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Curcuma xanthorrhiza Diluent's Effect on the Freezing and Thawing of Thin-Tailed Ram Sperm

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PIS, SB, and DTW all contributed to the writing of the work and the research that went into it. PIS and SB were responsible for the ideation, execution, supervision, and contribution to the manuscript. PIS analyzed the data and revised the paper. The study was conceived and supervised by all of the writers. The statistical work and paper evaluation were completed by PIS and SB, respectively. The research was directed by SB and DTW. Each author has seen and given their stamp of approval on the final version.

Key words
Antioxidant, *Curcuma xanthorrhiza* rhizome, Pre-freezing, Temulawak, Thin-tailed ram

ABSTRACT

The process of making frozen semen and preserving semen will increase sperm damage due to the activity of the free radicals that are formed. *Curcuma xanthorrhiza* rhizome has native antioxidant properties, such as curcumin, tumerol, and xanthorrhizol, which can be used for ram semen cryopreservation. This study examined the effects of *C. xanthorrhiza* extract on semen parameters after the freeze-thaw treatment in frozen semen processing. Four healthy and mature ram was conducted to collect the fresh semen in this research. Each donor's ejaculate will be separated into five wells and diluted in an egg yolk citrate (2.9% (v/v)) extender containing 1%, 2%, 3%, and 4% (P1; P2; P3; P4), and control/P0 which will then be evaluated for the quality of the semen produced, both in low temperatures (5° C) and frozen in straws that will be thawed later. The data obtained in this study were statistically analyzed using a completely randomized design (CRD). The results showed that the addition of *C. xanthorrhiza* improved the motility and viability of spermatozoa both in freeze-thawed semen ($p < 0.05$). P3 (3% supplementation) resulted in the highest motility (76.14±1.35% pre-freezing; 60.00±1.63% after thawing) and viability (85.00±2.00% pre-freezing and 70.14±1.57% after thawing) $p<0.05$). Regression tests using cubic curves showed that the R square^2 values on motilities and viabilities were higher than 0.85, and could be used as a predictor of the higher addition of *Curcuma xanthorrhiza*. In conclusion, the addition of 3% C. xanthorriza in semen diluent significantly enhanced post-thaw sperm quality in thin-tailed ram semen by reducing free radical-mediated oxidative stress during pre-freezing or after thawing.

INTRODUCTION

In recent years, artificial insemination (AI) technology has often been used in Indonesia. Artificial insemination limits the spread of infectious illnesses when organisms are near or share the same environment. Sperm Cryopreservation one of AI procedures, enables the broad transmission of significant genetic material, which also enhances the spring number in livestock for a short time. However, the application of frozen sperm for artificial insemination is hampered by poor fertility rates in sheep after the treatment or protocol. Freezing and thawing of spermatozoa is followed with diminished cell motility, viability, and fertility (Atessahin *et al*., 2008; Al-Mutary, 2021). Biochemical and physical alterations in spermatozoa membrane caused by oxidative damage during the freezing process can also interfere with spermatozoa function and even cause cell death. Furthermore, it is known that during the freezing process, it has the capacity to create lipid peroxide and reactive oxygen species (ROS). Which cause a deterioration in the quality of spermatozoa via a redox mechanism (Peris-Frau *et al*., 2020). ROS damage spermatozoa DNA by exposing sperm cells at high oxygen pressure and lowering sperm quality such as motility and viability (Jumintono *et al*., 2021). ROS-induced oxidative stress in oxygenated sperm caused by oxygen-containing ROS can damage cells and tissues. Spermatozoa are highly sensitive to oxidative stress because of their high fatty acid content, which is not balanced by adequate antioxidant levels. Antioxidants neutralize ROS and protect cells from

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oxidative damage; hence, sperm with high ROS levels have increased motility, concentration, and morphology, and reduced DNA damage (Jumintono *et al*., 2021). A fast reduction in temperature and pressure during the freezing process also causes physical and chemical damage to the spermatozoa membrane (Aitken and Drevet, 2020; Jumintono *et al*., 2021). Overall, the whole process that occurs during the preparation of frozen semen results in damage to sperm cells both due to physical factors (the freezing process that occurs quickly) and chemically (the formation of ROS during the process) which directly reduces the quality of frozen semen produced. Therefore, the addition of certain antioxidant substrates is needed to inhibit or reduce the negative impact of the sperm freezing process (Priyanto *et al*., 2023).

