Effect of adding Hops *Humulus lupulus* extract to tris extender on the percentage of abnormal sperm in the local Iraqi goats under cooling conditions

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Abstract

The current study aimed to investigate the effect of adding different concentrations of hops' alcoholic extract to Tris extender on abnormal sperm in local Iraqi goats. Three mature local Iraqi bucks were used in this study. Semen samples were collected from all males, pooled, and divided into two parts. One part of the semen was washed with a PBS solution (9.869 g/L) (1:5) and then centrifuged at 2500 rpm for 15 minutes. The seminal plasma was discarded, and the pellet was re-diluted with the washing solution. The washing process was repeated twice. Following two washes, the pellet was resuspended with Tris extender. Both parts were diluted with Tris extender, and three aliquots were prepared for each part. The tubes were treated with different levels of hops alcoholic extract: C (Control), T1: 30 µL/mL hops alcoholic extract, T2: 50 µL/mL hops alcoholic extract. All semen samples were subjected to cooling preservation. The results showed that no significant differences were observed between the washing status, treatments, time points, and their interaction in all studied abnormalities. However, midpiece abnormalities showed an essential difference between time 0 and 4, as well as interactions between washing status and time point, time point and concentration, and between washing status, time point, and hop alcoholic extract levels. In conclusion, unwashing semen did not increase the sperm abnormalities, and T2 hops alcoholic extract has a promising significant outcome. Further studies are warranted to evaluate the effects of hops' alcoholic extracts on antioxidant parameters.

Keywords: Hops, Local Iraqi goat, Sperm Abnormalities, sustainability, production

Introduction

Goats play a fundamental role in livestock units in many parts of the world, particularly in light of the increasing risk of climate change. They play a vital role in the livelihood and food security of rural populations. Despite their long-standing presence in the Arab region and the implementation of selection and breeding improvement programs, further efforts are needed to improve their genetic composition and environmental adaptation [1]. Artificial insemination is one reproductive technique in goat that is essential for enhancing reproductive efficiency and production levels [2,3]. This technique needs cooled or cryopreserved semen to prolong the viability of the sperm. However, with extended storage, sperm viability decreases, including individual

motility, live sperm, and abnormal sperm [4]. Improving the quality of the goat sperm and the normality of cryopreserved semen is one of the basic signs of the success of this technology [5]

Male goat fertility is a key factor significantly influencing the reproductive efficiency of the herd [6]. Their impact directly affects conception rates in females, ultimately leading to increased economic returns in livestock [7]. Chilled semen is widely used in goat artificial insemination due to its cost-effectiveness, ease of handling, transport, and the ability to generate more AI doses per ejaculate [8]. However, a significant issue with chilled goat semen is its limited preservation capacity, i.e., short half-life, attributed to susceptibility to oxidative stress caused by the generation of reactive oxygen species (ROS) [9]. ROS increments typically reduce the antioxidant systems in the seminal plasma, leading to a decline in semen quality and fertilization potential during storage [10]. Antioxidants help scavenge free radicals and reduce the risk of sperm damage during cold storage and artificial insemination [11]. Given their relevance to sustainable development, they are essential when derived from natural, eco-friendly sources. One such plant is hops (*Humulus lupulus*) [12].

Hops are rich in phenolic compounds, which act as primary antioxidants by inhibiting lipid peroxidation and protecting cell membranes from oxidative stress [13]. A study by Wang and his colloquies (2022) showed that the antioxidant activity of hops alcohol extract varies depending on beta-acid concentration levels (30%, 50%, 70%, 90%, and 100%) [14]. These polyphenols help maintain the activity of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase, and protect cells from ROS-induced damage in vivo [15]. However, despite these benefits, to our knowledge, no prior studies have assessed the effects of the hops alcoholic extract on abnormal sperm in any farm animal species. Thus, this study aimed to evaluate the impact of adding different concentrations of hops' alcoholic extract to Tris extender on abnormal sperm in local Iraqi goats.

