-warmed (37 °C) extender. First aliquot was subjected to extend with TEYCF dilutor without any antioxidant and served as control. In second, third, fourth and fifth aliquots supplemented with ethanolic extract of *Tinospora cordifolia* at the rate of 100, 300, 500 and 700 µg per ml, respectively in extended semen. These semen aliquots were cooled from 37 °C to 4 °C at a rate of 0.2–0.3 °C/min and maintained at 4 °C during liquid storage up to 96 h. When the temperature of aliquots reached

at 4 °C, it was considered as 0 h. The total sperm abnormality was assessed following standard procedure.

2.5 Total sperm abnormality evaluation

Sperm abnormalities were determined by using the eosin–nigrosin staining technique (Swanson & Bearden, 1951) [50]. The stain was prepared using eosin (1 gm) and nigrosin (5 gms) in 100 ml of buffer solution (2.94% sodium citrate dihydrate solution in double glass distilled water). The solution was heated in water bath at 37 °C for 30 min and filtered after cooling through whatman's filter paper No. 40 and stored at room temperature for further use. In brief, for preparation of slide, small drop (30 µl) of semen was placed on clean, grease free slide and added equal amount of eosin–nigrosin dye mixed well with blunt end fine glass rod. After a min, a thin smear from the mixture was prepared on glass slide, air dried and total abnormal sperm percentage was assessed by counting 300 spermatozoa in different microscopic fields at 100X. The morphological deformity in head, body or tail was considered as abnormality in spermatozoa (Evans and Maxwell, 1987) [11].

2.6 Statistical Analysis

The data obtained in the study were analysed statistically by using one-way analysis of variance test (ANOVA) with the help of SPSS version-20 (Snedecor and Cochran, 2004) [56].

3. Results

The present study was undertaken to assess the effect of addition of ethanolic extract of Giloy (*Tinospora cordifolia*) in TCYF diluter for preservation of ram semen at refrigerator temperature. In the current experiment, the mean values of total sperm abnormality percentage in ram semen at different hours of preservation are presented in table 1. At 0, 24 and 48 hrs of preservation, the mean percentage of total sperm abnormality didn't differ significantly among the all groups. At 72 hours of preservation, the mean percentage of total sperm abnormality differed significantly ($p<0.05$) among all groups and found significantly lower in all the treatment groups. Statistical analysis revealed no significant difference between TC100 and TC700 as compared to control. At 96 hrs of preservation, the mean percentage of total sperm abnormality differed significantly ($p<0.05$) among all the groups. In treatment groups, the mean percentage of total sperm abnormality was significantly ($p<0.05$) lower in TC300 and TC100 groups as compared to control. The mean percentage of total sperm abnormality didn't differ significantly between TC100 and TC300 as well as among TC100, TC500 and TC700 groups.

Table 1: Mean (±S.E) total sperm abnormality (%) in different groups during different hrs of preservation at refrigeration temperature (4 °C)

Duration of Preservation	Groups				
	Control	TC100	TC300	TC500	TC700
0 Hr	1.34±0.42	1.34±0.49	1.17±0.48	0.84±0.31	1.17±0.31
24 Hr	2.99±0.32	2.52±0.39	1.67±0.49	2.67±0.56	2.17±0.40
48 Hr	3.92±0.35	3.52±0.49	2.55±0.40	3.34±0.56	3.84±0.40
72 Hr	$5.72±0.27^{b}$	$4.54±0.42^{ab}$	$3.54±0.30^{a}$	$4.07±0.36^{a}$	$4.67±0.56^{ab}$
96 Hr	$7.14±0.23^{c}$	$4.99±0.45^{ab}$	$4.17±0.21^{a}$	$5.67±0.49^{bc}$	$5.94±0.28^{bc}$

Means having different superscript in a row differ significantly ($p<0.05$).