Curcuma xanthorrhiza rhizome, often known as Javanese turmeric or temulawak (Family: Zingiberaceae), is a commonly used as herbal cure and potentially have antioxidant function (Pujimulyani *et al*., 2018). The primary constituents of *C. xanthorrhiza* include starch (50-60%) and volatile oils (3-12%), including phelandren, camphor, tumerol, sineol, borneol, xanthorrhizol (1.48-1.63 %), sesquiterpenes (-curcumene, ar-curcumene, bisabolane, lactone germacone), and flavonoids (Rohman *et al*., 2020). *C. xanthorrhiza* rhizomes contain a plethora of the active compounds terpenoids and curcuminoids (Zhang *et al*., 2014; Rahmat *et al*., 2021) which has antibacterial and antioxidan effects (Rosidi *et al*., 2016; Widyastuti *et al*., 2021). DPPH (1,1-diphenyl-2-picrihydazine), superoxide anion, iron-reducing antioxidant power (FRAP), and metal bonding activity were used in previous investigations to determine *C. xanthorrhiza's* antioxidant capacity (Devaraj *et al*., 2014). According to the experiment's findings, curcumin, which contains phenolic and methoxy groups on its phenyl rings and diketone moiety, is an active component of *C. xanthorrhiza's* antioxidant function (Masuda *et al*., 2001; Priyadarsini, 2014). As is well known, curcumin blocks free radicals by neutralizing lipid peroxyl beforehand, which threatens membrane PUFA (Rahmat *et al*., 2021). These natural chemicals or compounds may be termed antioxidants because of their activities as (i) scavengers of reactive species, (ii) chelators of heavy metal ions such that the metals are unable to form reactive species, (iii) quenchers of singlet oxygen, (iv) breakers of free radical chain reactions, and (v) reducing agents (Rohman *et al*., 2020). The search for natural compounds with significant antioxidant activity seems to be a helpful preventative approach for phenotypes associated with free radicals (Jakubczyk *et al*., 2020), therefore there are emerging increasingly restrictive rules on the use of chemical antioxidants that have the potency to damaging the environment in the manufacturing process.

A substance of *C. xanthorrhiza* was not previously employed in ram sperm diluent cryopreservation. The present research was conducted using thin-tailed ram sperm to evaluate the cryoprotective advantages of *C. xanthorrhiza* as a semen diluent due to its widespread availability in tropical environments and its potential antioxidant properties. The main objectives of this study were to standardize the optimal dose of *C. xanthorrhiza* to be added to the semen extender and to evaluate the effects of adding *C. xanthorrhiza* as a semen diluent on cryocapacitation associated and apoptotic-like alterations in spermatozoa after equilibration and freezing-thawing.

MATERIALS AND METHODS

Animal experimental design and semen collection

Four mature and heathy thin-tailed rams (35-40 kg body weight; 2-5 y.o) were employ in this research. Rams have been kept at the Physiology and Reproduction Laboratory, Faculty of Animal Science, Gadjah Mada University, which is then inspected for livestock health and found to be clinically free of infectious diseases and external or internal parasites. The ejaculates were collected at 7-8 am twice weekly, and rams were regularly used for semen collection before (Gupta *et al*., 2022). All rams were individually fed the same concentrate mixture (CP 16%; 2.8%/BW), 10%/BW forage, and kept in individual pens. Since there were no invasive assessments of the animals, ethics committee approval was not necessary for this research.

Semen processing and evaluation

The quality and profile of the sperm was analyzed shortly after collection such as motility, viability, pH, sperm concentration, odor and semen colour to determine whether this fresh semen was appropriate to be used as a freezeable semen raw material. A Neubauer hemocytometer was used to examine the quality of fresh semen. Fresh sperm (100 µL) was positioned on a glass slide, examined under the microscope, and graded on a rating system ranging from 0 (no motility) to 100 (excellent motility), the assessment process was conducted by three experts who were experienced in assessing sperm quality to ensure the objectivity of the assessment. Spermatozoa viability (%) and abnormalities (%) were defined using a fixed smear stained using the eosin-nigrosin method. Nigrosin enhances the differentiation between the surrounding area and the sperm's head, rendering it easier to make observations. Eosin dyes just deceased sperm, rendering them a dark pink color, while the living ones appeared in white colours or do not absorb eosin. The proportions of both alive and death sperm were determined, and all

primer or secondary sperm abnormality could also be determined (El-Zeftawy *et al*., 2020). This study analyzed semen with > 80% progressive motile spermatozoa and > 90% viability for subsequent examination.

Yolk citrate (2.9% (v/v) sodium citrate dihydrate, 100 ml aquadest mixed with egg yolk 20%, 8% (v/v) glycerol (Merck, Germany), 1.000 IU/ml Penicillin and streoptomycin as antibiotics was used as the base semen diluent (freezing extender). The semen was dissolved with diluent until it reaches 50 mg/mL. Five equal aliquots of pooled ejaculate were divided and diluted (37°C) with diluent containing *C. xanthorrhiza* (1%, 2%, 3%, 4% called P1, P2, P3, and P4) and a base diluent as a control (P0). Then the fresh diluent semen was evaluated as the before frozen quality. The straws were afterwards adjusted at 5°C for 4 h. The equilibrated semen was collected and encapsulated to 0.25 mL straws. Straw had been placed in nitrogen vapor 5 cm over liquid nitrogen for ten minutes, and sperm was stored in liquid nitrogen. Following being stored for 24 h, each of the straws were independently thawed for semen assessment. subsequently freezing, each sample of sperm was promptly evaluated for its sperm quality. This study compared the efficacy of sperm before and after freezing in order to evaluate the effects of *C. xanthorrhiza* extract supplementation thoroughly.

