Effect of Silymarin on various Seminal Characteristic in pre-freeze *Hardhenu* crossbred bull semen

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Abstract

The present study was conducted to evaluate the effect of silymarin in semen extender for cryopreservation of Hardhenu crossbred bull semen. The semen with good physicochemical characteristics semen was diluted in Tris egg yolk glycerol extender (TEYGE) having different concentrations of silymarin (0, 25, 50, 100, 200, and 400 µg/mL denoted by C, S-25, S-50, S100, S200 and S-400). We studied different sperm motion characteristics through CASA in prefreeze semen samples like Total motility (TM, %), progressive motility (PM, %), straight linear velocity (VSL, µm/s), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), average lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), straightness (STR, %), distance average path (DAP, µm), distance curvilinear (DCL, µm), distance straight line (DSL, µm), motile concentration (million/ml), linearity (LIN, %), wobble (WOB) and distal mid piece reflex (DMR %) of the spermatozoa. The supplementation of silymarin up to 100 µg/ml did not affect the sperm motility and other CASA parameters. However, at higher concentration (200 and 400 µg/ml), there was negative effect on sperm motility and other CASA parameters in pre-freeze samples.

Key Words: Silymarin; CASA; *Hardhenu;* Bull; Semen

Introduction

The *Hardhenu* crossbred cattle represent a scientifically developed synthetic breed, developed by the Department of Animal Genetics and Breeding at Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, India. This has been genetically designed to enhance lactation performance while maintaining adaptability to the agro-climatic conditions prevalent in northern India, including the states of Haryana, Punjab, Rajasthan, and Uttar Pradesh. The genetic composition of Hardhenu comprises 62.5% exotic inheritance from Holstein Friesian and 37.5% indigenous inheritance derived from Sahiwal and Hariana breeds. Hardhenu cattle were created through inter-se mating over six to eight generations. The herd strength at LUVAS is approximately 300 animals, Cross breeding with Hardhenu bulls is recommended for areas with good management practices to enhance milk production in low-yielding indigenous or crossbred cattle (Yadav *et al.,* 2020; Dhaka *et al.,* 2023).

The widespread adoption of AI has significantly contributed to advancements in animal genetics, herd health, and overall economic returns in the livestock sector (Salisbury and Vandemark, 1961; Vale *et al.* 2014). The artificial insemination (AI) is a crucial technology in India's agricultural sector, particularly in dairy farming. But, still AI coverage of bovines in the country is about 30 percent and around 65 percent animals are still bred through natural service either because the services are not available at farmers' doorstep or they are not convinced with the efficacy of the existing services (Parsad and Kumar, 2022). Despite its long history, dating back over a century, cryopreservation still presents challenges, improving semen quality is difficult (Kumar *et al.*, 2023) with only about 50% of spermatozoa retaining post-thaw viability and 50 % lost their viability due to various cellular damages like oxidative stress and membrane disruption (Watson, 2000; Lemma, 2011; Sharafi *et al.,* 2022).

The supplementation of antioxidants in the semen extender could decrease the impact of oxidative stress and therefore improve post thaw sperm quality (Berra and Rizzo, 2009). Natural antioxidant (SOD, GPx) exerts a protective effect preserving the metabolic activity and cellular viability of cryopreserved bovine spermatozoa (Camara *et al.,* 2011).

Nowadays, the use of herbal natural product has gained interest worldwide. Among these, silymarin is an important antioxidant and is extracted from the seeds and fruits of milk thistle silybum marianum that contains the flavonolignans silybin which is the major active component (Khan *et al.,* 2013; El – Sheshtawy and El – Nattat, 2017). Silymarin is a strong antioxidant used as a remedy for liver protection against oxidative stress and also as protectant for testicular tissue and improving semen quality through elevation of blood testosterone level (Luangprion *et al.,* 2013).

The silymarin is being studied as a hepato-, neuro-, nephro- and cardio-protective ingredient due to its strong antioxidant and tissue regenerative properties Milić *et al*., 2013; Madrigal-Santillán *et al*., 2014; Vargas-Mendoza *et al*., 2014).

