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Original article

Impact of Aqueous Extracts of *Urtica dioica, Scabiosa atropurpurea* L. and *Silybum marianum* on Ram Sperm Motility During Refrigeration

Hela Derbali [a], Samia Ben Said [a,*], Wijden Niama [a] & Mokhtar Mahouachi [a]

[a] Université de Jendouba, Ecole Supérieure d'Agriculture du Kef, Laboratoire d'Appui à la Durabilité des Systèmes de Production au Nord-Ouest, 7119 Le Kef, Tunisie

Abstract

Plant extracts are recognized by improving sperm function during refregiration. This study aimed to evaluate the effects of incorporating aqueous extracts of *Urtica dioica, Scabiosa atropurpurea L.* and *Silybum marianum* to the preservation medium on chilled ram semen quality. Plant extracts were prepared from dried parts of the plants and added in a commercial dilution medium. Three doses were tested for each plant extract: 1g/l, 2g/l and 5g/l for *Urtica dioica* and *Scabiosa atropurpurea* and 2g/l, 5g/l and 10g/l for *Silybum marianum* and control (0g/l). The individual motility of the sperm was then assessed at different time intervals 6, 24, 48 and 72 hours after semen collection at 5°C. The results showed that the incorporation of *Urtica dioica* revealed variable levels of motility, with peaks at 2g/l and 5g/l, after 24 hours this dose still maintained relatively high motility (58.0% and 56.4% respectively). The addition of *Scabiosa atropurpurea* showed a significant improvement especially at 1% which preserved individual motility over time with 74.8% after 6 hours and 39.2% after 48 hours, but also a significant decline at higher concentrations. *Silybum marianum* maintained the best motility with the highest doses at 10% with 57.8% after 24 hours of semen collection indicating prolonged efficacy for sperm preservation. This study concludes that aqueous extracts of *Urtica dioica, Scabiosa atropurpurea* and *Silybum marianum* have significant benefits on sperm preservation, with specific concentrations optimizing individual motility.

Keywords: Ram semen, refrigeration, *Urtica dioica, Scabiosa atropurpurea L., Silybum marianum, Motility.*

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* **Corresponding author:**

Samia Ben Said. is an associate professor in the Department of animal poduction at the High school of agriculture of Kef , Jendouba university , Tunisia. Her research interests include the Biology, animal physiology and biotechnology and Genetic. She has lived, worked, and studied in Tunisia and France. Email: sabensaid@gmail.com

INTRODUCTION

Semen refrigeration is an essential technique in animal reproduction biotechnology, particularly in short-term preservation protocols aimed at optimizing artificial insemination outcomes [1,2]. It slows sperm metabolism, reduces the risk of premature deterioration prior to freezing [3] and prolongs sperm viability for several hours to a few days [4]. However, refrigeration is often associated with significant physiological stress, leading to oxidative damage that may reduce sperm motility and alter membrane integrity [5]. In fact, lipid oxidation, caused by the generation of free radicals during cryopreservation is a major factor contributing to loss of sperm viability [6]. To minimize the negative effects associated with semen refregiration such as membrane disorders, cholesterol efflux and DNA fragmentation [7], adding natural antioxidants to preservation media is a promising strategy [8] as natural antioxidants can neutralize free radicals and protect spermatozoa from oxidative damage [9]. Accordingly, plant extracts are very rich in natural antioxidants, due to their availability, relatively low cost and proven protective effects. *Urtica dioica, Scabiosa atropurpurea L*. and *Silybum marianum* are known for their antioxidant and cytoprotective properties [9-11]. These plants are used to provide antioxidant, anti-inflammatory and revitalizing benefits. They have also been shown in various studies to improve sperm quality in animals: enhancing sperm viability, protecting cell membranes from oxidative damage, and improving sperm motility and DNA integrity [12-14]. These positive effects may have implications for the improvement of assisted reproduction programs, in particularly ram semen preservation.

Thus, the aim of the current study was to evaluate the effect of supplementation of dilution medium with different doses of *Urtica dioica, Scabiosa atropurpurea L*. and *Silybum marianumon* on ram's sperm motility during 72 hours after semen collection at 5°C.

MATERIALS and METHODS

The present study was carried out at the experimental station of the Ecole Supérieure d'Agriculture du Kef (ESAK), located in the north west of Tunisia.

The aerial parts of Urtica dioica and Silybum marianum were collected during the stage of stem elongation (in Ferbruary), Scabiosa atropurpurea leaves, stems, roots, inflorescences were collected during the flowering stage (in May) at Kef area. Collected samples were dried in shade at room temperature for one month and then ground into fine powder using a grinder (SK 100 standard, Retsch, Germany). Aqueous plant extracts were obtained by macerating the dried plant in distilled water (at an initial concentration of 12.5 g/L) for 4 hours according to the methods described by Niama et al [11]. To obtain a clear extraction, the mixture was filtered in a first time using Whatman paper n°1 and then using micro filters (Millex® syringe filters, PVDF pore size 0.22 µm, diam. 33 mm, sterile, hydrophilic) to produce a concentrated aqueous extract stock. The filtered extracts were kept in -20°C until further use.

