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Curcumin and L cysteine mitigates cryopreservation-induced cryodamage in Barbari buck semen

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ABSTRACT

The study aimed to evaluate effect of different concentration of cucumin and L cysteine HCL in mitigation of cryopreservation-induced cryodamage in Barbari buck semen. The present experiment was carried out on four Barbari bucks, 2-3 years of age. Twenty-four ejaculates (six from each buck) were collected and diluted with Tris-Egg-Yolk-Citric acid-Fructose-Glycerol extender containing no additives (CON; control), Curcumin 1 and 2µg/ml (CUR1 and CUR2), L cysteine HCL, 2 and 5mM/L (LC2 and LC5) at 37 ºC and cryopreserved following standard protocols, processed, frozen, and evaluated for various seminal markers. As compared to control, the motility (50.50±0.42, 53.50±0.50, 52.50±0.50 and 51.33±0.42%), liveability 77.67±0.98, 81.50±1.05, 81.00±0.96 and 77.67±0.88%) and HOS reactivity (39.83±1.10, 44.00±0.96, 43.00±0.51 and 41.17±0.98%) was significantly ($P<0.05$) improves, whereas abnormality (11.83±1.07, 7.66±0.55, 8.66±0.49 and 11.00±1.26%) and enzyme leakage i.e. GOT (156.5±6.42, 140.0±5.19, 147.8±4.67, 151.8±5.73µmole/L), GPT (243.7±7.67, 231.5±6.22, 234.2±6.21 and 241.8±6.94 µmole/L), ACP (49.50±2.07, 47.33±1.87, 49.33±1.76 and 51.83±1.85KAU/100 ml) and AKP (140.5±5.28, 134.8±4.76, 137.0±5.02 and 145.5±5.98 KAU/100 ml) significantly decline in CUR1, CUR2, LC2 and LC5 groups respectively.In conclusion,supplementation of 2µg/ml Curcumin and 2mM/L seems to be beneficial in mitigating cryopreservation-induced cryodamagein barbari buck semen.

Keywords: Barbari Buck, Cryodamage, Cryopreservation, Curcumin, Extender, L Cysteine, Semen

Cryopreservation makes genetic material for artificial insemination (AI) in goats more accessible. However, frozen-thawed semen has declined motility and viability due cryopreservation and thawing induced deleterious changes in sperm's ultrastrucure and its membrane (Watson, 2000) due to excessive production of free radicals. To mitigate cryopreservation induced cryodamage battery of substances has been tried and tested as a semen additives with varying success in domestic animals (Ashutosh *et al*. 2025; Kumar *et al*. 2026[a]; Kumar *et al*. 2026[b]). Furthermore, Turmeric contain a biologically active polyphenolic compoundcalled as curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], is an excellent free radical scavenger. Curcumin suppressed the production of free radicals (e.g. superoxide anions, H_2O_2 and nitrite radicals) by activated macrophages *in vitro*, both of which are critical in inflammation (Mathuria Verma 2008). Many researchers opined that curcumin supplementation mitigate cryopreservation induced cryo-damge in sperm cells of many species (Khalil *et al.* 2025). Furthermore, cysteine supplementation mitigates crypreservation-induced damage in semen of Bull (Perumal *et al*. 2011; Hayanti *et al*. 2022), buffalo bull (Arunpandian *et al.* 2023; Panwar *et al.* 2024) and Zaraibi bucks (Altyeb *et al*. 2022). Moreover, cysteine supplementation protects boar sperms from ROS induced damage during liquid (Zhu *et al*. 2022.) and chilled storage (Dos Santos *et al.* 2024). The antioxidant property of cysteine protects enhance quality of chilled stored semen of dromedary camel (Seyedasgari *et al*. 2024). In this study, the authors hypothesized that curcumin and L cysteine supplementation mitigates cryopreservation-induced cryodamage of barbari buck semen. To test this hypothesis, we investigated the effects of different concentration curcumin and L cysteine on seminal attributes of frozen-thawed barbari buck.

MATERIALS AND METHODS

Animals and sampling: The experiment was conducted on four Barbari bucks (2-3 years), managed at DFS lab, C.V.Sc. & A.H., ANDUAT, Ayodhya-224229, Uttar Pradesh; under similar feeding and management conditions. The six ejaculates were harvested from each buck (total 24 ejaculates) using artificial vagina (at 39 to 41°C). After collection, pre-freez and post-thawed semen was assessed

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for various seminal parameters.