However, there are few studies in which effects of silymarin are studied on semen following cryopreservation. The silymarin also used as a natural additive to semen extenders, has been shown to improve the preservability of frozen Holstein bull semen (Ali *et al.,* 2022) and cattle bull semen (El-Sheshtawy RI *et al.,* 2017). With these facts the present study was carried out to evaluate the effect of silymarin in semen of *Hardhenu* crossbred bull.

Materials and Methods

Ejaculates were collected from *Hardhenu* crossbred bulls maintained at cattle farm LUVAS, Hisar. The ejaculate from 3 Hardhenu crossbred bulls having mass activity +3 and above and individual motility more than 70% was selected for the study. After calculating the sperm concentration and dilution semen was diluted in Tris - egg yolk extender. Semen was diluted with extender to make the sperm concentration 80 million/ml. A stock solution of 50 mg silymarin/5ml was prepared. Further, extended semen sample divided into six different groups having different concentration of silymarin (0, 25, 50, 100, 200 and 400 µg/ml denoted by C, S-25, S-50, S100, S200 and S-400 respectively). In treatment groups we added silymarin in extended semen sample. Sperm kinetic and motility as well as morphological anomalies were assessed using the computer assisted sperm analyser (CASA) system. For each sample, 5 optimal field were selected from each of the eight chambered Leja slide (depth 20 µm). The motion characteristics of sperm recorded were Total motility (TM, %), progressive motility (PM, %), straight linear velocity (VSL, µm/s), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), average lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), straightness (STR, %), distance average path (DAP, µm), distance curvilinear (DCL, µm), distance straight line (DSL, µm), motile concentration (million/ml), linearity (LIN, %), wobble (WOB) and distal mid piece reflex (DMR %) of the spermatozoa.

Statistical analyses were carried out using IBM SPSS Statistics software (IBM Corporation, USA) for windows. Statistical significance was set at 0.05 probability level. Data were analysed using one-way ANOVA and comparison of means were done by Duncan multiple range test (DMRT). Results are expressed as mean ± standard error (SE).

Results

The changes in sperm motility and abnormality parameters of spermatozoa in pre-freezing semen supplemented with silymarin shown in Table 1.

In the pre-freezing samples, sperm motility parameters such as motile sperm (%) and progressive sperm (%) in the treatment groups (S-25, S-50, and S-100) showed no significant difference ($p>0.05$) from the control group (65.02 ± 2.13 and 62.22 ± 2.22, respectively) as shown in table 10. However, motile sperm (%) and progressive sperm (%) were decreased significantly ($p<0.05$) at higher concentrations of silymarin (S-200 and S-400) compared to the control. Further, motile sperm (%) and progressive sperm (%) were significantly ($p<0.05$) higher in S-200 (44.07 ± 3.27 and 41.53 ± 3.25) compare to S-400 (30.37 ± 3.31 and 25.11 ± 3.48). This indicates that motility parameters decreased significantly from S-200 to S-400. These results show that silymarin negatively impacts pre-freeze semen motility parameters at higher concentrations (200 and 400 µg/ml). There were no significant differences ($p>0.05$) in sperm abnormality parameters such as bent tail (%) and distal droplet (%) in the pre-freezing samples treated with silymarin (S-25, S-50, and S-100) compared to the control group (0.45 ± 0.11 and 1.67 ± 0.20, respectively). However, these sperm abnormality parameters increased significantly ($p<0.05$) at higher concentrations of silymarin (S-200 and S-400) compared to the control. Further, bent tail (%) and distal droplet (%)were lower in S-200 (1.01 ± 0.13 and 2.99 ± 0.31) compared to S-400 (1.55 ± 0.33 and 3.28 ± 0.29). In the pre-freezing samples, abnormality parameters such as coiled tail (%) and distal midpiece reflex (DMR%) did not show any significant differences ($p > 0.05$) in the groups treated with silymarin (S-25, S-50, S-100, S-200, and S-400) compared to the control group (0.17 ± 0.08 and 3.72 ± 0.54, respectively).