Materials and Methods

Experimental animals

The approval of the ethical status was granted by Al-Qasim Green University (3155, 2024). This experiment was carried out at the Animal Field of the College of Agriculture, Al-Qasim Green University, Babil Province, from January 18, 2025, to March 10, 2025. Four local buck goats, aged 1.5 to 2 years and weighing 35–40 kg, were used in this study. The bucks were housed in semi-open pens and fed a concentrate diet at 2% of their body weight, along with free access to roughage. Clean drinking water was provided continuously, and veterinary care was available throughout the study.

Semen collection

Prior to semen collection, the Tris extender was prepared (Tris = 3.63 g, Citric acid monohydrate = 1.99 g, glucose = 1 g, Egg yolk = 10 mL, antibiotics, and 100 mL of double-distilled water) and placed in a water bath at 37°C. Bucks were previously trained for semen collection by using an artificial vagina (prepared in our laboratory for more than 10 days [16]. Following training, semen samples were collected from all the bucks once per week. Following Semen collection, samples were transferred immediately to the

lab and kept in a water bath at 37°C until processing. Collected ejaculates were pooled and divided into two parts. One part of the semen was washed with a PBS solution (9.869 g/L) (1:5 ratio) and then centrifuged at 2500 rpm for 15 minutes. The seminal plasma was discarded, and the pellet was re-diluted with the washing solution. The washing process was repeated twice. Following two washes, the pellet was resuspended with Tris extender [17]. Both parts were diluted with Tris extender (Table 1), and three aliquots (tubes) were prepared for each part. The tubes were treated with different levels of hops alcoholic extract (extracted by the Science and Technology department): C (Control), T1: 30 µL/mL hops alcoholic extract, T2: 50 µL/mL hops alcoholic extract. All tubes were subjected to cooling preservation. Briefly, following the treatments, the samples were placed in a beaker at 30 °C and then stored in a refrigerator until the temperature reached 4 °C. Evaluations were performed at both 0 °C and 4 °C.

Abnormal sperm assessment

The percentage of abnormal sperm slides was prepared according to the method described by [18]. A drop of each treated semen sample was mixed with Eosin-Nigrosin stain on a clean, warm (37°C) glass slide, smeared, and left to dry. Two smears were prepared for each sample to assess sperm abnormalities using the Swanson and Bearden method. The evaluation process was repeated at time 0 and after 4 hours of storage at 5°C. Abnormal sperm were counted, with approximately 200 sperm observed under a microscope at 40x magnification. The classification of the abnormal sperm was according to [19]. Head abnormalities were classified into Pyriform, Dwarf, Narrow, Giant, Twin, and Detached. Regarding the midpiece, abnormalities were classified into swollen, twin, and protoplasmic droplets. The tail abnormalities were classified into Coiled, Bent, and Twin. The percentage of abnormal sperm was calculated according to the equation below:

$$\text{Abnormal sperm \%} = \frac{no.abnormal\ sperm}{Total\ no.of\ sperm} X100$$

Statistical analysis

Data were analyzed using the Statistical Analysis System (SAS, 2018) to assess the effects of washing status, hops' alcoholic extract, and time point, and their interactions using a factorial experiment in a completely randomized design (CRD) [20]. Significant differences among means were evaluated using Duncan's multiple range test [21]. For more accuracy, the current research was repeated five times.

Results and Discussion

Effect of washing local Iraqi goat semen on the sperm abnormalities (head, Tail, and midpiece).

In the current study, statistical analysis revealed no significant differences between washed and unwashed goat semen in terms of sperm abnormalities (head, tail, and midpiece) (Table 1).

Table 1. Effect of washing status local Iraqi goat semen on the sperm abnormalities (Mean ± SE)

Washing Status	Head Abnormalities (Mean ± SE)	Midpiece Abnormalities (Mean ± SE)	Tail Abnormalities (Mean ± SE)
Unwashed	0.61 ± 0.07 A	0.54 ± 0.07 A	1.83 ± 0.19 A
Washed	0.48 ± 0.06 A	0.50 ± 0.06 A	1.43 ± 0.16 A

(mean ± SE) local Iraqi goats following washing. Means in the same column with the same letters are not significantly different from each other according to Duncan's multiple range test at a 5% significance level.

Effect of adding hops' alcoholic extract levels on local Iraqi goat sperm abnormalities (head, Tail, and midpiece).