Semen evaluation

The data observed were the concentration, motility, viability abnormalities, and plasma membrane integrity/HOST of the spermatozoa before and after freezing. Thawing procedure was conducted by applying warm water to the frozen semen straw for 10 min until the semen is completely thawed. The evaluation of thawed sperm was conducted in the same manner as the evaluation of raw sperm described above. The hypoosmotic swelling test was conducted through exposing 0.1 ml of semen with 1 ml of 150 M hypoosmotic solvent for 30 min at 37°C. Following incubation, 0.2 ml of the solution was disseminated employing a cover sheet on slide glass. A fluorescent field microscope was utilized to investigate 200 spermatozoa at a magnification of 1000x. It has been observed that spermatozoa have a protruding or curved tail. This test measures the functional integrity of the sperm membrane. (Bucak *et al*., 2012).

Statistical analysis

The outcome variables are represented as the mean standard error of the mean (Mean±SEM). The influence of *C. xanthorrhiza* extract administration in each circumstance before and after freezing procedure was determined through a multifactorial analysis of all data. In addition, the results for each condition were analyzed using one-way analysis of variance followed by Tukey's post hoc test to determine whether or not there were statistically significant differences in all parameters among the groups. In a regression evaluation, a predictive equation was established with the dependent variable adjusted supplementation and the independent variable sperm quality. For all statistical analyses, SPSS statistics software (version 26.0; IBM Corp., Chicago, IL, USA) was utilized (Kumaresan *et al*., 2020).

RESULTS

In five distinct experiments, the implications of addition *C. xanthorrhiza* on sperm qualities prior to and following freezing and thawing were determined. All qualities parameters such as motility, viability, abnormalities, and plasma membrane integrity/HOST (%) of sperm from thin-tailed rams supplemented with different dosages of *C. xanthorrhiza* (Temulawak) were better than the control P0 (Table I). The addition of 3% *C. xanthorrhiza* extract resulted in the most outstanding motility both in freezing treatment (76.14±1.35%

Table I. Sperm parameters (Mean±SE) (%) in pooled semen supplemented with different *Curcuma xanthorrhiza* concentration of ram semen following freeze/thawing.

Treatment	Motility		Viabiliy		Abnormality		Plasma membrane integrity	
	Pre-freezing	Post thawing	Pre-freezing	Post thawing	Pre-freezing	Post thawing	Pre-freezing	Post thawing
P0	69.00±2.24*^{a}	54.14±1.35*^{a}	78.43±0.98*^{a}	65.14±2.91*^{a}	12.29±1.60*	18.00±1.15*^{a}	69.43±2.44*	63.14±1.57*
P1	72.00±2.45*^{bc}	56.00±1.41*^{ab}	80.14±3.02*^{a}	67.29±2.29*^{ab}	12.14±1.07*	19.00±1.15*^{ab}	69.57±2.57*	65.00±1.53*
P2	73.57±1.51*^{bc}	58.00±1.63*^{bc}	82.00±1.41*^{ab}	69.14±1.35*^{c}	11.86±1.35*	20.14±1.57*^{b}	70.86±2.41*	66.00±2.24*
P3	76.14±1.35*	60.00±1.63*^{c}	85.002.00*	70.14±1.57*^{c}	12.57±1.13*	19.00±1.15*^{ab}	67.86±1.57*	64.14±1.07*
P4	73.14±0.00*^{bc}	57.00±1.15*^{b}	82.29±1.25*^{b}	67.14±1.35*^{ab}	13.43±1.13*	20.00±1.29*^{b}	68.71±2.75*	63.71±2.43*
R^2	0.96	0.98	0.94	0.99	0.96	0.78	0.97	0.88
P values	0.107	0.55	0.117	0.97	0.291	0.165	0.449	0.216

^{abcd} total means with different superscripts within a row differs significantly (p<0.05). *total means with different superscripts within a row differs significantly (p<0.05). P_0, without *C. xanthorrize*; P_1, 1% of *C. xanthorrize*; P_2, 2% of *C. xanthorrize*; P_3, 3% of *C. xanthorrize*; P_4, 4% of *C. xanthorrize*.

pre-freezing; 60.00±1.63% after thawing) and viability percentage (85.00±2.00% pre-freezing and 70.14±1.57% after thawing) $p<0.05$). The data showed significant differences in pre-freezing and post-thawing in ram semen diluted with *C. xanthorrhiza* at different concentrations, as shown in Table I.

DISCUSSION

Effect of C. xanthorrhiza *on semen quality*

The data showed that addition of *Curcuma xanthorrhiza* 3% resulted in the highest motility in both freezing treatments, which is supported by earlier studies showing that curcumin (the antioxidant active ingredient of *C. xanthorrhiza)* has similar effects, which increased semen quality after the addition of curcumin in cattle (Gupta *et al*., 2022), bull (Tvrda *et al*., 2012, 2016), buffalo (Herbowo *et al*., 2019), rams and rats (Soleimanzadeh and Saberivand, 2013; Omur and Coyan, 2016), goat (Bucak *et al*., 2010), and boars (Chanapiwat and Kaeoket, 2015). However, administration of *C. xanthorrhiza* to any species, notably goats, has never been conducted in an earlier study.

