**How to Cite**:
Ali, J. S., Hussain, S. O., & George, R. (2022). Influence of different concentrations of Silymarin and steps of freezing on frozen semen properties of Holstein Bulls born in Iraq. *International Journal of Health Sciences*, *6*(S3), 6767–6780.
https://doi.org/10.53730/ijhs.v6nS3.7528

# Influence of different concentrations of Silymarin and steps of freezing on frozen semen properties of Holstein Bulls born in Iraq

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***Abstract*---**To explore the effect of silymarin on bull spermatozoa during cooling and cryopreservation. Semen was collected by artificial vagina from Holstein bulls born in Iraq and diluted by Tris-Citrate-Fructose egg yolk diluents. Diluted spermatozoa were treated with silymarin (0.018 mg/50ml, 0.027 mg/50ml, 0.036 mg/50ml), and packaged in 0.25 ml straws, which were further cooled to 5°C before freezing in liquid nitrogen. Thawing was carried out at 37°C for 30 sec, and the progressive motility%, dead%, abnormality% and acrosome integrity% were assessed, concentrations of addition to the control (0.0 mg/ml) reaching a final volume of 10 ml in each tube. The results proved after cooling and freezing, Individual motility% of sperm was significantly ($p< 0.05$) higher in T1 which differed significantly ($p< 0.05$) from T2 and T3 and all these values significantly ($p< 0.05$) different from control mean while values of dead sperm% show the lowest in T1 which is significantly ($p< 0.05$) different from control after cooling while after freezing lowest value in T1 and T2 and highest in T3 and control with significant ($p<0.05$) different. Abnormal sperm% was lowest in T1 after cooling with significant different ($p< 0.05$) from T2, T3 and control. After freezing lowest value in T1 and T2 with significant ($p< 0.05$) different from control and T3. Abnormal acrosome% show no significant different between groups after cooling, but after freezing lowest values in T1 and T2 with significant difference ($p< 0.05$) from T3 and control group. Regarding the effect of cooling and freezing on semen quality of bull semen, the result show addition of silymarin act as protective factor to sperm to withstand the

detrimental effect of cooling and freezing in liquid nitrogen by preventing the significant ($p< 0.05$) decrease of sperm individual% and also prevent the significant increase of dead, abnormal and defect acrosome% in after freezing compared to after cooling.

***Keywords*---**Silymarin, Cryopreservation, semen freezing, antioxidant, sperm protective.

## Introduction

Artificial Insemination (A.I) is significant technology that has been utilized to advance livestock farming, allowing for accelerated genetic progress and selection (Mediros *et al.*, 2002) where successful semen cryopreservation improves the efficiency and success rate of AI. Sperm cryopreservation procedures are not always sufficient because a large number of sperm suffer physiological damage which leads to the loss of fertility following freezing and thawing (Baily *et al.*, 2000). Sperm cryopreservation is critical for livestock production because it enables and accelerate the spread of genetic diversity and it facilitates the distribution of genetically superior animals around the world (Nijs *et al.*, 2009).During semen cryopreservation a lot of damage occurs to sperm characteristics in which the production of reactive oxygen specie also plays a role ( Said and Agarwal., 2010). The supplementation of antioxidants in the semen extender could decrease the impact of oxidative stress and therefor improve sperm quality following freeze – thawing process (Berra and Rizzo, 2009).Natural antioxidant exert 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 herbs silymarin is extract from the seeds and fruits of milk thistle silybum marianum that contains the flavonolignans silybim which is the major active component (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 (Fakurazi *et al.*, 2008; Luangprion *et al.*, 2013). Therefore, the use of natural antioxidants could be a possible strategy for reducing oxidative stress in body. Silymarin is extracted from milk thistle (Silybum marianum) seeds (Khan *et a*l., 2009). This compound is a polyphenolic flavonoid with a potent antioxidant property which not only acts as free radical scavenger but also increases the capacity of cell antioxidant enzymes (Kohno *et al.,* 2002; Kiruthiga *et al.,* 2007 ; Soto et al., 2003). This plant antioxidant might therefore be considered as an alternative to ameliorate the toxic effect of arsenide and infertility mediated by this pollutant (Eskandari and Momeni, 2016).

