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Comparative study between silymarin (Silybum Marianum), melatonin and a combination of them for improving frozen sperm properties of Holstein Bulls Born in Iraq

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***Abstract*---**This study aims to identify whether adding silymarin and melatonin separately or together to the tris diluent in maintaining the fertile viability of the sperm after cooling and freezing in liquid nitrogen. Semen was collected by artificial vagina from Holstein bulls born in Iraq and diluted by Tris-Citrate-Fructose egg yolk diluents. Diluted spermatozoa was treated with tris diluent without any addition (control), silymarin 0.018gm/50ml tris (T1), combination of silymarin and melatonin (T2) and melatonin 0.05gm/100ml tris alone (T3), and packaged in 0.25 ml straws, which were further cooled to 5°C before freezing in liquid nitrogen, and the progressive motility%, dead sperm%, abnormality% and acrosome defects% were assessed after cooling and freezing. Thawing was carried out at 37°C for 30 sec. The results indicate that the addition of silymarin and melatonin had a role in improving and maintaining the sperm properties whether after cooling or freezing, but silymarin was better than melatonin alone or combination of them in reducing sperm damage after cooling and freezing. The results also showed that freezing had a negative effect on the sperm properties, but it was less or no effect of the diluent contain silymarin alone. Hence, the overall results of the present study confirmed that 0.018mg silymarin supplementation to the semen extender can improve the motility and viability of spermatozoa and reduce the number of apoptotic sperm in the frozen-

thawed semen of bulls which used in artificial insemination center in Iraq.

***Keywords*---**silymarin and melatonin, frozen semen, antioxidants, bulls born in Iraq.

Introduction

Good quality of cryopreserved semen is required to get positive artificial insemination outcomes (Oliveira *et al.*, 2012). However, the quality of semen can be compromised by various factors such as cryopreservation process (Rasul *et al.*, 2001; Bailey *et al.*, 2003; Khan *et al.*, 2009), types of extender used (Crespilho *et al.*, 2012; Harahap et al., 2020). ROS causes lipid peroxidation on the sperm plasma membrane (da Silva Maia *et al.*, 2010; AL-Badry *et al.*, 2016), impairing membrane fluidity and the activity of membrane enzymes and ion channels and therefore resulting in poor sperm motility and fertility(Pons-Rejraji *et al.*, 2009). One of the main causes of increased apoptotic spermatozoa is excessive ROS and oxidative stress (Carmody and Cotter, 2001; Wang, 2003). Moreover, the indigenous antioxidants are dramatically reduced during freeze-thaw cycle (Bilodeau *et al.*, 2000). interacting with antioxidant enzymes (Deng *et al.*, 2017).

The antioxidants are compounds that could decrease the harmful effects of oxidative stress on spermatozoa during the sperm preservation process (Wafa *et al.*, 2021). Melatonin has been used as an antioxidant in the semen of different mammals for the improvement of the post-thawed semen quality (Succu et al., 2011; Ashrafi *et al.*, 2013; El-Raey *et al.*, 2015; Karimfar *et al.*, 2015). Natural antioxidants exert a protective effect preserving the metabolic activity and cellular viability of cryopreserved bovine spermatozoa (Câmara *et al.*, 2011). Alevra et al., (2022) conclude that the use of moderate concentrations of melatonin are beneficial to semen preservation, and the mechanisms through which melatonin acts positively on spermatozoa need to be further investigated to establish improvement protocols for cryopreservation in fish species. Nowadays, the use of herbal natural product has gained interest worldwide. Many of the herbs have been developed into herbal supplement which are claimed to assist in healthy life style. Among these herbs Silymarin is an extract from the seeds and fruits of the milk thistle silybum marianum that contains the flavonolignans silybin which is the major active component. Silymarin is a strong antioxidant used as a remedy for liver protection against oxidative stress and also as a protectant for the testicular tissue and improving semen quality through elevation of blood testosterone level (Fakurazi *et al.*, 2008; Luangpirom *et al.*, 2013).