Preparation of diluter: The glycerolated egg yolk tris dilutor was prepared (Gangwar *et al*. 2015) on the day of semen collection. The dilutor was prepared in two parts. Part A contained 3.604 g of Tris buffer (Sigma-Aldrich), 1.902 g of citric acid monohydrate (Merck, USA), 1.00 g of anhydrous fructose (Merck, USA), 6 ml of glycerol, 100,000 units of penicillin G sodium, 100 mg of dihydrostreptomycin sulphate and glass distilled water up to a final volume of 100 ml. The resulting dilutor showed a pH of 6.8-6.9 and a final osmolarity of 410 mOsm/litre. Ejaculates selected (ocular motility ≥ 70%) were diluted in a Tris-Egg-Yolk-Citric acid-Fructose-Glycerol extender. Post-dilution, the ejaculates were classified as CON (No additive), CUR1 and CUR2 (1.0 and 2.0 µg/mL curcumin (w/v) (Sigma-Aldrich, USA); LC2 and LC5 (2 and 5 mM/L L-Cysteine hydrochloride (w/v) (USB Corporation Cleveland, USA). Afterwards, French mini straws were prepared using automatic filling and sealing machine (ISEVO, IMV, France).

Cryopreservation of semen: After extension and filling (0.25 mL, 80 million sperms per straw), the straws were equilibrated (at 4ºC for 4h) in cold cabinet followed by exposure of straws to liquid nitrogen vapour for 10 minutes (the straws were kept at 2.5 cm above liquid nitrogen level in Styrofoam ice box); finally straws were transferred in to LN2 conatiner for 24h, then frozen straws were thawed at 37ºC for 30 sec for post-thaw evaluation .

Evaluation of post-thaw sperm quality: In frozen-thawed semen, motility, viability, morphology and HOS reactivity was assessed. Seminal plasma from freshly diluted and frozen-thawed samples was separated by centrifugation at 3000 rpm for 20 min and used to test enzyme leakage Glutamic oxaloacetic transaminase (GOT), Glutamate pyruvate transaminase (GPT), Alkaline phosphatase (AKP) and Acid phosphatase (ACP) using standard kits (CliniQuant TM micro) and a Merilizer (Meril Diagnostic Pvt. Ltd.).

Statistical analysis: The data (mean±SE) were analyse dusing Graph Pad Prism 5 software The factorial model included the effect of curcumin and L cysteine as independent variables and post thaw seminal attributes as dependent variables. An ANOVA was used to assess differences among the treatment groups. Post hoc Tukey's test was conducted to know the significant difference between different curcumin and L cysteine concentrations at $P<0.05$.

RESULTS AND DISCUSSION

Effects on cyto-morphological seminal parameters in pre-freeze and post-thaw semen : The significant ($P<0.05$) increment in initial motility and viability, and decline in abnormality was noted in post-thaw (PT) samples in all groups except CON (Table 1).

Progressive motility: The significant ($P<0.05$) decline in motility was noted in CON group, whereas supplementation of Curcumin and L cysteine seems to be beneficial in mitigating cryopreservation-induced decline in motility, with the highest effect noted in CUR2 group. The mean value of PT motility in the current study was in line with earlier studies (Anghel *et al.* 2010). Unlike present findings, many researchers observed higher (Soleimanzadeh

Table 1. Effect of different concentrations of Curcumin (CUR1 and CUR2) and L Cysteine (LC2 and LC5) on Seminal attributes (Mean± SE) of Barbari buck semen