Silymarin is one of the most frequently studied bioflavonoid in the class of flavonols. It is a polyphenolic flavonoid anti- oxidant isolated from the fruits and seeds of milk thistle. An active constituent of milk thistle (Silybum marianum) is basically a flavolignan, silymarin is a major active constituents among four isomeric fluonoids such as silibinin, isosilibinin, silydianin and silychristin (Kaur and Agarwal, 2007). Silymarin possesses special biological effects, such as an

antioxidative activity (Jang *et al.,* 2011). Flavonolic antioxidants such as silymarin and quercertin are reported to quench oxygen derived free radicals as well as substrate-derived free radicals by notation a hydrogen atom or an electron to free radical, protect cell constituents against oxidative damage (Fra- schi *et al.*, 2002). Silymarin has also shown a protective action against oxidative damage and revealed an iron-chelating effect and remarkable effect in inhibition of Cu2+ mediated LDL oxidation (Marco *et al.*, 2001).Although there is plenty of information on beneficial and biological properties of silymarin. In view of this and since no available literature in Iraq regarding the application of silymarin as an antioxidant supplement to semen diluent of bulls, during cryopreservation, the present project planned to study the ability of silymarin as a additive to Tris diluent at different concentrations on improving sperms capability to withstand the effect of cooling and freezing in liquid nitrogen.

**Materials and Methods**

This study was carried out at the Artificial insemination center – Abu Ghraib in Baghdad – province / Iraq on (4) Holstein bulls born and reared in Iraq. All bulls of similar age (3-4 years) , and the bulls reared under the same healthy management (feeding and watering) and healthy environment ; Semen was collected weekly from each bull by artificial vagina. After collection. The routine evaluation of fresh semen was done as described by Alam *et al.*, (2005) and Shaha *et al.*, (2008). Volume of fresh semen was recorded from the graduated mark of the semen collecting tube. Mass activity was evaluated by placing a drop (25µl) of semen onto a pre-warmed (+37℃) glass slide without cover slip, sperm motility was assessed by observing motion under 10× magnifications using (100× optical systems) ,sperm concentration $x10^6$/ ml (set by using photometer system used in A.I center). Determination of sperm individual motility; single drop of diluted fresh semen on pre warmed glass slide was taken and cover slip was placed over it. The individual motility percentage of sperm was visualized with a compound microscope via 400× at 37°C (Qureshi, 2011). Under microscopy, 100 spermatozoa were counted at five different fields. All those sperms, which showed any type of movement, were considered as motile spermatozoa while those sperm, which move in straight direction, were considered as progressive motile.

Dead sperm% (Blom, 1950), abnormal sperm % (Evans and Maxwell, 1987) and acrosome integrity% (Kovács and Foote 1992) and the method for discrimination as clarified according to (Kumar *et al.,* 2018). Semen samples was diluted by tris which used by center consist from [ Tris 2.24 gr ; citric acid 1.25 gr ; fructose 1gr ; glycertol 7ml% ; egg-yolk and antibiotic 20 ml%], which divided in to four parts. Control groups ( Tris ), T1 Tris + 0.018 mg/50mL Silymarin, T2 Tris + 0.027 mg/50 mL Silymarin, T3 Tris + 0.036mg/50 mL Silymarin, all diluted samples exposed to cooling at 5 °C for ( 2 ) hours and Physical evaluation ( after cooling ) included individual motility% , dead sperms %, abnormal sperms% and Acrosome integrity% .During the last end of cooling period all samples packing into straws 0.25 ml and freezing at -196 °C in liquid nitrogen for ( 48 hrs. ) and thawing all frozen semen in thawing rate (37 $C^0$ for 30 sec). Physical evaluation ( post-thawing) included individual motility% , dead sperms% , abnormal sperms% and Acrosome integrity%.

**Statistical analysis**

Statistical analysis of data was performed using SAS (Statistical Analysis System – version 9.1). Tow – way ANOVA and least significantly differences (LSD) post hoc test were performed to assess significantly differences among means.. $p<0.05$ is considered statistically significant.