Animal management and semen collection

Semen of five clinically healthy and fertile rams of the Queue Fine de l'Ouest breed, aged between five and seven years with a mean weigh of 61.9 ± 2.8 kg, were collected, the rams were kept under similar management conditions, exposed to daylight, ambient temperature and relative humidity. They were maintained in confinement with ad libitum access to forage and water during the entire trial period.

Before semen collection, the preservation medium was prepared using stock aqueous plant extract diluted with a commercial diluent (Andromed® CSS, HUVE Search, BC- Overpelt, Europalaan 11 BP12, 3900 Pelt – Belgium). Three final doses were obtained for each plant extract: 1g/L, 2g/Land 5g/Lfor Urtica dioica and Scabiosa atropurpurea, 2g/L, 5g/L and 10g/Lfor Silybum marianum with a control medium refers to the use of the commercial extender alone.

Semen was collected once a week using an artificial vagina during two months with two collections per session. After collection, the ejaculate volume was immediately measured using graduated Pyrex tubes. Then, the mass motility score (on a scale from 0 to 5) using undiluted sperm, and the individual motility (on a scale from 0 to 100%), after dilution in physiological saline were assessed as described by Baril et al.[15]. Sperm concentration was determined after dilution using a spectrophotometer [15]. The collected ejaculates having at least 75% individual sperm motility, and 2.5 x109/ml sperm concentration were pooled to obtain a homogeneous sample and used in the experimental work. A total number of 10 dilutions medium (3 doses per each plant) + control group were prepared and used (Table 1). Treated and control (n=64) fractions were stored at 5°C. Individual sperm motility was assessed at 6, 24, 48 and 72 hours after dilution.

Table 1. Composition of control and experimental semen extenders

Treatments	Composition (%)
Control	20% Andromed + 80% distillated water
1 g/L	20% Andromed + 8% Concentrated aqueous extract of plant X + 72% distillated water
2 g/L	20% Andromed + 16% Concentrated aqueous extract of plant X + 64% distillated water
5 g/L	20% Andromed + 40% Concentrated aqueous extract of plant X + 40% distillated water
10 g/L	20% Andromed + 80% Concentrated aqueous extract of plant X

Plant X : Urtica dioica or Scabiosa atropurpurea L. or Silybummarianum

Stasitical analysis

An analysis of variance (ANOVA) was performed using SPSS software (IBM SPSS Statistics 22) to compare the effects of the different treatments on individual sperm motility at each time interval. A one-factor ANOVA was used to determine whether there were significant differences in mean motility percentages between the different groups (plant extracts and control). The Duncan test was applied to

evaluate differences between groups. Descriptive statistics are presented as mean ± standard error of the mean(Mean ± SEM). The results of statistical analyses were evaluated with a maximum error margin of 5%.

RESULTS and DISCUSSION

The results of the study showed that the average of the ejaculate volume and sperm concentration were respectively 1.3 ± 0.1 ml and 3.7 ± 0.3 billions spz/ml. Mass motility and individual motility were 4.1 ± 0.2 and 84.3 ± 2.20%, respectively. These values indicate a well-established baseline for assessing subsequent treatment effects.

The evaluation of the effect of the incorporation of aqueous extracts of *Urtica dioica, Scabiosa atropurpurea* and *Silybum marianum* in the extender of semen preservation on the individual ram sperm motiltiy during refrigeration significantly enhanced it over time when stored at 5°C. The results demonstrate that the 3 extracts maintained individual sperm motility at a high level at least 24 hours after storage:

Effect of aqueous extract of *Urtica dioica* on ram sperm motility during chilled semen storage

The aqueous extract of Urtica doica significantly conserved the high individual motility level during time, after 6 hours of refrigeration, the 2g/l and 5g/l treatments significantly retained the highestindividual sperm motility compared with the control group (68.2% and 68.7% vs. 52.8%, P = 0.02). At 24 hours, the 2g/Land 5g/L groups continued to preserve significantly higher individual sperm motility than the control (58% and 56.4% vs. 39.6%, p = 0.025). after 48 hours of refrigeration, a highly significant difference (p = 0.009) was also observed between the groups, the 5g/L treatment was the most effective, with an individual sperm motilityof 44.2%, in marked contrast to the lower values observed in the control (32.8%) and 1g/L (28%) groups. However, after 72 hours, the difference between the groups was not statistically significant. (Table 2)

This significant improvement in individual sperm motility after incorporation of the Urtica dioica aqueous extract during the refrigeration period could be explained by the richness of the plant in flavonoids and phenolic acids (quercetin and caffeic acid) [16], [17], known for their ability to neutralize reactive oxygen species which protecting sperm membranes, who are particularly sensitive to oxidative stress due to their high polyunsaturated fatty acid content [18], [12] so it plays a crucial role as a natural antioxidant protecting spermatozoa from damage [19]. At moderate concentrations (2g/L and 5 g/L), aqueous extract of Urtica doica improved sperm motility, which is in line with previous studies underlying its antioxidant properties [12] by limiting lipid peroxidation that can help to maintain membrane integrity and mitochondrial activity, essential for cell motility [16].. This observation

suggests that the optimal use of nettle must be carefully calibrated to maximize its benefits while minimizing potential risks