Parameters	Pre-freeze					Post-thaw				
	CON	CUR 1 (1 µg/ml)	CUR 2 (2 µg/ml)	LC 2 (2 mM/L)	LC 5 (5mM/L)	CON	CUR 1 (1 µg/ml)	CUR 2 (2 µg/ml)	LC 2 (2 mM/L)	LC 5 (5mM/L)
Motility (%)	79.50± 0.76^{a}	80.83± 0.70^{ab}	82.17± 0.83^{b}	80.17± 0.54^{ab}	80.50± 0.56^{ab}	48.67± 0.49^{a}	50.50± 0.42^{b}	53.50± 0.50^{c}	52.50± 0.50^{cd}	51.33± 0.42^{bd}
Liveability (%)	87.50± 0.76^{a}	88.83± 0.65^{ab}	89.67± 0.55^{b}	85.50± 0.95^{ab}	88.67± 0.76^{ab}	74.50± 0.76^{a}	77.67± 0.98^{b}	81.50± 1.05^{c}	81.00± 0.96^{c}	77.67± 0.88^{b}
Abnormality (%)	8.83± 0.65^{b}	7.50± 0.50^{ab}	6.66± 0.66^{a}	7.66± 0.76^{ab}	7.83± 0.60^{ab}	13.50± 1.33^{c}	11.83± 1.07^{bc}	7.66± 0.55^{a}	8.66± 0.49^{ab}	11.00± 1.26^{bc}
HOS (%)	50.17± 1.07^{a}	50.17± 0.87^{a}	54.00± 0.57^{b}	53.67± 0.55^{b}	53.00± 0.68^{c}	34.33± 1.35^{a}	39.83± 1.10^{b}	44.00± 0.96^{c}	43.00± 0.51^{bc}	41.17± 0.98^{bc}
GOT (µmole/L)	64.50± 2.01	59.17± 1.64	58.17± 2.07	58.00± 2.11	58.83± 1.10	165.8± 6.30^{c}	156.5± 6.42^{bc}	140.0± 5.19^{ab}	147.8± 4.67^{bc}	151.8± 5.73^{abc}
GPT (µmole/L)	116.8± 1.72^{c}	113.3± 2.01^{bc}	107.5± 1.28^{a}	108.5± 1.60^{ab}	112.3± 1.83^{abc}	250.2± 6.37	243.7± 7.67	231.5± 6.22	234.2± 6.21	241.8± 6.94
ACP (KAU/100 mL)	28.00± 1.63^{c}	23.67± 1.20^{b}	21.00± 1.46^{a}	20.50± 1.08^{a}	24.00± 1.12^{b}	56.00± 2.01^{b}	49.50± 2.07^{ab}	47.33± 1.87^{a}	49.33± 1.76^{ab}	51.83± 1.85^{ab}
AKP (KAU/100 mL)	59.17± 2.46	54.67± 2.59	50.00± 2.98	53.17± 2.93	56.83± 2.22	153.5± 6.64^{a}	140.5± 5.28^{abc}	134.8± 4.76^{bc}	137.0± 5.02^{c}	145.5± 5.98^{ac}

Means bearing different superscripts (a-c) in a row differed significantly ($p<0.05$)

and Saberivand, 2013, Ismail *et al.* 2020) and lower PT motility in 125 µmol/L curcumin supplemented and cessation of motility was recorded at 250 µmol/L curcumin supplemented in human or rat semen (Naz, 2011). Curcumin appears to inhibit progressive motility by acidifying spermatozoa intracellularly and hyperpolarizing their membrane as a result of a concentration-dependent reduction in intracellular pH from 7.33 to 6.81 in humans and mice (Naz, 2014). The beneficial effect of curcumin might be due to its ability to boost antioxidant status, mitochondrial function and membrane integrity while reducing apoptosis (An *et al.* 2025). It seems that curcumin has dose-dependent effect on semen; beneficial at lower concentrations, a detrimental impact higher concentration.

Like present findings, beneficial effects of cysteine supplementation also has been reported in mammalian sperm cells (Zhu *et al.* 2017, Jannatifar *et al.* 2019, Koohestanidehaghi *et al.* 2021, Hayanti *et al.* 2022, Arunpandian *et al.* 2023, Tudu *et al.* 2023, Kafi *et al.* 2024, Panwar *et al.* 2024). Cysteine, an α-amino acid, is a precursor in the production of intracellular glutathione (GSH), which neutralizes the reactive species of oxygen and catalyzes hydrogen or other superoxide detoxification (Memon *et al.* 2011); can protect the sperm membrane function from the damaging effect of free radicals by directly scavenging free radicals (Bilodeau *et al.* 2001), thus improving post-thawed seminal quality of buck semen (Adel *et al.* 2024).