Table 1: Sperm motility and abnormality parameters of pre-freeze semen samples supplemented with silymarin (Mean ± SE)

Parameters	C	S-25	S-50	S-100	S-200	S-400
Motile sperm %	65.02^{c} ±2.13	66.74^{c} ±2.24	65.85^{c} ±2.04	64.83^{c} ±1.61	44.04^{b} ±3.27	30.37^{a} ±3.31
Progressive motile %	62.22^{c} ±2.22	64.43^{c} ±2.32	62.93^{c} ±2.11	61.77^{c} ±1.69	41.53^{b} ±3.25	25.11^{a} ±3.48
Bent tail %	0.45^{a} ±0.11	0.58^{ab} ±0.09	0.42^{a} ±0.11	0.42^{a} ±0.13	1.01^{b} ±0.13	1.55^{c} ±0.33
Coiled tail %	0.17 ±0.08	0.21 ±0.07	0.14 ±0.03	0.16 ±0.06	0.38 ±0.12	0.41 ±0.11
Distal droplet %	1.67^{a} ±0.20	1.49^{a} ±0.15	2.35^{ab} ±0.42	2.35^{ab} ±0.36	2.99^{bc} ±0.31	3.28^{c} ±0.29
Distal Midpiece Reflex (DMR) (%)	3.72 ±0.54	4.43 ±0.46	4.77 ±0.48	4.94 ±0.48	4.87 ±0.45	5.34 ±0.38

Mean ±SE value with different superscript in row differ significantly ($p \leq 0.05$), C (1): control, S-25: silymarin 25 µg/ml, S-50: silymarin 50 µg/ml, S-100: silymarin 100 µg/ml, S-200: silymarin 200µg/ml and S-400: silymarin 400 µg/ml