Sperm motility is crucial for the process of transporting spermatozoa to the ovum and is a significant factor in spermatozoa penetrating the cumulus cells and zona pellucida of the ovum for fertilization (Bucak *et al*., 2012). In this study, *C. xanthorrhiza* addition significantly enhanced the motility and viability spermatozoa compared to the control ($P<0.05$) in both frozen and thawed semen, which is consistent with other studies in cattle and goats using diluent containing curcumin (Bucak *et al*., 2012; Kumar *et al*., 2014). Curcumin addition in pre-freezing and post-thawed ram spermatozoa markedly improved CASA motility and viability. The present investigation determined which the addition of *C. xanthorrhiza* to sperm diluent effectively maintains more than 40% of the sperm's post-thawing motility (PTM), which is suitable for insemination (Table I). This was due to curcumin improved capacitation and the acrosome response, both of which enhance sperm motility in human sperms. This was accomplished by increasing the amount of the transcriptional nuclear component Nrf2 (Nuclear factor E2-related factor 2), which is an inducible transcription factor essential for maintaining redox signal transmission against oxidative stress in spermatogonia and sperm cytoplasm in mammals (He *et al*., 2022). The bZIP protein NRF2 controls the regulation of promoters expressing compounds involved in detoxifying ROS to shield cells from oxidative damage, Nrf2 induces the transcription of a series of antioxidant enzymes directly dependent on the antioxidant response element (ARE) promoter (Aydos *et al*., 2021). However, the effects on small ruminants have not been clarified yet. The only conclusion that can be drawn from this study is that the antioxidant activity of curcumin present in *C. xanthorrhiza* actively minimizes the detrimental effects of ROS, which means that it will generally maintain sperm quality, especially after thawing (Santonasato *et al*., 2021). Curcumin and xanthorhizol (XNT) in *C. xanthorrhiza* acted as antioxidant agents to suppress the growth of ROS (Carapina da Silva *et al*., 2019), which could maintain sperm quality in this study. This preservation due to XNT decreased the oxidative damage caused by free radicals (Lim *et al*., 2005). XNT antioxidant characteristics demonstrate powerful neuroprotective benefits by inhibiting hydrogen peroxide (H_2O_2)-induced lipid peroxidation, glutamate-induced neurotoxicity, and ROS production in cells. Additionally, the presence of a phenolic hydroxyl group (sesquiterpene phenol) on the bisabolene skeleton of XNT likely contributed to its powerful antioxidant capabilities by chelating Cu^{2+}. This may inhibit the onset of LDL oxidation and the production of free radicals inside the lipoprotein (Jantan *et al*., 2012; Oon *et al*., 2015), and depress cell death in this study. According to this study, curcumin acts as a potent antioxidant that reduces the consequences of oxidative stress. This is supported by a previous study using the DPPH method (Rosidi *et al*., 2016), which evaluated the antioxidant activity of the ethanol extract of C. *xanthorrhiza* using the liquid-liquid extraction technique in hexane. With an IC_{50} value of 86 ppm, the *C. xanthorrhiza* extract was shown to have a reasonably potent antioxidant effect. It reduces oxidative damage through engaging with various biochemical processes, such as its capacity to neutralize metals that are toxic and modulate the functions of a large number of enzymes (Jakubczyk *et al*., 2020). Moreover, curcumin which also cointain in C. *xanthorrhiza* has a higher antioxidant capacity than vitamins E and C, and beta-carotene (Rosidi *et al*., 2016; Septiana *et al*., 2020). Curcumin is expected to be used as a semen supplement because of its capacity for antioxidants, as indicated by earlier research. Curcumin ingredients in *C. xanthorrhiza* diluent semen scavenge ROS and suppress LPO, enhancing semen quality through reducing the oxidative damage in sperm cells (Bucak *et al*., 2012; Tvrda *et al*., 2012).

The lack of antioxidants in order to decrease the amount of the generation of ROS resulted in decreased motility and viability in the control group. This results in the acrosome response affecting the sperm's lifespan, sperm create and export ROS to the extracellular environment, most of which are produced by mitochondria in response to flagellar activity and leads early sperm maturation and derivate the fertilization sperm ability or accelerate sperm death (Riesco *et al*., 2021). Under normal circumstances,

naturally produced antioxidants or antioxidant enzymes protect sperm in a decreasing microenvironment. Elevated oxidative stress causes modifications in lipids, proteins, and nucleic acids, which ultimately contribute to a decrease in mitochondrial function and cell mortality (Tan *et al*., 2018; Jakubczyk *et al*., 2020).

C. xanthorrhiza suplementation had a better protective impact on functional membrane integrity than the control both in pre-freezing and after thaw semen, although not significantly different (P>0.05). Additionally, the data showed that the addition of *C. xanthorrhiza* led to a higher HOST score, although it was not significantly different (*p>0.05*) from that of the control. These characteristics are linked to the control of proinflammatory cytokines, nitric oxide synthase (iNOS) enzymes, cyclooxygenase-2 (COX-2), lipoxygenase, xanthine oxidase, and reduction of malondialdehyde (MDA) (Alizadeh and Kheirouri, 2019; MS *et al*., 2020).