In the current study, the data revealed no significant differences among treatments in all types of sperm abnormalities (head, tail, and midpiece). However, a slight numerical increase in head abnormalities was observed at the 15 µL concentration (0.42, 0.60, 0.43 for 0, 15, and 25 µL, respectively). Similarly, tail abnormalities showed a minor numerical increase in the control group (1.77, 1.37, 1.15), while midpiece abnormalities slightly declined with increasing hops alcoholic extract levels (0.60, 0.50, 0.40) (Table 2).

Table 2. Effect of adding different hops alcoholic extract levels on local Iraqi goat sperm abnormalities (Mean ± SE)

Hops Extract Level (µL)	Head Abnormalities (Mean ± SE)	Midpiece Abnormalities (Mean ± SE)	Tail Abnormalities (Mean ± SE)
C	0.49 ± 0.09 A	0.60 ± 0.08 A	1.80 ± 0.22 A
T1	0.60 ± 0.09 A	0.50 ± 0.08 A	1.68 ± 0.24 A
T2	0.55 ± 0.08 A	0.46 ± 0.08 A	1.41 ± 0.22 A

(Mean ± SE) local Iraqi goat semen treated with hops alcoholic extract, Control= C, 30 µL hops alcoholic extract=T1, 50 µL hops alcoholic extract=T2. Means in the same column with the same letters are not significantly different from each other according to Duncan's multiple range test at a 5% significance level.

Effect of storage time (0 and 4 hours) on local Iraqi goat sperm abnormalities (head, Tail, and midpiece).

Our results showed significant differences in sperm abnormalities following storage at 0 and 4 hours in local Iraqi goat diluted semen (Table 3). The midpiece abnormalities increased at time 4 ($P \leq 0.05$) compared to time 0 (0.63 ± 0.05 vs. 0.40 ± 0.16, respectively). However, the data showed no significant difference between times for head and tail abnormalities (Table 3).

Table 3. Effect of storage time (0 and 4 hours) on local Iraqi goat sperm abnormalities (Mean ± SE).

Time (hr)	Head Abnormalities (Mean ± SE)	Midpiece Abnormalities (Mean ± SE)	Tail Abnormalities (Mean ± SE)
0	0.54 ± 0.07 A	0.41 ± 0.07 B	1.63 ± 0.16 A
4	0.55 ± 0.07 A	0.63 ± 0.06 A	1.64 ± 0.20 A

(Mean ± SE) local Iraqi goat semen stored at (0, 4 °C). Means with different letters in the same column are significantly different from each other according to Duncan's multiple range test at a 5% significance level.

Effect of interaction between washing status and adding hops' alcoholic extract levels on local Iraqi goat semen on the sperm abnormalities (head, Tail, and midpiece).

The data showed that there was no significant difference between washing status and adding hops' alcoholic extract levels on local Iraqi goat semen in terms of sperm abnormalities (head, Tail, and midpiece) (Table 4).

Table 4. Effect of interaction between washing status and adding hops' alcoholic extract levels on local Iraqi goat sperm abnormalities (Mean ± SE).

Washing status	Hops Extract Level (µL)	Head Abnormalities (Mean ± SE)	Midpiece Abnormalities (Mean ± SE)	Tail Abnormalities (Mean ± SE)
Unwashed	C	0.55 ± 0.11 A	0.60 ± 0.15 A	1.83 ± 0.21 A
	T1	0.60 ± 0.14 A	0.50 ± 0.10 A	2.00 ± 0.42 A
	T2	0.68 ± 0.11 A	0.53 ± 0.13 A	1.68 ± 0.37 A
Washed	C	0.43 ± 0.10 A	0.60 ± 0.08 A	1.78 ± 0.39 A
	T1	0.60 ± 0.14 A	0.50 ± 0.14 A	1.38 ± 0.22 A
	T2	0.43 ± 0.11 A	0.40 ± 0.11 A	1.15 ± 0.22 A

(Mean ± SE) local Iraqi goat semen following washing and treatments with hops alcoholic extract, Control = C, 30 µL hops alcoholic extract=T1, 50 µL hops alcoholic extract=T2. Means with the same letters within the same column are not significantly different from each other according to Duncan's multiple range test at a 5% significance level.

