

Effect of Adding Aqueous Extract of Kalgan Seeds to Tris Buffer on Some Semen Characteristics of Holstein Bulls After Freezing

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ABSTRACT

The study was conducted in the artificial insemination department from September 2024 to December 2024 with the aim of demonstrating effect of adding different concentrations of the aqueous extract of the seeds of the Kalgan plant to the Tris diluent on some traits of the semen of Holstein bulls after freezing. Four Holstein bulls were used in this experiment. Semen was collected from the four bulls every week at a rate of one ejaculation per bull. The semen of four bulls was mixed and mixed together (Pooled Semen) to eliminate individual differences between bulls. The experiment was divided into four treatments: The first treatment was the control treatment (T1) Tris diluted without adding antioxidant, T2: (Tris diluted + 501 µg of Kalgan seed extract / 10 ml diluted), T3: (Tris diluted + 250 µg of Kalgan seed extract / 10 ml diluted), (T4: Tris diluted + 350 µg of Kalgan seed extract / 10 ml diluted). The results showed that adding 250 µg of Kalgan seed aqueous extract / 10 ml / Tris diluted in treatment (T3) led to a highly significant increase ($P>0.01$) in the percentage of plasma membrane integrity compared to the rest of the treatments after freezing and liquefaction after 48 hours and after one month. The results also showed a highly significant decrease ($P>0.01$) in the concentration of malondialdehyde in the T3 treatment after freezing and liquefaction compared to the rest of the treatments. The results also showed a highly significant increase in the percentage of intact genetic material and the level of total antioxidants in the seminal plasma in T3 after freezing and liquefaction compared to the rest of the treatments. We conclude from this study that the addition of the aqueous extract of Kalgan seeds (250 µg/ml Tris diluted) led to an improvement in some semen traits after freezing of Holstein bulls (plasma membrane integrity, percentage of intact genetic material and level of total antioxidants in seminal plasma) and a decrease in the level of malondialdehyde in the seminal plasma.

1. INTRODUCTION

Artificial insemination is one of the most widely applied and widespread biotechnology techniques in the world and is used for the purposes of genetic improvement of reproductive and productive performance of animals. Cryopreservation has contributed to reducing the obstacles and factors that hinder the process of obtaining semen from genetically distinct bulls, such as geographical location and time (Al-Jashmi, 2017). Cryopreservation in bulls stimulates an additional source of reactive oxygen species that harm sperm and lead to a decrease in the activity of antioxidant enzymes, and the sperm membrane becomes more susceptible to lipid oxidation (El-Sisy et al., 2007), thus affecting the permeability of the sperm membrane (Awda et al., 2009), in addition to the destruction of genetic material and a decrease in sperm motility, vitality and fertilization ability (Sarıözkan et al., 2009). The addition of natural plant extracts to semen diluents protected the sperm of agricultural animals from reactive oxygen species by improving their qualitative traits after freezing in Holstein bulls (Al-Zaidi, 2018) due to their high content of antioxidants. The aqueous extract of the seeds of the Kalgan plant (*Silybum marianum*) is rich in Silymarin, a powerful antioxidant that has the ability to inhibit free radicals and provides protection from lipid oxidation in the cell membrane. It is used in the treatment of many conditions such as protecting the liver from oxidative stress and protecting testicular tissues and improving the quality of semen by raising the level of testosterone in the blood (Fakurazi Luangpirom et al., 2008 et al., 2013). Therefore, the current study aims to demonstrate effect of adding different concentrations of the aqueous extract of the seeds of the Kalgan plant to Tris diluent on some traits of the semen of Holstein bulls after freezing, including the percentage of integrity of the plasma membrane of sperm, measuring the level of malondialdehyde as a vital characteristic of oxidative stress, the level of total antioxidants in seminal plasma, and estimating the percentage of damage to the genetic material.