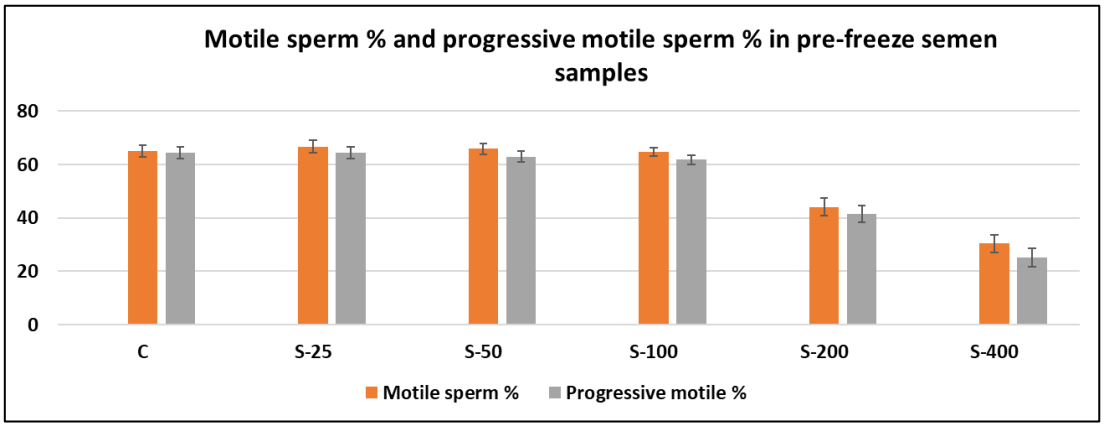

Fig 1: Sperm motility parameters in pre-freeze semen samples

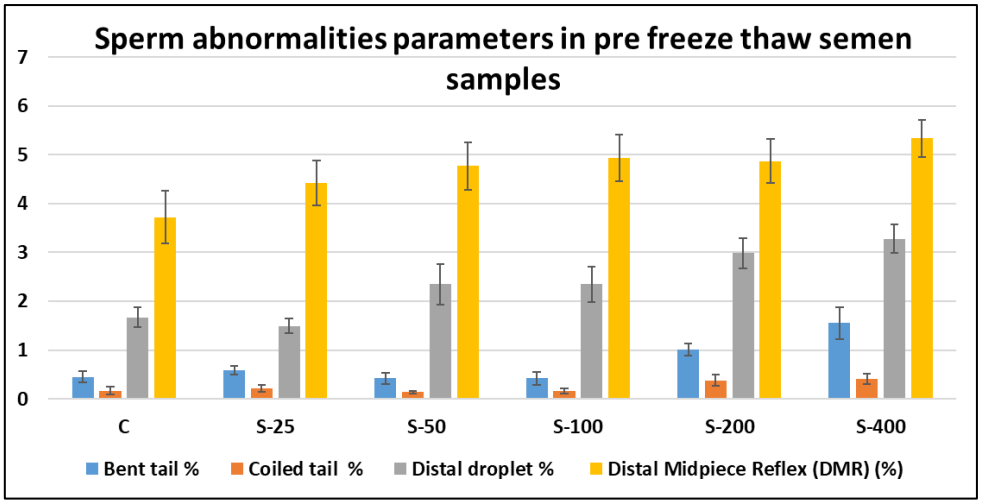

Fig 2: Sperm abnormality parameters in pre-freeze semen samples

In the pre-freezing samples, sperm motility parameters such as motile sperm (%) and progressive sperm (%) in the treatment groups (S-25, S-50, and S-100) showed no significant difference ($p>0.05$) from the control group (65.02 ± 2.13 and 62.22 ± 2.22, respectively) as shown in table 10. However, motile sperm (%) and progressive sperm (%) were decreased significantly ($p<0.05$) at higher concentrations of silymarin (S-200 and S-400) compared to the control. Further, motile sperm (%) and progressive sperm (%) were significantly ($p<0.05$) higher in S-200 (44.07 ± 3.27 and 41.53 ± 3.25) compare to S-400 (30.37 ± 3.31 and 25.11 ± 3.48). This indicates that motility parameters decreased significantly from S-200 to S-400. These results show that silymarin negatively impacts pre-freeze semen motility parameters at higher concentrations (200 and 400 µg/ml). There were no significant differences ($p>0.05$) in sperm abnormality parameters such as bent tail (%) and distal droplet (%) in the pre-freezing samples treated with silymarin (S-25, S-50, and S-100) compared to the control group (0.45 ± 0.11 and 1.67 ± 0.20, respectively). However, these sperm abnormality parameters increased significantly ($p<0.05$) at higher concentrations of silymarin (S-200 and S-400) compared to the control. Further, bent tail (%) and distal droplet (%) were lower in S-200 (1.01 ± 0.13 and 2.99 ± 0.31) compared to S-400 (1.55 ± 0.33 and 3.28 ± 0.29). In the pre-freezing samples, abnormality parameters such as coiled tail (%) and distal midpiece reflex (DMR%) did not show any significant differences ($p > 0.05$) in the groups treated with silymarin (S-25, S-50, S-100, S-200, and S-400) compared to the control group (0.17 ± 0.08 and 3.72 ± 0.54, respectively).

Discussion

Cryopreservation of bovine semen is an essential tool in artificial insemination and genetic improvement programs. However, the freeze–thaw process inevitably causes structural and functional damage to spermatozoa due to cold shock, ice crystal formation, and, notably, oxidative stress (Kumar *et al.,* 2019; Khan *et al.,* 2021; Hai *et al.,* 2024).

In recent years, the incorporation of natural antioxidants into semen extenders has gained interest as a strategy to counteract oxidative stress. In this context, silymarin, a polyphenolic flavonoid complex extracted from *Silybum Marianum* (milk thistle), has shown promise due to its strong free radical scavenging and antioxidant properties (Luangpirom et al., 2013; Surai, 2015; Heidari et al., 2023). silymarin is known to stabilize cell membranes, enhance antioxidant enzyme activity, and inhibit lipid peroxidation, which may translate to improved post-thaw sperm quality (El-Raghi et al., 2025). There is limited literature available on evaluation of effect of silymarin on sperm motility, viability, membrane integrity, acrosome integrity and antioxidant effect on post thaw semen with inconsistent findings.