The addition of 3% *C. xanthorrhiza* (P3) resulted in a slight preservation of the percentage of membrane integrity, motility, and viability both in pre- and post-thawing semen in all groups. This demonstrated that the addition of up to 3% *C. xanthorrhiza* extract substantially preserved the characteristics of thin-tailed ram spermatozoa prior to and after thawing. In the presence of curcumin, cysteine, or hyaluronan in the cryogenic state, there was no reduction in MDA levels in ram and goat spermatozoa (Bucak and Tekin, 2007; Bucak *et al*., 2008, 2010).

This study found an interesting data that supplementation with 4% *C. xanthorrhiza* (P4) resulted in reduced sperm quality. An intriguing finding in this study was that sperm quality metrics decreased after freezing-thawing with the addition of 4 % *C. xanthorrhiza*, which is in line with a previous study showing a detrimental impact of curcumin at high concentrations on these parameters (Gupta *et al*., 2022). After freezing and thawing, excessive curcumin in *C. xanthorrhiza* contain in semen may promote sperm intracellular acidification and hyperpolarization of the membrane, which may explain why sperm activity is reduced (Naz, 2014). The potential of acidification within cells and sperm metabolism damage cannot be ruled out. *C. xanthorrhiza* and their curcumin amounts and reactions may be influenced by organisms, the sort of semen enhancer used, the method of processing of the semen, and freezing techniques (Tvrdá *et al*., 2016). However, curcumin has both negative and positive effects on sperm function, according to the amount present in the sperm extender (Naz, 2011). Therefore, it is essential to note the formulation with curcumin added to the diluent. Regression test using cubic curve showed the R^2 values on motilities and viabilities had higher than 0.85 and could be used as a predictor on the higher addition of *C. xanthorrhiza*.

Effect of pre-freezing and after thawing on semen diluted with Curcuma xanthorriza

Cryopreservation or freezing of sperm is one of the most crucial techniques for advancing bioprocesses for assisted reproduction (Yánez-Ortiz *et al*., 2021). This study found that all parameters after thawing showed reduced sperm quality due to the cryopreservation mechanism (Table I). Cryopreservation-induced oxidative stress caused by free radicals is the primary cause of apoptosis in sperm cells (Gupta *et al*., 2004, 2022). Previous investigations have shown that spermatozoa undergo apoptotic-like changes when frozen and thawed (Martin *et al*., 2004), including decreased mitochondrial transmembrane potential, chromatin crosslinking, and DNA fragmentation, all of which lead to decreased viability and motility. Pre-post thaw, there is a nearly two-fold increase in apoptosis-like spermatozoa, which lowers the sperm quality (Shah and Andrabi, 2021). The freeze-thaw process targets sperm DNA and the protamine process. A previous study found that freezing-thawing of goat spermatozoa increased DNA fragmentation. DNA fragmentation was evident in chromatin dispersion (the halo surrounding the nucleus) and loss of protamine, which we observed in the aberrant sperm cell population in this investigation (deprotamination). DNA fragmentation in sperm cells is associated with increased deprotamination, which increases the risk of infertility (Kritaniya *et al*., 2020). *C. xanthorrhiza* addition of up to 3 % in this study was shown to protect sperm quality throughout the freezing-thawing process, and this study's findings support other studies (Tvrdá *et al*., 2016). Curcumin was previously added to the semen extender in bull spermatozoa, and similar effects on the mitochondrial transmembrane potential were observed (Tvrda *et al*., 2012). The antioxidant curcumin protects sperm mitochondria from oxidative stress, resulting in sperm mitochondrial function and protection (Omur and Coyan, 2016). At higher doses, *C. xanthorrhiza* did not preserve sperm DNA, protamine, or membrane phosphatidylserine, which may have been related to curcumin toxicity at higher concentrations (> 3 %). This finding is similar to that of another study that used curcumin as semen diluent (Kumaresan *et al*., 2020). This is further supported by the graph below, which shows a regression trend for motility (R^2 = 0.95; P = 0.055) and viability (R^2 = 0.88; P = 0.097) after thawing (Fig. 1; Table I). Consequently, discovering and utilizing the optimal concentrations of *C. xanthorrhiza* in the extender at 3 % might help protect ram sperm from harm throughout the cryopreservation storage procedure.

CONCLUSION

In conclusion, the supplementation of 3 % *C. xanthorrhiza* to the semen diluent significantly enhanced pre-post thaw semen quality in thin-tailed ram semen by reducing free radical-mediated oxidative stress. The overall improvement in the feezability of ram sperm was attributable to a reduction in cryocapacitation-associated alterations, apoptotic-like changes, and protein carbonyls following freezing-thawing. *C. xanthorrhiza* demonstrated dual effects, improving sperm quality at lower doses (up to 3 %) and degrading spermatozoa at higher doses (≥ 4 %); consequently, the amount of *C. xanthorrhiza* must be considered prior to its use as an additive.

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IRB approval

Not applicable.