2. MATERIALS AND METHODS

Semen was collected from four bulls per week, with one ejaculation per bull. The semen of four bulls was mixed and mixed together (Pooled Semen) in each replicate to eliminate individual differences between bulls. The necessary tests were performed to evaluate the semen and then diluted using Tris diluent, which was prepared in advance according to the method of (2000) and Maxwell Salamon. The experiment was divided into four groups and the diluent was added to the semen samples gradually. The distribution of treatments was as follows: (T1) Tris diluent without adding antioxidant, T2: (Tris diluent + 501 µg of Kalgan seed extract / 10 ml diluent), T3: (Tris diluent + 250 µg of Kalgan seed extract / 10 ml diluent), (T4: Tris diluent + 350 µg of Kalgan seed extract / 10 ml diluent). The tests were performed after freezing and liquefaction at 37°C for 30 seconds.

Traits studied

1: Percentage of intact sperm plasma membrane

The percentage of sperm with intact plasma membrane was estimated according to the method of Jeyendran et al.,(1984)

2: Estimation of malondialdehyde concentration in seminal plasma

The concentration of malondialdehyde in seminal plasma was estimated according to the method described by Kumaresan et al.,(2006)

3: Estimation of total antioxidant activity in seminal plasma

The principle of the test was based on what was stated by Brand-Williams et al.,(1995)

4: Estimation of percentage of damage to sperm genetic material

Based on what was indicated by Tejada et al.,(1984)

Statistical analysis

The statistical program Statistical Analysis System -SAS (2018) was used to analyze the data to study effect of different treatments on the studied traits according to a complete random design (CRD), and the significant differences between the averages were compared using Duncan's multinomial test (1955).

3. RESULTS AND DISCUSSION

Effect of adding different levels of aqueous extract of Kalgan seeds on the percentage of plasma membrane integrity of sperm after cooling, freezing and liquefaction for 48 hours and after a month

The results of Table (1) showed highly significant differences ($P < 0.01$) in the percentage of plasma membrane integrity after freezing and liquefaction for 48 hours, where treatment (T3) excelled, which recorded (± 0.24 79.40) compared to treatments T1, T2 and T4, which recorded (± 0.37 76.80), (± 0.45 77.00) and (± 0.20 75.20) respectively. As for the period after freezing and liquefaction by one month, the results in the mentioned table showed highly significant differences ($P < 0.01$) in the percentage of plasma membrane integrity, where treatment (T3) was excelled, which recorded (± 0.20 79.20) compared to treatments T1, T2 and T4, which recorded (± 0.51 76.40), (± 0.37 76.20) and (± 0.00 75.00) respectively. The superiority of the Kalgan treatments over the control treatment may be attributed to the fact that this excelled may be evidence that silymarin is characterized by its antioxidant properties, as it improved the antioxidant capacity of the system and may have increased the stability of the membranes (Feitosa et al., 2008), and Aghashahi et al., 2020 expressed that silymarin improves the antioxidant defense system in human semen, as it increased the activity of antioxidant defense enzymes of sperm such as superoxide dismutase (SOD), glutathione peroxidase, and catalase (CAT). Freezing leads to physical, chemical, and mechanical changes in the sperm membranes of all mammalian species (El-Sheshtawy, 2014 and El-Nattat), as these changes are attributed to differences in temperature, lipid oxidation, production of reactive oxygen species, osmotic pressure, and others (Camara et al., 2011), and since sperm are highly susceptible to oxidative damage due to the presence of large quantities of unsaturated fatty acids in the membrane Plasma (Laudat et al., 2002), sperm are more susceptible to lipid oxidation, so oxidative stress leads to damage to the membrane structure and reduces the activity of enzymes and membrane ion channels (Griveau et al., 1997),

Table 1: Effect of adding different levels of aqueous extract of Kalgan seeds on the percentage of integrity of the plasma membrane of sperm after freezing and liquefaction for 48 hours and after one month

Mean $\pm$ Standard Error			treatments
Level of Significance	After freezing and liquefaction for a month	After freezing and liquefaction for 48 hours	

*	76.40 ±0.51 B b	76.80 ±0.37 B b	T1
**	76.20 ±0.37 B b	77.00 ±0.45 B b	T2
NS	79.20 ±0.20 A a	79.40 ±0.24 A a	T3
**	75.00 ±0.00 C b	75.20 ±0.20 C b	T4
---	**	**	Level of Significance
T1 = Tris diluted control treatment without antioxidant addition, T2 = 150 µg Kalgan seed extract/10 ml diluted, T3 = 250 µg Kalgan seed extract/10 ml diluted, T4 = 350 µg Kalgan seed extract/10 ml diluted Means with different letters within the same column are significantly different from each other. ** (P≤0.01.)			