Effect of interaction between washing status and storage time (0 and 4 hours) on local Iraqi goat semen on sperm abnormalities (head, Tail, and midpiece).

As shown in Table 5, there was a significant increase ($P \leq 0.05$) in midpiece abnormalities between the samples washed at time 4 (0.65 ± 0.07) and those washed at time 0 (0.35 ± 0.09) (Table 5). However, no significant differences were found at either time point in any of the abnormalities before washing. A slight numerical increase was observed at 4 hours in all categories, but it was not statistically significant (Table 5).

Table 5. Effect of interaction between washing status and storage time (0 and 4 hours) on local Iraqi goat sperm abnormalities (Mean ± SE).

Washing Status	**Time (hr)**	**Head Abnormalities (Mean ± SE)**	**Midpiece Abnormalities (Mean ± SE)**	**Tail Abnormalities (Mean ± SE)**
Unwashed	**0**	0.57 ± 0.07 A	0.48 ± 0.11 AB	1.73 ± 0.12 A
	4	0.65 ± 0.10 A	0.62 ± 0.10 AB	1.93 ± 0.38 A
Washed	**0**	0.52 ± 0.10 A	0.35 ± 0.09 B	1.52 ± 0.31 A
	4	0.45 ± 0.09 A	0.65 ± 0.07 A	1.35 ± 0.14 A

(Mean ± SE) local Iraqi goat semen following washing and stored at (0, 4 °C). Means with different letters in the same column are significantly different from each other according to Duncan's multiple range test at a 5% significance level.

Effect of interaction between adding hops' alcoholic extract levels and storage time (0 and 4 hours) on local Iraqi goat sperm abnormalities (head, Tail, and midpiece).

The data showed that there were no significant differences between the addition of hops' alcoholic extract levels and the time of storage (0 and 4 hours) (Table 6). However, the data showed a significant increase ($P \leq 0.05$) in the midpiece abnormalities at time 4 in T1 (0.72 ± 0.09) compared to time 0 in C and T1 (0.35 ± 0.12, 0.27 ± 0.09), respectively (Table 6).

Table 6. Effect of interaction between adding hops' alcoholic extract levels and storage time (0 and 4 hours) on local Iraqi goat sperm abnormalities (Mean ± SE).

Time (hr)	Hops Extract (µL)	Head Abnormalities (Mean ± SE)	Midpiece Abnormalities (Mean ± SE)	Tail Abnormalities (Mean ± SE)
0	C	0.50 ± 0.08 A	0.60 ± 0.13 AB	2.05 ± 0.38 A
	T1	0.60 ± 0.13 A	0.28 ± 0.10 B	1.45 ± 0.22 A
	T2	0.53 ± 0.13 A	0.35 ± 0.13 B	1.38 ± 0.21 A
4	C	0.48 ± 0.13 A	0.60 ± 0.11 AB	1.55 ± 0.21 A
	T1	0.60 ± 0.15 A	0.73 ± 0.10 A	1.93 ± 0.44 A
	T2	0.58 ± 0.09 A	0.58 ± 0.11 AB	1.45 ± 0.40 A

(Mean ± SE) local Iraqi goat semen following treatments with hops alcoholic extract stored at (0, 4 °C), Control = C, 30 µL hops alcoholic extract=T1, 50 µL hops alcoholic extract=T2. Means with different letters in the same column are significantly different from each other according to Duncan's multiple range test at a 5% significance level.

Effect of interaction between washing status, adding hops' alcoholic extract levels, and storage time (0 and 4 hours) on local Iraqi goat semen on sperm abnormalities (head, Tail, and midpiece).

Table 7 illustrates that there were significant differences in midpiece abnormalities (Table 7). Washed semen in T1 at time 4 was significantly greater ($P \leq 0.01$) (0.80 ± 0.15) compared to washed T1 at time 0 (0.20 ± 0.15) and washed T2 at time 0 (0.15 ± 0.06) (Table 7). However, there were no significant triple interactions found between hop extract levels, storage time, and semen washing status in head and tail abnormalities. The observed numerical changes in all categories were minor and did not reach statistical significance.