4. Discussion

Quality of ram sperm deteriorates rapidly at room temperature, this makes transportation fresh semen difficult to remote areas (Shamsuddin *et al.*, 2000) [43]. The use of chilled semen considered is one of the solution to prevent decline in fertility of frozen semen and is more effective, cheap (Sri *et al.*, 2012) [48] and without the need for liquid nitrogen as compared to frozen semen (Gadea *et al.*, 2005) [13]. During liquid storage, oxidative damage of sperm resulted from reactive oxygen species (ROS) generated by the cellular components of semen. This is possibly one of the main cause for the decline in motility and fertility during storage, the other cause may be low temperature on the destabilization of sperm membrane structure (Bucak and Tekin 2007) [5]. Efforts to improve the preservation of cooled ram semen quality over the storage period have been focused recently on alteration of extenders (Marti *et al.*, 2003) [25] as well as the addition of specific components to maintain membrane integrity, prevent oxidative stress or preserve motility of spermatozoa in ram (Watson and Anderson, 1983; Maxwell and Stojanov, 1996; Upreti *et al*., 1998; Sanchez-partida *et al*., 1997) [53, 26, 52, 40]. A wide range of antioxidant and additives have been tested to minimize the damage caused by cooling and freezing-thawing in ram semen (Spalekova *et al*., 2011; Rather *et al.*, 2016) [46, 36]. Hydro-ethanolic extract of *Tinospora cordifolia* contained tinocordioside, cordifolide A, palmatine, quercetin, β-sitosterol, heptacosanol, syringing which have excellent antioxidant activities during various antioxidant assays (Kumar *et al.*, 2018) [22].

The mean percentage of total abnormality was significantly ($p<0.05$) lower in TC300 and TC500 groups as compared to control preserved for 72 hrs whereas it also recorded significantly ($p<0.05$) lower in TC100 and TC30 groups as compared to rest of groups preserved for 96 hrs at refrigerator temperature (4 °C). In the support of present findings Allai *et al.* (2016) [4] observed significant ($p<0.05$) decrease in sperm abnormality after addition of *Opuntia ficus indica* cladodesextract (ACTEX) in TEY extender preserved for 72 hrs in Boujaad ram semen during liquid storage at 5 °C. Similarly, Zaenuri *et al.* (2014) [55] showed significantly ($p<0.05$) higher percentages of normal sperm in Boer cross buck by addition of crude extract of Fig fruit (6%) in semen extender preserved for 6 days. El-Harairy *et al.* (2016) [8] also observed significant ($p<0.05$) decrease in percentages of abnormal sperm in Rahmani ram by adding aqueous extract *Arctium lappa* roots (@ 1000 µg/ml) to the semen extender preserved at 5 °C. The findings of present study are accordance with observation of Hammad *et al.* (2019) [18] who resulted that incorporation of aqueous *Moringa oleifera* leaves extract (@ 300 µg/ml) or methanolic *Moringa oleifera* leaves extract (@100, 200 and 300 µg/ml) significantly decreased sperm abnormality after cryopreservation of extended bull semen. Contrary to present findings, supplementation of Green tea (Mehdipour *et al.*, 2016) [28], Pomegranate (Mehdipour *et al.*, 2017) [29], Propolis (El-Harairy *et al.*, 2018) [9], Rosemary or Echinacea (Yavas and Yavas, 2018) [54] and Fennel (Najafi *et al.*, 2019) as antioxidant in ram semen didn't differ significantly for the

total sperm abnormality percentage. This improvement in viability could be explained by the lower effect of ROS on sperm membranes due to the excellent antioxidant activities of ethanolic extract of *Tinospora cordifolia* which is reported by Kumar *et al.* (2018) [22]. Allai *et al*. (2016) [4] also stated that the decrease in sperm abnormality in semen during liquid storage at 5 °C is possibly due to antioxidative effects of acetone extract from *Opuntia ficus-indica* cladodes. Agro climatic influences, differences in methodology used or breed difference might be responsible for the variation in these results (Saxena and Tripathi, 1986) [42].

5. Conclusion

From this study, it was concluded that addition of different concentrations of *Tinospora cordifolia* extract in semen extender improved the liquid semen storage quality at 4 °C and this improvement might be due to the antioxidant property of *Tinospora cordifolia*. The concentration of *Tinospora cordifolia* extract @300 µg/ml showed optimum results.

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