Effect of adding different levels of aqueous extract of Kalgan seeds on the level of malondialdehyde in seminal plasma

The results of Table 2 showed highly significant differences in the level of malondialdehyde in seminal plasma, as it decreased in treatment T3, which recorded (± 0.75 41.04) compared to treatments T1, T2 and T4, which recorded (± 3.10 74.13), (± 0.59 69.85) and (± 1.87 75.60) respectively. Regarding the decrease in the level of malondialdehyde in treatment T3. The reason for the decrease may be attributed to the fact that during cryopreservation of sperm, lactate is produced (O'SHEA, 1966) and Wales), and lactate oxidation stimulates calcium uptake by increasing intracellular NADH levels in sperm (Vijayaraghavan et al., 1989), the increase in calcium uptake coincides with increased oxygen consumption and hence accelerates the metabolic rate of sperm (Simpson et al., 1987), in addition, there is a relationship between calcium accumulation and lipid oxidation (Toskulkao, 1988) and Glinsukon), and just as silymarin increases ATP levels and modifies intracellular calcium levels (Burczynski et al., 2013). This may also be attributed to the fact that silymarin is important in increasing effectiveness of glutathione (Esmaeil, 2017).

Table 2: Effect of adding different levels of aqueous extract of Kalgan seeds on the level of malondialdehyde in seminal plasma

Mean ± Standard Error MDA	treatments
74.13 ±3.10 a	T1
69.85 ±0.59 b	T2
41.04 ±0.75 c	T3
75.60 ±1.87 a	T4
**	Level of Significance
T1 = Tris diluted control treatment without antioxidant addition, T2 = 150 µg Kalgan seed extract/10 ml diluted, T3 = 250 µg Kalgan seed extract/10 ml diluted, T4 = 350 µg Kalgan seed extract/10 ml diluted Means with different letters within the same column are significantly different from each other. ** (P≤0.01.)	

Effect of adding different levels of aqueous extract of Kalgan seeds on the level of total antioxidants in seminal plasma

The results of Table 3 showed highly significant differences (P≤0.01) in the level of total antioxidants in seminal plasma, as treatment T3 was excelled, which recorded (± 0.00005 0.00054) compared to treatments T1, T2 and T4, which recorded (± 0.00054 0.00021), (± 0.00006 0.00033) and (± 0.00002 0.00025) respectively. The excelled of the total antioxidant level in seminal plasma in the T3 treatment may be due to the addition of the aqueous extract of Kalgan seeds, as it is one of the most

medicinal plants that have antioxidant properties and has a very clear effect, as it begins its work in cells by stopping or delaying free radicals and reducing oxidative stress. In addition, it is not only an antioxidant, but it also stimulates the production of specific antioxidants, which helps increase the level of glutathione (Mohira, 2021). Silymarin stimulates the activity of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (El-Nattat and El-Sheshtawy, 2017). Silymarin increases effectiveness of glutathione, which sweeps away the hydrogen peroxide radical H_2O_2 and inhibits lipid oxidation (Esmail, 2017). Silymarin improves the antioxidant defense system in human semen, as it increased the activity of antioxidant defense enzymes of sperm such as superoxide dismutase (SOD), glutathione peroxidase (GPO) and catalase (CAT). (Aghashahi et al., 2020).