Ethics statements

There were no invasive assessments of animals, and ethics committee approval was not necessary for this study.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Aitken, R.J. and Drevet, J.R., 2020. The importance of oxidative stress in determining the functionality of mammalian spermatozoa: A two-edged sword. *Antioxidants*, **9**: https://doi.org/10.3390/antiox9020111

Alizadeh, M. and Kheirouri, S., 2019. Curcumin reduces malondialdehyde and improves antioxidants in humans with diseased conditions: A comprehensive meta-analysis of randomized controlled trials. *Biomedicine,* **9**: 10–22. https://doi.org/10.1051/bmdcn/2019090423

Al-Mutary, M.G., 2021. Use of antioxidants to augment semen efficiency during liquid storage and cryopreservation in livestock animals: A review. *J. King Saud Univ. Sci*., **33**: 101226. https://doi.org/10.1016/j.jksus.2020.10.023

Atessahin, A., Bucak, M.N., Tuncer, P.B. and Kizil, M., 2008. Effects of anti-oxidant additives on microscopic and oxidative parameters of Angora goat semen following the freeze-thawing process. *Small Rumin. Res*., **77**: 38–44. https://doi.org/10.1016/j.smallrumres.2008.03.002

Aydos, S.O., Yunus, Y., Dunya, A., Asuman, S. and Kaan, A., 2021. Relationship between functional Nrf2 gene promoter polymorphism and sperm DNA damage in male infertility. *Syst. Biol. Reprod. Med*., **67**: 399-412. https://doi.org/10.1080/19396368.2021.1972359

Bucak, M.N., Ateşşahin, A. and Yüce, A., 2008. Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze-thawing process. *Small Rumin. Res*., **75**: 128–134. https://doi.org/10.1016/j.smallrumres.2007.09.002

Bucak, M.N. and Tekin, N., 2007. Protective effect of taurine, glutathione and trehalose on the liquid storage of ram semen. *Small Rumin. Res*., **73**: 103–108. https://doi.org/10.1016/j.smallrumres.2006.12.001

Bucak, M.N., Başpinar, N., Tuncer, P.B., Çoyan, K., Sariözkan, S., Akalin, P.P., Büyükleblebici, S. and Küçükgünay, S., 2012. Effects of curcumin and dithioerythritol on frozen-thawed bovine semen. *Andrologia*, **44**: 102–109. https://doi.org/10.1111/j.1439-0272.2010.01146.x

Bucak, M.N., Tuncer, P.B., Sariözkan, S., Başpinar, N., Taşpinar, M., Çoyan, K., Bilgili, A., Akalin, P.P., Büyükleblebici, S., Aydos, S., Ilgaz, S., Sunguroğlu, A. and Öztuna, D., 2010. Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: Antioxidants protect DNA integrity against cryodamage. *Cryobiology*, **61**: 248–253. https://doi.org/10.1016/j.cryobiol.2010.09.001

Carapina da Silva, C., Pacheco, B.S., das Neves, R.N., Dié Alves, M.S., Sena-Lopes, Â., Moura, S., Borsuk, S. and de Pereira, C.M.P., 2019. Antiparasitic activity of synthetic curcumin monocarbonyl analogues against *Trichomonas vaginalis*. *Biomed. Pharmacother*., **111**: 367–377. https://doi.org/10.1016/j.biopha.2018.12.058

Chanapiwat, P. and Kaeoket, K., 2015. The effect of *Curcuma longa* extracted (curcumin) on the quality of cryopreserved boar semen. *Anim. Sci. J*., **86**: 863–868. https://doi.org/10.1111/asj.12395

Devaraj, S., Ismail, S., Ramanathan, S. and Yam, M.F., 2014. Investigation of antioxidant and hepatoprotective activity of standardized *Curcuma xanthorrhiza* rhizome in carbon tetrachloride-induced hepatic damaged rats. *Sci. World J*., 2014. https://doi.org/10.1155/2014/353128

El-Zeftawy, M., Mahmoud, G.B. and Hassan, M., 2020. Impact of thermal stress exposure on seminal quality, antioxidant defence system, TNF-α and TIMP-3 in Ossimi ram. *Reprod. Domest. Anim*., **55**:

870–881. https://doi.org/10.1111/rda.13697

Gupta, S., Kumar, A., Mahajan, A., Sharma, P., Sachan, V., Aggrawal, J., Yadav, S., Saxena, A. and Swain, D.K., 2022. Curcumin in a tris-based semen extender improves cryosurvival of Hariana bull spermatozoa. *Andrologia*, **54**. https://doi.org/10.1111/and.14255

He, C., Sun, J., Yang, D., He, W., Wang, J., Qin, D., Zhang, H., Cai, H., Liu, Y., Hua, J. and Peng, S., 2022. Nrf2 activation mediates the protection of mouse Sertoli cells damage under acute heat stress conditions. *Theriogenology*, **177**: 183-194. https://doi.org/10.1016/j.theriogenology.2021.10.009

Herbowo, M.T., Arifiantini, R.I., Karja, N.W.K. and Sianturi, R.G., 2019. Cryopreservation of swamp buffalo semen in skim milk yolk-based diluent with two different cryoprotectants. *Trop. Anim. Sci. J.*, **42**: 13–18. https://doi.org/10.5398/tasj.2019.42.1.13