Also, it prevents acetaminophen induced liver injury through restoration of glutathione level (Câmara *et al.*, 2011). 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 thiethele (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). Flavonolic antioxi- dants such as silymarin is 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). Besides its several well-known physiologic roles, melatonin has a significant antioxidant potential through direct free radical scavenging properties. It may also exert indirect stimulatory effects on several antioxidant enzymes such as glutathione peroxidase/reeducates, catalase, and superoxide dismutase (Bhattacharya *et al.*, 2019). The protective effects of melatonin supplementation of the cryopreservation media on the porcine, bovine, ovine, equine and, human frozen-thawed spermatozoa have been previously reported in several independent studies (Medrano *et al.*, 2017).

The protective effects of melatonin against oxidative stress has also been reported for human (Du Plessis *et al.*, 2010), boar (Jang *et al.*, 2010), buffalo (Li *et al.*, 2012), rat (Rao and Gangadharan, 2008), and for mouse (Sarabia *et al.*, 2011) sperm. In recent studies on mammals, the supplementation of freezing extenders with melatonin improved the post-thaw sperm functions of ram (Ashrafi *et al.*, 2011; Succu *et al.*, 2011), bull (Perumal *et al.*, 2013), buffalo (El-Raey *et al.*, 2014), stallion (Izadpanah *et al.*, 2015), and of human (Karimfar *et al.*, 2015) sperm. Melatonin (N – acetyle – 5 – methoxy tryptamine) is an indole derivative endogenous compound that is secreted rhythmically by the pineal gland and plays a major role in regulating circadian clock and seasonal reproduction in mammals. Also, melatonin as well as its metabolites has indirect antioxidant action with powerful direct scavenging ability of free radicals (Kapadiya *et al.*, 2016). Several studies in world and in Iraq has been the application of silymarin and melatonin as an antioxidant supplement to semen diluent of bulls for improving sperm viability, but no found any study to compare between the best concentration of them or add together of these concentrate for this purpose the present study aims to find out which is better silymarin, melatonin or add them together best.

Materials and Methods

This study was carried out at the Artificial insemination center – Abu-Ghraib in Baghdad – province / Iraq on (3) 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). Determination of sperm individual motility (Qureshi, 2011), dead sperm % (Blom, 1950), abnormal sperm % (Evans and Maxwell, 1987) and acrosome integrity% (Kovács and Foote (1992) was clarified according to (Kumar *et al* 2018). Semen samples was diluted in rate 1:15 Tris diluent the same which used by A.I center –Iraq and divided in to (4) parts: Tris control, Tris + 0.018 gm/50ml Silymarin (T1), Tris + 0.018 gm/50 ml Silymarin +0.05 gm/100ml melatonin (T2), Tris + 0.05gm/100 mL Melatonin (T3), all samples cooling the diluted samples at 5 °C for (2) hours and Physical evaluation (after cooling) including 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 (37C^{o} for 30sec). Physical evaluation (post-thawing)

including 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). Two – way ANOVA and least least significantly differences (LSD) post hoc test were performed to assess significantly differences among means.. $p< 0.05$ is considered statistically significant.

Result

Table 1: Result after cooling proved
Highest sperm progressive motility was recorded in T1 which differed significantly ($P < 0.05$) from T3 and control group which attained the lowest values . Meanwhile similar trend was achieved after freezing. Steps of freezing (after cooling and freezing) has no significant effect in T1, T2 and T3 but the significant ($P < 0.05$) decrease in individual motility depicted in control group only.

Table 1
Effect of silymarin, silymarin in combination with melatonin hormone and melatonin alone on individual motility of bull sperms after cooling and freezing

Physical Characteristic	Treatment	After Cooling	After Freezing
Individual motility %	Control	53.80± 0.83 A C	44.20 ± 0.85 B c
	T1 Silymarin	60.60 ± 0.72 A a	58.80 ± 0.71 A a
	T2 Silymarin and melatonin	58.00 ± 0.64 A ab	56.20 ± 0.70 A ab
	T3 melatonin	56.20 ± 0.59 A b	54.40 ± 0.78 A b
LSD	2.94		

Means with a different small letters in the same column are significantly ($P < 0.05$) different . Means with a different capital letters in the same row are significantly ($P < 0.05$) different .