Table 3: Effect of adding different levels of aqueous extract of Kalgan seeds on the level of total antioxidants in seminal plasma

Mean $\pm$ standard error TAO	treatments
0.00021 $\pm$ 0.00054 c	T1
0.00033 $\pm$ 0.00006 b	T2
0.00054 $\pm$ 0.00005 a	T3
0.00025 $\pm$ 0.00002 bc	T4
**	Level of Significance
T1 = Tris diluted control treatment without antioxidant addition, T2 = 150 μ g Kalgan seed extract/10 ml diluted, T3 = 250 μ g Kalgan seed extract/10 ml diluted, T4 = 350 μ g Kalgan seed extract/10 ml diluted	
Means with different letters within the same column are significantly different from each other. ** (P $\leq$ 0.01.)	

Effect of adding different levels of aqueous extract of Kalgan seeds on the percentage of genetic material damage after freezing and liquefaction for 48 hours.

The results of Table 4 showed highly significant differences in the percentage of genetic material integrity, where treatment T3 excelled, recording ($\pm$ 0.18 98.74) compared to treatments T1, T2 and T4, which recorded ($\pm$ 0.24 96.06), ($\pm$ 0.12 96.94) and ($\pm$ 0.45 95.93) respectively. The excelled in the percentage of intact genetic material in the T3 treatment may be due to the addition of the aqueous extract of Kalgan seeds at a level of 250 μ g/10 ml dilution. Fats are large molecules sensitive to peroxidation and are found in the plasma membrane of sperm in the form of unsaturated fatty acids (PUFA) which contain more than two double bonds between carbon and carbon. These fatty acids (PUFA) maintain the flexibility of the plasma membrane of sperm (Leahy and Gadella, 2011). Reactive oxygen species (ROS) attack unsaturated fatty acids (PUFA) which cause a series of chemical reactions called peroxidation of fats which leads to loss of flexibility of the plasma membrane and decreased activity of membrane enzymes and channel ions and thus inhibits the membrane mechanism required for the fertilization process (Agarwal and Allamanen, 2006). Lipid peroxidation (LPO) in sperm is a reaction Self-replicating unless stopped by antioxidants. In semen, the role of the aqueous extract of Kalgan seeds is to inhibit free radicals, enhance the capacity of antioxidant enzymes, and reduce oxidative stress (Eskandari and Momeni 2016a). One of the hypotheses that researchers have found in the damage to genetic material is that DNA damage in sperm is a natural result of oxidative stress (Agarwal and Said 2003). Which occurs as a result of the imbalance between the strength and potential of antioxidants present in semen and the production of ROS, which leads to the formation of oxidative products (Bungum et al., 2011), as the sperm membrane is rich in unsaturated fatty acids, which makes it easy to attack by ROS with more effects on the nuclear membrane and then damage the sperm DNA (Aitken, 2001 and Krausz), Silymarin is characterized by its antioxidant properties as it works to improve the antioxidant system's ability and maintains the stability of membranes (Feitosa et al., 2008), Silymarin also works to improve the antioxidant defense system in semen by increasing the activity of antioxidant defense enzymes of sperm such as superoxide dismutase (SOD), glutathione peroxidase and catalase (CAT), thus preserving the traits of semen, including genetic material. (et al.,(2020 Aghashahi.

Table 4: Effect of adding different levels of aqueous extract of Kalgan seeds on the percentage of genetic material damage after freezing and liquefaction for 48 hours

Mean $\pm$ Standard Error		treatments
percentage of genetic material damage	percentage of intact genetic material	
3.94 $\pm$ 0.24 ab	96.06 $\pm$ 0.24 bc	T1
3.06 $\pm$ 0.12 b	96.94 $\pm$ 0.12 b	T2
1.26 $\pm$ 0.18 c	98.74 $\pm$ 0.18 a	T3
4.07 $\pm$ 0.45 a	95.93 $\pm$ 0.45 c	T4
**	**	Level of Significance
T1 = Tris diluted control treatment without antioxidant addition, T2 = 150 μ g Kalgan seed extract/10 ml diluted, T3 = 250 μ g Kalgan seed extract/10 ml diluted, T4 = 350 μ g Kalgan seed extract/10 ml diluted		
Means with different letters within the same column are significantly different from each other. ** (P $\leq$ 0.01).		

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