**Results**

**Influence of different concentration of silymarin on sperms properties oF Holstein bulls born in Iraq**

in (Table:1), Highest sperm individual motility% was attained in T1 which differed significantly ($p<0.05$) from T2 and T3 and all these values differed significantly ($p< 0.05$) from control in after cooling and freezing. Steps of freezing affect significantly ($p< 0.05$) control group only with significant decrease of individual motility.

Table 1
Effect of different concentrations of Silymarin on individual motility % of sperms for Holstein bulls born in Iraq during different steps of freezing (Mean ± SE)

| Physical Characteristic | concentration of Silymarin mg/50ml | Steps of freezing | |
|---|---|---|---|
| | | After cooling | Post-thawing |
| Individual motility % | T Control | 52.40 ± 1.19 A c | 43.20 ± 0.81 B c |
| | T1 = 0.018 | 59.20 ± 0.75 A a | 57.40 ± 0.82 A a |
| | T2 = 0.027 | 56.00 ± 0.81 A b | 54.40 ± 0.65 A b |
| | T3 = 0.036 | 55.00 ± 1.08 A b | 53.60 ± 0.83 A b |

**LSD 2.46**

Within column different small letters for each parameter differed significantly ($P< 0.05$). Within row different capital letters for each parameter differed significantly ($P< 0.05$). in (Table:2), Lowest dead sperm% was proved T1 which differed significantly ($p< 0.05$) from control group after cooling mean while after freezing lowest value in T1 and T2 and highest in T3 and control group, steps of freezing affect significantly ($p< 0.05$) in control and T3 groups.

Table 2
Effect of different concentrations of silymarin on Dead % of sperms for Holstein bulls born in Iraq during different steps of freezing. (Mean ± SE)

| Physical Characteristic | concentration of silymarin mg/50ml | Steps of freezing | |
|---|---|---|---|
| | | After cooling | Post-thawing |
| | Control | 10.88 ± 1.08 | 31.86 ± 2.18 |

| | | | |
|---|---|---|---|
| Dead Sperm % | | B a | A a |
| | T1 = 0.018 | 6.20 ± 0.55<br>A b | 8.54 ± 1.05<br>A c |
| | T2 = 0.027 | 8.16 ± 0.72<br>A ab | 10.38 ± 0.96<br>A c |
| | T3 = 0.036 | 8.88 ± 0.78<br>B ab | 18.48 ± 1.06<br>A b |

**LSD 3.20**

Within column different small letters for each parameter differed significantly (P< 0.05). Within row different capital letters for each parameter differed significantly (P< 0.05). in (Table:3), Lowest abnormal sperm% was found in T1 after cooling which is significantly (p< 0.05) different from T2, T3 and control. While after freezing lowest value in T1 and T2 and highest in T3 and control with significant different (p< 0.05). Steps of freezing increased significantly the values in T3 and control.

Table 3
Effect of different concentrations of silymarin on Abnormality % of sperms for Holstein bulls born in Iraq during different steps of freezing. (Mean ±SE)

| Physical Characteristic | concentration of Silymarin mg/50ml | Steps of freezing | |
|---|---|---|---|
| | | After cooling | Post-thawing |
| Sperm Abnormalities % | Control | 5.90 ± 0.39<br>B a | 18.72 ± 0.37<br>A a |
| | T1 = 0.018 | 3.80 ± 0.23<br>A c | 4.08 ± 0.55<br>A b |
| | T2 = 0.027 | 4.0 ± 0.33<br>A b | 4.10 ± 0.5<br>A b |
| | T3 = 0.036 | 5.12 ± 0.27<br>B ab | 17.50 ± 0.56<br>A a |

**LSD 1.19**

Within column different small letters for each parameter differed significantly (P< 0.05). Within row different capital letters for each parameter differed significantly (P< 0.05). Within (Table:4), After cooling, there is no significant (p< 0.05) different between control and other groups, regarding the value of in abnormal acrosome% after freezing highest value was in control which differed significantly (p< 0.05) from other groups and lowest value in T1 and T2. The steps of freezing increased significantly the values of acrosomal defect in T3 and control group.