Viability: The supplementation of 2µg/ml Curcumin and 2mM/L L-cysteine seems equally effective in mitigating cryopreservation-induced loss of membrane function. The supplementation of 1µg/ml Curcumin and 5mM/L L-cysteine also significantly improved PT viability but to a lesser extent than the other doses of Curcumin and L-cysteine. The mean values of PT viability in untreated control were similar to the observation of Kulaksiz *et al*. (2020). However, lower PT-viability was reported by Memon *et al*. (2011) cysteine supplemented semen and higher PT-viability (Ismail *et al.* 2020) in curcumin supplemented buck semen.

The curcumin may play a role in mitigating the H2O2 -induced injury to sperm (Zhang *et al.* 2017). N-acetyl-cysteine (NAC), a derivative of the amino acid L-cysteine, helps in glutathione (GSH) production (Rushworth and Megson 2014) and may assist in restoring the depleted GSH pool, which is caused by oxidative stress and inflammation (Wang *et al.* 2014).

Sperm abnormality: The incorporation of curcumin and L-cysteine HCl significantly (P<0.05) decline PT abnormality than those of CON (Table 1). The highest decline was recorded CUR2 followed by LC2, LC5,and CUR1. The corrent observations corroborated well with previous study (Abdelnour *et al*. 2020). Cysteine is functions as an antioxidant directly or indirectly through intracellular antioxidants that protect from ROS-mediated deleterious effects (Perumal *et al.* 2011).

Hypo-osmotic swelling reactivity: The incorporation of additives (Cucumin and L-cysteine HCl) significantly (P<0.05) enhanced the post-thaw HOS reactive sperm in all the bucks. The PF HOS reactive sperms were significantly higher in CUR2 and LC2, followed by LC5, as compared to CON and CUR1. Likewise, PT HOS positive values were significantly higher in the Curcumin and L-cysteine-supplemented group compared with the control. The present findings are in agreement with many previous studies (Bucak *et al.* 2012; Elsheshtawy, 2020). There is a positive correlation between plasma membrane integrity and mitochondrial activity as measured by the HOS test (Zuge *et al.* 2008). The findings of the present study demonstrate that supplementation with L-cysteine and curcumin can effectively enhance plasma membrane integrity (viability) and functionality (HOS test). Supplementation of curcumin and its nanoparticle improves post thaw seminal attributres in buffalo bull (Khalil *et al.* 2025) and rabbit semen (Abdelnour *et al.* 2020).

Effects on enzymatic activity in pre-freeze and post-thaw semen: The different enzymatic activity at the pre-freeze and the post-thaw stages are depicted in Table 1. The PF GOT activity did not differ significantly among all groups, whereas PT GOT activity was decreased significantly (p<0.05) in the all group except CON. The PF GPT activity did not differ significantly (p>0.05) in CON, CUR1 and LC5 groups, whereas maximum siginificant decline was noted in CUR2 and LC2. In contrast, PT GPT activity did not differ significantly among all groups. The PF ACP and AKP activity did not differ significantly among all groups, but a significant decline in PT ACP and AKP activity was noted in the CUR2 group.

The PT GOT and GPT activity declined significantly curcumin (2µg/ml) and cysteine (2mM/ L) supplemented semen than those of other groups. Similarly, Chaudhary *et al*. (2022) also reported reduced significantly GOT activity in curcumin (25µM) supplemented extenders as compared to control. Due to sperm membrane disruption and the ease of enzyme leaking from spermatozoa, there is a high concentration of transaminase in the extracellular fluid (Gundogan, 2006). Cysteine is an intracellular glutathione precursor (Uysal and Bucak, 2007) penetrate the cell membrane easily, boosting the intracellular reduced glutathione (GSH) biosynthesis both *in vivo* and *in vitro* and preserving membrane lipids and proteins *via* indirect radical scavenging properties.

The PF ACP activity was significantly (p<0.05) declined in CUR2 and LC2, followed by CUR1 and CON. However, the PT ACP activity was significantly (p<0.05) reduced only in CUR2 group as compared to others. It seems that 2µg/ml curcumin supplementation is effective in mitigating cryopreservation-induced cryodamage, as evidenced by an increase in motility, viability and HOS rates.

The PF AKP values did not differ significantly among all groups. However, the PT AKP activity was significantly (p<0.05) reduced in all groups except control. In conclusion, incorporation of cucumin (2µg/ml) and L-cysteine HCl (2mM/L) in semen extender significantly

mitigates cryopreservation induced cryodamage of Barbari buck semen.

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