In pre-freezing sample, we demonstrated only computer assisted semen analysis (CASA) parameters such as; sperm motility, sperm abnormalies, actual kinematic and relative kinematic of sperm. The motility parameters i.e. motile sperm % and progressive motile sperm % showed no significant ($P<0.05$) variation among S-25, S-50 and S-100 compare to control. However, motility parameters decreased significantly in dose dependent manner in S-200 and S-400 groups as compare to control. The findings of motility parameters of present study are in contradict with previous finding of El-Sheshtawy RI *et al.,* (2017). They studied the Impact of silymarin enriched semen extender on bull sperm preservability and revealed that sperm motility of the groups with Silymarin concentrations 180 µg/ml, 360 µg/ml and 540µg/ml after 8 day of chilling were significantly ($P<0.02$) higher than control. Also, Khorsand *et al.,* (2024) revealed that silymarin concentrations of 75 µg/mL and 100 µg/mL significantly increased progressive motility ($p < 0.05$) in stallion sperm. The exact machanism of action of

silymarin on sperm is still unknown. However, silymarin increased the expression of Bax, phosphorylated (p)-JNK and p-p38, and cleaved poly-ADP ribose polymerase, and decreased the levels of Bcl-2 and p-ERK1/2 in a concentration-dependent manner in AGS human gastric cancer cells (Kim *et al.,* 2019; Kim *et al.,* 2021; Koushki *et al.,* 2023). Moreover, ERK1/2 stimulation is downstream to protein kinase C (PKC) activation, which is also present in the human sperm tail (PKCβI and PKCϵ). ERK1/2 stimulates and p38 inhibits forward and hyperactivated motility, respectively (Almog *et al.,* 2008; Wang *et al.,* 2021; Du *et al.,* 2024). Thus indirect inhibition of ERK1/2 and stimulation of p38 proteins seems to be the reason behind decreased motility parameters in silymarin treated groups with higher concentrations.

The sperm abnormality parameters assessed by CASA i.e. bent tail (%) and distal droplet (%) in the S-25, S-50 and S-100 groups did not show any significant difference ($p>0.05$) from the control group. However, bent tail (%) and distal droplet (%) increased significantly ($P<0.05$) in dose dependent manner in S-200 and S-400 groups as compare to control. Moreover, sperm abnormality parameters such as coiled tail (%) and DMR (%) not showed significant difference among all silymarin treated groups as compare to control. To best of our knowledge, there is no study available to compare our results of sperm abnormality parameters in pre freeze semen treated with silymarin.

The sperm distance related actual kinematic parameters such as (DAP, DCL and DSL) and velocity related actual kinematic parameter such as (VAP, VCL and VSL) in the S-25, S-50 and S-100 groups high and not showed any significant difference ($p>0.05$) from the control group. However, distance and velocity related actual kinematic parameter decreased significantly ($P<0.05$) in dose dependent manner in S-200 and S-400 groups as compare to control. Moreover, actual kinematic parameters such as ALH and BCF in the S-25, S-50 and S-100 groups high and not showed any significant difference ($p>0.05$) from the control group. But ALH is significantly higher in group S-400 and BCF became lowest in the S-400 group as compare to control. The sperm relative kinematic parameters such as WOB%, LIN% and STR% in the S-25, S-50, S-100 and S-200 groups became high and not showed any significant difference ($p>0.05$) from the control group. However, WOB%, LIN% and STR% decreased significantly ($P<0.05$) in S-400 group as compare to control. To best of our knowledge, there is no study available to compare our results of actual and relative kinematic parameters of sperm in pre freeze semen treated with silymarin.

Conclusion

The supplementation of silymarin up to 100 µg/ml did not affect the sperm motility and other CASA parameters. However, at higher concentration (200 and 400 µg/ml), there was negative effect on sperm motility and other CASA parameters in pre-freeze samples.

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