Table 4
Effect of different concentrations of silymarin on Abnormal acrosome% of sperms for Holstein bulls born in Iraq during different steps of freezing (Mean ±SE)

| Physical Characteristic | concentration of Silymarin mg/50ml | Steps of freezing | |
|---|---|---|---|
| | | After cooling | Post-thawing |
| Acrosomal defect % | Control | 3.42 ± 0.26 B a | 8.42 ± 0.66 A a |
| | T1 = 0.018 | 2.34 ± 0.22 A a | 3.42 ± 0.32 A c |
| | T2 = 0.027 | 2.72 ± 0.22 A a | 3.74 ± 0.50 A c |
| | T3 = 0.036 | 2.86 ± 0.23 B a | 6.98 ± 0.57 A b |

**LSD 1.14**

Within column different small letters for each parameter differed significantly (P< 0.05). Within row different capital letters for each parameter differed significantly (P< 0.05).

**Discussion**

The result of influence of different concentration of Silymarin on sperms properties of Holstein bulls born in Iraq, are proved because Silymarin, which has a potent antioxidative feature, is a flavonoid isolated from the fruits and seeds of the milk thistle (S. marianum) (Fraschini *et al.*, 2002; Zeisel, 2004). In addition, several recent studies have shown the potential hepatopre- ventive and therapeutic efficacy of Silymarin in different animal models and cell culture systems (Kaur and Agarwal, 2007; Polyak *et al.*, 2010; Wagoner *et al.*, 2010). It's positive effects have been ascribed to the putative anti-oxidative, anti-inflammatory and anti-proliferative properties based on the modulation of specific signaling pathways, transcription factors and gene expression (Katiyar *et al.*, 2005). Silymarin efficiently prevents mitochondrial ROS formation under basal conditions indicating that as well as at early time points upon secondary oxidative stress induced by $H_2O_2$ exposure. However, the exact mechanism of silymarin effects on mitochondrial function, motility and membrane integrity remains unclear. However, these effects may be related to its ant oxidative properties (Jang *et al.,* 2011).

Cryopreservation causes wide-ranging physical, chemical and mechanical injures to sperm membranes of all mammalian species (Fakurazi *et al.*, 2008). Which are attributed to temperature changes, alterations in the transition from the lipid phase, production of reactive oxygen species (ROS) and osmotic stress (Câmara *et al.*, 2011 ; El-Sheshtawy *et al.*, 2014). Moreover, the over accumulation of ROS causes a state of oxidative stress that involves structural damage of sperm membranes, fall of intracellular ATP levels causing decreasing in the viability and motility of cryopreserved sperm (Ortega *et al.*, 2009; Baumber *et al* ., 2000). To decrease the harmful effects of ROS, seminal plasma possesses powerful source of

ROS scavengers which offer protection for sperm, including enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and small molecular antioxidants such as ascorbic acid and a-tocopherol(Agarwal *et al* ., 2005 ; Aitken *et al.*, 2004). The herbal remedies contain antioxidants to counteract the deleterious action of reactive oxygen species (ROS). Sperm motility was kept high in chilled semen at the concentrations of silymarin 0.018, 0.027, 0.036 mg/50mL during cooling stage before freezing samples. This indicates that chilled extended semen with these concentrations could be used in A.I.

Addition of silymarin maintain post thawing alive%, and prevent the significant ($p < 0.05$) rise of sperm abnormalities, percent of post thawing abnormal acrosome and maintained the percent of sperm motility. The improved semen quality in both cooling and frozen semen is due to the strong antioxidant capacity of silymarin (Luangpirom *et al.*,2008 ; Luangpirom *et al* .,2013). Wellington and Jarvis**,** (2001) postulated that the mechanism of action of silymarin is through the stimulation of ribosomal RNA protecting the cell membrane from oxidative damage. They stated also that silymarin stimulates the activity of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase. It could be concluded that silymarin as a natural additive to semen extenders improved preservability in both chilled and frozen bull semen. However, the success of AI largely depends on the efficiency of *in vitro* semen storage protocols in maintaining spermatozoa quality (Lone *et al.*, 2017; Skidmore *et al.*, 2018). Semen is preserved in an extender containing Tris, glycerol, glucose, citric acid, water, egg yolk, and antibiotics at refrigerated temperatures for several days during liquid storage (Al-Bulushi *et al.*, 2019) and at −196 °C for several months or even years during cryopreservation (Gangwar *et al.*, 2018 ; Prell *et al.*, 2020). Typically, seminal plasma contains both enzymatic and non-enzymatic antioxidants; however, their protective actions against oxidative stress are significantly weakened following semen dilution in the extender prior to storage (Peris-Frau *et al.*, 2020).