Table 7. Effect of interaction between washing status, adding hops' alcoholic extract levels, and storage time (0 and 4 hours) on local Iraqi goat semen on sperm abnormalities (head, Tail, and midpiece) (Mean ± SE).

Washing	Hops Extract (µL)	Time (hrs)	Head Abnormalities (Mean ± SE)	Midpiece Abnormalities (Mean ± SE)	Tail Abnormalities (Mean ± SE)
Unwashed	C	0	0.55 ± 0.09 A	0.50 ± 0.22 ABC	2.05 ± 0.23 A
		4	0.55 ± 0.22 A	0.70 ± 0.22 AB	1.60 ± 0.35 A
	T1	0	0.50 ± 0.18 A	0.35 ± 0.13 ABC	1.55 ± 0.15 A
		4	0.70 ± 0.22 A	0.65 ± 0.13 ABC	2.45 ± 0.82 A
	T2	0	0.65 ± 0.19 A	0.55 ± 0.22 ABC	1.60 ± 0.20 A
		4	0.70 ± 0.09 A	0.50 ± 0.18 ABC	1.75 ± 0.78 A
Washed	C	0	0.45 ± 0.15 A	0.70 ± 0.15 AB	2.05 ± 0.76 A
		4	0.40 ± 0.15 A	0.50 ± 0.08 ABC	1.50 ± 0.26 A
	T1	0	0.70 ± 0.20 A	0.20 ± 0.15 BC	1.35 ± 0.43 A
		4	0.50 ± 0.21 A	0.80 ± 0.15 A	1.40 ± 0.20 A
	T2	0	0.40 ± 0.19 A	0.15 ± 0.06 C	1.15 ± 0.35 A
		4	0.45 ± 0.15 A	0.65 ± 0.13 ABC	1.15 ± 0.29 A

Means with different letters are significantly different from each other according to Duncan's multiple range test at a 5% significance level.

The decrease in the percentage of midpiece deformities in the T1 and T2 treatments with hops alcoholic extract control in washed semen was observed in the current study at times 0 and 4 following dilution. This may be due to the hops extract, which helps protect the sperm goat membranes, rich in unsaturated fatty acids, from oxidative stress.

The cell membrane, which contains free fatty acids, is susceptible to damage caused by free radicals formed during cryopreservation [5,22]. Hops are rich in phenolic compounds, which act as primary antioxidants by inhibiting lipid peroxidation and protecting cell membranes from oxidative stress [13]. These polyphenols help maintain the activity of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase, and protect sperm from ROS-induced abnormalities [15,23].

Regarding the washed status, the date showed there were no significant differences between washed and unwashed semen. These dates were not agreed upon [24,25], who found that washing the buck's seminal plasma before cryopreservation has a positive effect on the viability of semen post-thaw. This effect is due to the fact that the goat seminal plasma is toxic for goat semen diluted with egg yolk or milk extender [26]. This effect has an adverse impact on goat semen cryopreservation due to the enzymes secreted from the bulbourethral gland, which interact with egg yolk, leading to sperm coagulation. The presence of enzymes (bulbourethral secretion glycoprotein-60 and egg yolk coagulating enzyme) in the seminal plasma caused the harmful interactions between seminal plasma and egg yolk or milk [8]. This will catalyze the hydrolysis of egg yolk phosphatidylcholine (PC) into lysophosphatidylcholine (LPC), which has a toxic effect on goat male sperm and subsequently results in a low fertility rate [27]. In the current study, the washing and unwashing of buck sperm abnormalities did not affect even after dilution. This result was supported by [28,29], who found no beneficial effect of removing buck seminal plasma before cryopreservation. Therefore, our data agreed that washing buck semen plasma did not affect the viability of goat semen.

Conclusion

Based on the results of this study, it can be concluded that adding hops (*Humulus lupulus*) alcoholic extract, local Iraqi goat, at concentrations of 30 and 50 µL/mL might have a significant effect on sperm viability or longevity. Likewise, the semen washing process showed no significant influence on the studied characteristics of spermatozoa. Thus, the use of hops' extract at the tested concentrations does not appear to alter the physical parameters of abnormal sperm in local Iraqi goats.

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