Table 2: Lowest dead sperm percentage was observed in T1, T2 and T3 and all these values differed significantly ($P< 0.05$) from control group after cooling and freezing. Freezing process has no significant effect in T1, T2 and T3, in contrast only there is an increase in dead sperm percentage in control group with significant ($P < 0.05$) difference between after cooling and after freezing.

Table 2
Impact of silymovin , silymarin in combination with melatonin hormone and melatonin alone on dead sperm % of bulls after cooling and freezing

Physical Characteristic	Treatment	After Cooling	After Freezing

Dead Sperm %	Control	9.62 ± 1.03 B a	33.06 ± 2.12 A a
	T1 Silymarin	4.66 ± 0.46 A b	6.00 ± 0.98 A b
	T2 Silymarin and melatonin	6.24 ± 0.54 A b	8.64 ± 0.98 A b
	T3 melatonin	6.50 ± 0.71 A b	8.84 ± 1.00 A b
LSD	3.04		

Means with a different small letters in the same column are significantly (P < 0.05) different Means with a different capital letters in the same row are significantly (P < 0.05) different .

Table 3: This table indicated no significant (P < 0.05) different between T1, T2 and T3 in abnormal sperm% which recorded the lowest values. but there is significant (P< 0.05) difference with the control group only which indicated the highest value after cooling. After freezing, the same trend proved with highest value in control group and T3 and lowest in T1 with significant (P < 0.05) difference. Steps of freezing increased significantly (P < 0.05) the abnormalities% in control group only.

Table 3
Effectiveness of silymarin, silymarin combined. with melatonin and melatonin hormone alone on abnormal sperm% of bull after cooling and freezing

Physical Characteristic	Treatment	After Cooling	After Freezing
Sperm abnormalities%	Control	6.16 ± 0.33 B a	19.98 ± 0.47 A a
	T1 Silymarin	3.74 ± 0.21 A b	4.62 ± 0.46 A c
	T2 Silymarin and melatonin	4.76 ± 0.33 A b	5.06 ± 0.57 A bc
	T3 melatonin	4.84 ± 0.33 A b	6.01 ± 0.59 A b
LSD	1.20		

Means with a different small letters in the same column are significantly (P < 0.05) different . Means with a different capital letters in the same row are significantly (P < 0.05) different .

Table 4: Lowest value in T1 and T2 which differed significantly (P < 0.05) from control which indicated highest value in abnormal acrosome % (after cooling) , while after freezing no a significant (P < 0.05) differences between T1, T2 and T3 which recorded lowest value , but highest value in control group with significant (P < 0.05) different with other treated groups . Steps of freezing increased significantly (P < 0.05) the acrosomal defect % in control group only.

Table 4
Influence of silymarin, silymarin combination with melatonin and melatonin hormone alone on defected acrosome% of bull sperms after cooling and freezing

Physical Characteristic	Treatment	After Cooling	After Freezing
Acrosome defect %	Control	3.44 ± 0.14 B a	8.72 ± 0.60 A a
	T1 Silymarin	2.44 ± 0.13 A b	3.22 ± 0.28 A b
	T2 Silymarin and melatonin	2.88 ± 0.13 A b	3.54 ± 0.27 A b
	T3 melatonin	3.02 ± 0.14 A ab	3.94 ± 0.29 A b
LSD	0.81		

Means with a different small letters in the same column are significantly ($P < 0.05$) different. Means with a different capital letters in the same row are significantly ($P < 0,05$) different.

Discussion

Influence of silymarin, Melatonin and combination of them on sperms properties of Holstein bulls born in Iraq. The results of this study indicate that the motility, viability and membrane integrity of bull sperm exposed to silymarin alone improved post thawing alive% and was significantly higher than control and melatonin. Silymarin is an extract from the seeds and fruits of the milk thistle Silybum marianum which is a strong antioxidant (Luangpirom *et al.*, 2013). El-Sheshtawy and El-Nattat (2017) found that silymarin improved sperm preservability in frozen bull semen. 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). In addition, several recent studies have shown the potential hepatopreventive and therapeutic efficacy of silymarin in different animal models and cell culture systems (Kaur and Agarwal, 2007). The improved semen quality in both cooling and frozen semen is due to the strong antioxidant capacity of silymarin (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. In the present study also revealed that the motility parameters of spermatozoa, dead, abnormality and acrosome defect were improved when the semen extender was supplemented with melatonin.