Furthermore, high polyunsaturated fatty acid levels render the spermatozoon plasma membrane highly susceptible to oxidative stress and particularly to lipid peroxidation by reactive oxygen species (ROS) (Özer Kaya *et al.*, 2018), due to the imbalance between ROS levels and natural antioxidant activity of sperm (Hamilton *et al.*, 2016). The detrimental effects of ROS on spermatozoa decrease sperm viability and motility, impair fertilization, reduce implantation and pregnancy, minimize the cleavage rate, lower embryo quality, and inhibit blastocyst formation (Selvaraju *et al.*, 2008 ; Osman *et al.*, 2015 and Simon *et al.*, 2017). Consequently, the use of exogenous antioxidants to maintain ROS balance and protect spermatozoa from oxidative damage during preservation has recently garnered increased interest (Souza *et al.*, 2019 ; Al-Mutary *et al.*, 2020).During cryopreservation, any changes in mitochondrial membrane fluidity may result in the release of ROS and changes in the membrane potential (Said *et al* ., 2010). Hydrogen peroxide ($H_2O_2$), nitric oxide (NO), and superoxide anion ($O2–$) have positive effects on intracellular signaling, sperm capacitation, and acrosome reactions (Medeiros *et al* ., 2002). Although at the appropriate levels of these molecules play a significant role in sperm physiology, namely capacitation and acrosome reaction, they are detrimental to sperm function at high concentrations due to toxicity. The exact mechanism of ROS generation and function have not

been fully characterized in sperm (agarwal et al.,2005). However, it is known that these molecules are products of incomplete reduction of molecular oxygen, and the toxicity is associated with protein inactivation due to ionization, lipid peroxidation, and DNA damage. The improvement in semen extenders and cryopreservation techniques has significantly reduced the harmful effects of cryopreservation. However, cryopreservation still causes sperm damage in humans and various animals (deMenezes *et al.*, 2016 ; Makarova *et al.*, 2018).

Concerning the effect of steps of freezing (cooling and post-Thawing) on some semen properties of Holstein bulls born in Iraq, occur during cryopreservation, any changes in mitochondrial membrane fluidity may result in the release of ROS and changes in the membrane potential (Said *et al.*, 2010). Hydrogen peroxide ($H_2O_2$), nitric oxide (NO), and superoxide anion ($O_2^-$) have positive effects on intracellular signaling, sperm capacitation, and acrosome reactions (Medeiros *et al.*, 2002). The improvement in semen extenders and cryopreservation techniques has significantly reduced the harmful effects of cryopreservation. However, cryopreservation still causes sperm damage in humans and various animals (deMenezes *et al.*, 2016 ; Makarova *et al.*, 2018). There are statistically significant differences in the quality of bull semen before and after cryopreservation. Cooling and freezing of spermatozoa during process of cryopreservation causes reduction of temperature of the spermatozoa and its surrounding solution. This change in temperature induces sperm plasma membrane damage which leads to a state of sperm abnormal morphology such as abnormal acrosome (Khumran *et al.*, 2015) The damaged sperm plasma membrane allows influx of calcium ions which resulted in abnormally high amount of intracellular calcium ions in the spermatozoa. This phenomenon contributes to the premature capacitation of spermatozoa (Yimer et al., 2015).This individual differences particularly for bull semen have been recorded and to cover this difference, freezing protocols were adjusted for individual bulls or packaging straws with more spermatozoa for “poor freezers”. The effect of different temperature to the quality of spermatozoa may be varies for different individuals from the same species. Therefore, the accuracy of result may be reduced due to this factor (Al-Fatlawy *et al.*, 2020). The results in present study were included in table (1, 2, 3 and 4), and shows that cooling and post-thawing effect significantly ($P< 0.05$) on sperms properties in control group included individual motility %, dead%, abnormalities% , acrosome defects% in comparison with treated groups (T1**,** T2 and T3) these are show that steps of freezing effected significantly on characteristics of sperms ,this result is comparable with (Al-Badrany *et al.*, 2017). The fact that progressive motility is more affected by the freezing process implies that these parameters measure different aspects of cell physiology and in particular, that the physiological basis for the progressive motility parameter is more sensitive to cryobiological damage (Anel *et al.,* 2003). During cryopreservation spermatozoal mitochondria undergo damages (Gillan *et al.*, 2004 ; Peris *et al.*, 2004).