Jakubczyk, K., Drużga, A., Katarzyna, J. and Skonieczna-żydecka, K., 2020. Antioxidant potential of curcumin. A meta-analysis of randomized clinical trials. *Antioxidants*, **9**: 1–13. https://doi.org/10.3390/antiox9111092

Jantan, I., Saputri, F.C., Qaisar, M.N. and Buang, F., 2012. Correlation between chemical composition of *Curcuma domestica* and *Curcuma xanthorrhiza* and their antioxidant effect on human low-density lipoprotein oxidation. *Evid. Based Complement. Altern. Med.*, **2012**. https://doi.org/10.1155/2012/438356

Jumintono, J., Alkubaisy, S., Silva, D.Y., Singh, K., Jalil, A.T., Syarifah, S.M., Mustafa, Y.F., Mikolaychik, I., Morozova, L. and Derkho, M., 2021. Effect of cystamine on sperm and antioxidant parameters of ram semen stored at 4 °C for 50 h. *Arch. Razi Inst.*, **76**: 981–989.

Kritaniya, D., Yadav, S., Swain, D.K., Reddy, A.V., Dhariya, R., Yadav, B., Anand, M. and Nigam, R., 2020. Freezing-thawing induces deprotamination, cryocapacitation-associated changes; DNA fragmentation; and reduced progesterone sensitivity in buck spermatozoa. *Anim. Reprod. Sci.*, **223**: 106628. https://doi.org/10.1016/j.anireprosci.2020.106628

Kumar, S., Pandey, A.K., Razzaque, W.A.A. and Dwivedi, D.K., 2014. Importance of micro minerals in reproductive: Performance of livestock. *Vet. World*, **4**: 230–233. https://doi.org/10.5455/vetworld.2011.230-233

Kumaresan, A., Gupta, M.D., Datta, T.K. and Morrell, J.M., 2020. Sperm DNA integrity and male fertility in farm animals: A review. *Front. Vet. Sci.*, **7**: 1–15. https://doi.org/10.3389/fvets.2020.00321

Lim, C.S., Jin, D.Q., Mok, H., Oh, S.J., Lee, J.U., Hwang, J.K., Ha, I. and Han, J.S., 2005. Antioxidant and anti-inflammatory activities of xanthorrhizol in hippocampal neurons and primary cultured microglia. *J. Neurosci. Res.*, **85**: 3244–3253.

Martin, G., Sabido, O., Durand, P. and Levy, R., 2004. Cryopreservation induces an apoptosis-like mechanism in bull sperm. *Biol. Reprod.*, **71**: 28–37. https://doi.org/10.1095/biolreprod.103.024281

Masuda, T., Maekawa, T., Hidaka, K., Bando, H., Takeda, Y. and Yamaguchi, H., 2001. Chemical studies on antioxidant mechanism of curcumin: Analysis of oxidative coupling products from curcumin and linoleate. *J. Agric. Fd. Chem.*, **49**: 2539–2547. https://doi.org/10.1021/jf001442x

MS, S.A.B., Waldman, H.S., Krings, B.M., Lamberth, J., Smith, J.E.W. and McAllister, M.J., 2020. Effect of Curcumin supplementation on exercise-induced oxidative stress, inflammation, muscle damage, and muscle soreness. *J. Diet. Suppl.*, **17**: 401–414. https://doi.org/10.1080/19390211.2019.1604604

Naz, R.K., 2011. Can curcumin provide an ideal contraceptive? *Mol. Reprod. Dev.*, **78**: 116–123. https://doi.org/10.1002/mrd.21276

Naz, R. K. 2014. The effect of curcumin on intracellular pH (pHi), membrane hyperpolarization and sperm motility. *J. Reprod. Infert.*, **15**: 62–70.

Omur, A.D. and Coyan, K., 2016. Protective effects of the antioxidants curcumin, ellagic acid and methionine on motility, mitochondrial transmembrane potential, plasma membrane and acrosome integrity in freeze-thawed Merino ram sperm. *Vet. Med.* (Praha), **61**: 10–16. https://doi.org/10.17221/8677-VETMED

Oon, S.F., Nallappan, M., Tee, T.T., Shohaimi, S., Kassim, N.K., Sa'ariwijaya, M.S.F. and Cheah, Y.H., 2015. Xanthorrhizol: A review of its pharmacological activities and anticancer properties. *Cancer Cell Int.*, **15**: 1–15. https://doi.org/10.1186/s12935-015-0255-4

Peris-Frau, P., Soler, A.J., Iniesta-Cuerda, M., Martín-Maestro, A., Sánchez-Ajofrín, I., Medina-Chávez, D.A., Fernández-Santos, M.R., García-álvarez, O., Maroto-Morales, A., Montoro, V. and Garde, J.J., 2020. Sperm cryodamage in ruminants: Understanding the molecular changes induced by the cryopreservation process to optimize sperm quality. *Int. J. mol. Sci.*, **21**. https://doi.org/10.3390/ijms21082781

Priyadarsini, K.I., 2014. The chemistry of curcumin: From extraction to therapeutic agent. *Molecules*,

19: 20091–20112. https://doi.org/10.3390/molecules191220091

Priyanto, L., Herdis, H., Santoso, S., Anwar, R.I., Priyatno, T.P., Sitaresmi, P.I., Azhari, F., Gunawan, M. and Putranti, O., 2023. The reproductive success of simental bovine after sex-sorting under various incubation and centrifugation protocols. *Vet. World,* **16**: 631-637.