These findings are in accordance with other studies with melatonin supplementation in bull semen which found by Chaudhary *et al* (2021) summarized different studies such as (Ashrafi *et al.*, 2013; Kapadiya *et al.*, 2016;ChaithraShree *et al.*, 2019), buffalo bull semen (El-Raey *et al.*, 2014; El-Raey *et al.*, 2015; Abdel-Khalek *et al.*, 2016), mithun semen (Perumal *et al.*, 2013; Perumal *et al.*, 2016; Perumal *et al.*, 2018), ram semen (Ashrafi *et al.*, 2011; Succu *et al.*, 2011), and human semen (Karimfar *et al.*, 2015; Deng *et al.*, (2017) to improve the semen motility. These positive contributions for sperm motility

offered by melatonin have been explained by different authors in different ways. According to Succu *et al* (2011), Ashrafi *et al* (2013), Karimfar *et al* (2015), Chen *et al* (2016) and Deng *et al* (2017) melatonin supplementation, by decreasing the ROS level and the lipid peroxidation, avoid the oxidative damage to the spermatozoa membrane and maintain the sperm motility. According to El-Raey *et al* (2015), melatonin can preserve and maintain the ultrastructure integrity of the plasma membrane and mitochondrial arrangement of the cryopreserved sperm so that the sperm are less susceptible to the cryo-injury, and thus the motility can be maintained. Melatonin has also shown the positive effects on the antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione, by increasing their expression to improve the total antioxidant capacity (El-Raey *et al.*, 2014; Deng *et al.*, 2017) resisting the oxidative damage.

Several previous studies have reported an increase in the spermatozoa reactive oxidative species content concurrent with a decrease in antioxidant capacity (oxidative stress) during long-term cold storage in different animal species (Triwulanningsih *et al.*, 2008). Therefore, supplementation of antioxidants could ameliorate sperm oxidative stress and improve sperm quality measures during cold storage (Triwulanningsih *et al.*, 2008). This antioxidant alters the available free radicals to molecules that have less negative impacts on the cell. Its addition to semen diluents is expected to decrease or prevent the emergence of free radicals that can ruin the plasma membranes (Triwulanningsih *et al.*, 2008). Sperm cells are very susceptible to lipid peroxidation by free radicals such as hydrogen peroxide, superoxide anion, and hydroxyl radical which could later lead to the structural damage of sperm membranes during the aerobic storage of sperm (Sinha *et al.*, 1996). Cold shock during semen preservation poses stress on the sperm cell membrane via the free radicals leading to sperm damage (Sanocka, and Kurpisz., 2004; Thuwanut *et al.*, 2011).

The mechanism by which the free radicals do their damage was ascribed to their ability to catch the electrons from the nucleic acids, lipids and proteins causing the cell damage(Flora, 2009). Much work has been done to protect the integrity of the sperm cells of different species from physical and chemical damage during processing and preservation. During the cryopreservation process, with the cellular respiration of spermatozoa, a great amount of reactive oxygen species (ROS) was produce resulting in a decrease in endogenous antioxidants levels (O'Flaherty, 2014). 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 (H2O2), nitric oxide (NO), and superoxide anion (O2–) have positive effects on intracellular signaling, sperm capacitation, and acrosome reactions (Medeiros *et al.*, 2002).