Üstuner *et al.*, (2015) explain that the freezing process negatively affects ($P< 0.05$) the sperm parameters (individual motility, dead and abnormality), agreement with Hussain *et al.*, 2016) reported that significant decrease in individual motility and increase in dead and abnormalities percentage for both poor and good ejaculate during different steps, dilution, cooling and freezing of bull semen, this might be attributed to the fact that lactic acid which produced as

an end product of sperm metabolism, resulting in harmful lowering of PH which exerts toxic effect on sperm cell (Ball and Peter, 2004). The challenge during cryopreservation is not sperm cells' ability to resist the storage period in liquid nitrogen but crossing the intermediate temperature zone between −15°C and −60°C. The cells must go through these temperatures twice, first during cooling and then thawing, causing injurious effects on the integrity of the sperm plasma membrane, acrosome, and nucleus; mitochondrial function; and sperm motility (Medina-Robles *et al.*, (2020) and Buranaamnuay *et al.*, (2009).

The considerably reduced values for sperm motility, viability, morphology, and plasma membrane/acrosome integrity observed after cryopreservation of semen over fresh or pre-freeze stage (Chaudhari et *al.*, 2015), but when referring to the results shown in the tables above, it is clear that the studied characteristics, whether in Tris diluent control or addition different concentration of silymarin, are more deteriorated in post thawing compared to after cooling, but this effect is less when adding 0.018mg/ml and 0.027mg/50ml silymarin it had a role in protecting sperms from effect of freezing, where it was observed that there was no significant decrease in the individual motility of sperm% (Table 1) in the three different concentration of silymarin and it also appeared that the attributes were mention above as dead% (Table 2) abnormal sperm% (Table 3) ,acrosome integrity % (Table 4), were less affected by freezing compared to 0.036 mg/50ml and control. The results of the current study regarding this aspect summarized that addition silymarin to Tris diluent especially concentration 0.018mg/ml improve freezability of bull sperms .Successful sperm storage (liquid and frozen) requires slowing of the cell metabolism and thereby prolongs viability (Gibb and Aitken, 2016). and the concentration of the antioxidants present in the semen can be reduced by dilution, and as a result decreasing the beneficial effect of this endogenous antioxidative defense. Thus, the addition of antioxidants, even in small concentrations, can improve sperm function during preservation.

Frozen semen production consisted of a series of the process including semen dilution in extender, cold storage and post-thawing evaluation of sperm sample is routinely conducted to perform artificial insemination all these procedures (cryopreservation) produce reactive oxygen species (ROS) or free radicals in sperm samples (Saraswat *et al.*, 2016)so during cryopreservation, semen is exposed to cold shock and atmospheric oxygen, which increases their susceptibility to lipid peroxidation (LPO) and moreover excessive production of these reactive oxygen species (ROS), impairs the motility and fertilization capacity (Saraswat *et al.*, 2013). Increase ROS production by weakening spermatozoa can have detrimental effect on sperm function (Baumber *et al.*, 2000) Moreover, freeze-thaw cycle reduces the level of antioxidant in mammalian semen (Stradaioli *et al.*, 2007). It can be concluded from the present study that silymarin as a natural additive to semen extenders improved preservability of frozen bull semen, and this additive can decrease the negative effect of freezing in liquid nitrogen.

**Conflict of interest statement**

Conflict of interest statement Authors declare that there is no conflict of interest statement.

**Acknowledgements**

Author thanks all staff member of Artificial Insemination Center mainly the head of department Dr. Riyath George and Dr. Omar Adel Mohammed for their cooperation and afferent me indefinite facilities along experimental period in order to be performed conveniently.

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