Pujimulyani, D., Yulianto, W.A., Setyowati, A., Arumwardana, S. and Rizal, R., 2018. Antidiabetic and antioxidant potential of *Curcuma mangga* Val extract and fractions. *Asian J. Agric. Biol*., **6**: 162–168.

Rahmat, E., Lee, J. and Kang, Y., 2021. Phytochemistry, biotechnology, and pharmacological activities. *Hindawi Evid. Based Complement. Altern. Med*., **2021**: 15. https://doi.org/10.1155/2021/9960813

Riesco, M.F., Alvarez, M., Anel-Lopez, L., Neila-Montero, M., Palacin-Martinez, C., Montes-Garrido, R., Boixo, J.C., de Paz, P. and Anel, L., 2021. Multiparametric study of antioxidant effect on ram sperm cryopreservation from field trials to research bench. *Animals*, **11**: 1–14. https://doi.org/10.3390/ani11020283

Rohman, A., Widodo, H., Lukitaningsih, E., Rafi, M., Nurrulhidayah, A.F. and Windarsih, A., 2020. Review on *in vitro* antioxidant activities of *Curcuma* species commonly used as. *Fd. Res*., **4**: 286–293. https://doi.org/10.26656/fr.2017.4(2).163

Rosidi, A., Khomsan, A., Setiawan, B., Riyadi, H. and Briawan, D., 2016. Antioxidant potential of temulawak (*Curcuma xanthorrhiza* Roxb). *Pak. J. Nutr*., **15**: 556–560. https://doi.org/10.3923/pjn.2016.556.560

Santonasato, M., Mottola, F., Lovine, C., Colacuri, N. and Rocco, L. 2021. Protective effect of curcumin on the outcome of cryopreservation in human sperm. *Reprod. Sci*., **29**: 2895-2905. https://doi.org/10.1007/s43032-021-00572-9

Septiana, E., Yadi, Y. and Simanjuntak, P., 2020. Antioxidant activity of endophytic fungi isolated from turmeric flowers. *Biosaintifika J. Biol. Biol. Educ*., **12**: 268–273. https://doi.org/10.15294/biosaintifika.v12i2.24396

Shah, S.A.H. and Andrabi, S.M.H., 2021. A systematic review and meta-analysis of spermatozoa cryopreservation, *in vitro* and *in vivo* fertility practices in water buffalo. *Vet. Res. Commun*., **45**: 47–74. https://doi.org/10.1007/s11259-021-09789-0

Soleimanzadeh, A. and Saberivand, A.. 2013. Effect of curcumin on rat sperm morphology after the freeze-thawing process. *Vet. Res. Forum*, **4**: 185–189.

Tan, B.L., Norhaizan, M.E., Liew, W.P.P. and Rahman, H.S., 2018. Antioxidant and oxidative stress: A mutual interplay in age-related diseases. *Front. Pharmacol*., **9**: 1–28. https://doi.org/10.3389/fphar.2018.01162

Tvrdá, E., Tušimová, E., Kováčik, A., Paál, D., Greifová, H., Abdramanov, A. and Lukáč, N.. 2016. Curcumin has protective and antioxidant properties on bull spermatozoa subjected to induced oxidative stress. *Anim. Reprod. Sci*., **172**: 10–20. https://doi.org/10.1016/j.anireprosci.2016.06.008

Tvrda, E., Knazicka, Z. and Lukac, N., 2012. Selected heavy metals versus antioxidant parameters in bull seminal plasma. A comparative study. *J. environ. Sci. Hlth. A Toxic/Hazard. Subst. environ. Eng*., **47**: 1261–1266. https://doi.org/10.1080/10934529.2012.672117

Widyastuti, I., Luthfah, H.Z., Hartono, Y.I., Islamadina, R., Can, A.T. and Rohman, A., 2021. Aktivitas antioksidan temulawak (*Curcuma xanthorrhiza* Roxb.) dan profil pengelompokannya dengan kemometrik antioxidant activity of temulawak (*Curcuma xanthorrhiza* Roxb.) and its classification with chemometrics. *Indones. J. Chemom. Pharm. Anal*., **1**: 28–41. https://doi.org/10.22146/ijcpa.507

Yánez-Ortiz, I., Catalán, J., Rodríguez-Gil, J.E., Miró, J. and Yeste, M.. 2021. Advances in sperm cryopreservation in farm animals: Cattle, horse, pig and sheep. *Anim. Reprod. Sci*., **246**: 1–18. https://doi.org/10.1016/j.anireprosci.2021.106904

Zhang, C.M., Fan, P.H., Li, M. and Lou, H.X., 2014. Two new sesquiterpenoids from the rhizomes of *Curcuma xanthorrhiza*. *Helv. Chim. Acta*, **97**: 1295–1300. https://doi.org/10.1002/hlca.201300440