Effect steps of freezing on some semen properties of Holstein bulls born in Iraq

Zamiri, 2020, summarized that low temperature, especially sub-zero one, imparts physical and chemical changes in the cell membrane resulting in damages in

sperm functionality or even death. These damages are due to oxidative stress, and cold and osmotic shocks imparted on the sperm during cooling, freezing, and thawing procedures (Aitkin, 2020). As a result of these changes, sperm motility and fertility will be reduced (Salamon and Maxwell, 2000; Aisen *et al.*, 2005; Aitkin, 2020). Despite many years of research, the quality of cold or frozen spermatozoa has not improved as desired. During cold storage, reasonable sperm quality can be obtained only for few days, and cryopreservation in liquid nitrogen or on dry-ice induces substantial damage and death of spermatozoa in many animals. Many endogenous and exogenous factors impact on the quality of cryopreserved spermatozoa, including the species, breed, individual variations, seasonal variations in the quality of sperm, sensitivity of the acrosome to temperature, and intracellular ice crystal formation (La Falci *et al.*, 2002).

Low temperature, especially sub-zero one, imparts physical and chemical changes in the cell membrane resulting in damages in sperm functionality or even death (Zamiri, 2020). Oxidative stress is one of the most important factors contributing to poor post-thaw semen quality during sperm cryopreservation (Bucak *et al.*, 2010). Oxidative stress arises due to an imbalance between the production of ROS and a biological system's ability to detoxify it that is, when oxidants exceed antioxidants (Du Plessis *et al.*, 2008). It is often associated with an increased rate of cellular damage induced by oxygen (O2) and oxygen derived oxidants (Sikka, 1996). ROS broadly is represented by radicals such as the hydroxyl ion, superoxide, nitric oxide, peroxyl ion, and nonradicals like peroxynitrate, ozone, nascent oxygen, and hydrogen peroxide (Bansal and Bilaspuri, 2011).

Two ROS-generating systems are possibly involved, that is, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase at the level of sperm membrane (O'flaherty *et al.*, 2003), and sperm diaphorase (mitochondrial nicotinamide adenine dinucleotide–hydrogen, NADH-dependent oxidoreductase) at the level of mitochondria (Koppers *et al.*, 2008). In bovine semen, ROS are also generated by immature, dead, and defective spermatozoa through an aromatic amino acid oxidase pathway (Sarıozkan *et al.*, 2009). Leucocytes and radiation exposure contribute in a minor way to the formation of ROS (Agarwal *et al.*, 2008). Kumar *et al.*, 2019 explain that freeze–thaw stress enhances the sperm respiration or oxidative metabolism and excess formed electrons instead of completing the whole series of the electron transport chain in mitochondria, directly leak through mitochondrial inner membrane, and bind with oxygen resulting in the formation of free-radical superoxide (highly reactive molecule). Although ROS formation takes place during the whole cryopreservation procedure, excessive production of ROS during the freeze–thaw process mainly inflicts oxidative damage.

Due to their highly reactive nature, they can combine readily with other molecules, directly causing oxidation that can lead to structural and functional changes and finally cellular damage (Guerin *et al.*, 2001). All cellular components, including lipids, proteins, nucleic acids, and sugars are potential targets of oxidative stress (Agarwal *et al.*, 2008). ROS produced during the freeze–thaw process cause lipid hydroperoxides (4-hydroxynonenal, 4- HNE and various 2-alkenals), which have damaging effects to the membrane system (Bansal and Bilaspuri, 2011). They decrease membrane fluidity, electrical resistance, and change the phase properties of membranes. Peroxidative attack on

polyunsaturated fatty acids causes both structural and functional damage that is, the membrane becomes fragile and its semipermeable property is lost leading to membrane leaching. Cholesterol efflux, calcium influx, capacitation, and acrosomal reaction-like changes cumulatively depict cryocapacitation, which reduces viability and sperm fertility (Medeiros *et al.*, 2002). Membrane damage causes loss of adenosine triphosphate (ATP), proteins, and essential enzymes, which lead to reduction in motility of frozen–thawed spermatozoa. Toxic by-products also inhibit a large number of cellular enzymes such as glyceraldehyde 6-phosphate dehydrogenase (G-6-PDH) and adenosine triphosphatase (ATPase) that may also affect sperm movement (Pereira *et al.*, 2017). Conclussion: results of the present study confirmed that 0.018mg silymarin alone or with 1.5mM/ml melatonin to the semen extender can improve the motility and viability of spermatozoa and reduce the number of apoptotic sperm in the frozen-thawed semen of bulls which used in artificial insemination center in Iraq.

Conflict of interest statement

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

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