Table 2. Effect of *in vitro* incorporation of aqueous extract of *Urtica doica* on individual ram sperm motility (%) during chilled semen storage

Treatments	Storage period (hours)			
	6	**24**	**48**	**72**
Control	$52.8^{a} \pm 1.4$	$39.6^{a} \pm 1.3$	$32.8^{a} \pm 2.1$	14.2 ± 0.7
1g/L	$63.9^{a} \pm 1.7$	$43.2^{a} \pm 1.6$	$28^{a} \pm 1.9$	15.8 ± 1.2
2 g/L	$68.2^{b} \pm 1.3$	$58^{b} \pm 1$	$32.1^{a} \pm 1.3$	26 ± 1.3
5 g/L	$68.7^{b} \pm 1.6$	$56.4^{b} \pm 0.8$	$44.2^{b} \pm 0.9$	28 ± 1.6
p value	0.02	0.02	0.009	0.08

Values are means ± SEM semen fraction in each group, (n=48). Means with different superscripts (a and b) in the same column differ from each other (P<0.05).

Aqueous extract of *Scabiosa atropurpurea L.* on individual ram sperm motility during chilled semen storage

The *in vitro* incorporation of *Scabiosa atropurpurea L.* aqueous extract into the storage diluent induced a dose-dependent improvement in individual sperm motility during the first 24 h of storage, at 6 h, the 1g/L concentration produced a highly significant individual sperm motility (74.8 ± 0.8%) compared with the control (52.8 ± 1.4%; P = 0.023). Similarly, at 24 h, the same treatment maintained a high individual sperm motility (55.7 ± 1.3% vs. 39.6 ± 1.3%; P = 0.04), Over 24 hours (at 48 h and 72 h) the individual motility continues to decline sharply, with the lower concentrations especially 1g/L showing better performance than the higher concentrations and the control group but no significant difference was found between the treatments and the control) (Table 3).

These improvements in individual sperm motility observed especially over the first 24hours could be directly associated with the richness of *Scabiosa atropurpurea* in bioactive compounds that provides an exceptional antioxidant properties, due to its high polyphenol, flavonoid, phenolic acids and anthocyanes content known for their powerful antioxidant effect and ability to trap reactive oxygen species (ROS) [20] effectively protect sperm cell membranes [11]. At low concentrations, *Scabiosa* extract maintains high motility, which is particularly advantageous for sperm preservation by stabilizing membrane lipids, preserving mitochondrial integrity and reduce lipid peroxidation, which is the main cause of loss of viability in low-temperature spermatozoa [18]. In addition to its antioxidant properties, *Scabiosa* has anti-inflammatory and neuroprotective effects, offering further protection against cell damage[21]. Nevertheless, at higher concentrations, a progressive decrease in the viability of spermatozoa during the 72 hours of refrigeration is observed. However, the beneficial effect of the extract appears to be dose-dependent. Indeed, although low doses improve viability, it is well documented that high concentrations of antioxidants can cause increased generation of free radicals

leading to reduced motility [22]. The potential of *Scabiosa* to improve fertility and reduce cell damage makes it promising for semen cryoconservation applications.

Table 3. Effect of *in vitro* incorporation of aqueous extract of *Scabiosa atropurpurea L.* on individual sperm motility during semen cooling

Treatments	Storage period (hours)			
	6	24	48	72
Control	$52.8^{a} \pm 1.4$	$39.6^{a} \pm 1.3$	32.8 ± 2.1	14.2 ± 0.7
1 g/L	$74.8^{b} \pm 0.8$	$55.7^{b} \pm 1.3$	39.2 ± 1	27.3 ± 0.9
2 g/L	$65.7^{a} \pm 1.5$	$46^{a} \pm 1.2$	33.7 ± 0.8	22.8 ± 1
5 g/L	$55^{a} \pm 1$	$37.1^{a} \pm 1.1$	24.2 ± 1.3	15.5 ± 1
***p* value**	0.023	0.04	0.127	0.189

Values are means ± SEM semen fraction in each group, (n=48). Means with different superscripts(a and b) in the same column differ from each other ($P<0.05$).

Aqueous extract of *Silybum marianum* on ram individual sperm motility during chilled semen storage

The aqueous extract of *Silybum marianum* groups showed a higher individual motility comparable to the control group at all concentrations after 6 hours especially with 5g/Land 10g/L (67.3% ± 1.9 and 70 % ± 1.9 respectively). After 24 hours, the incorporation of aqueous extract of *Silybum marianum* at 2g/L, 5g/L and 10 g/Lmaintained a higher individual motility than the control.

At 48 hours, *Silybum marianum* aqueous extracts continue to show relatively stable individual motility, with the 2g/L group maintaining higher motility (46.4% ± 1.4) than the control group. At 72 the higher concentrations of *Silybum marianum* incorporated in the extender continued to perform best in terms of individual motility, confirming its prolonged efficacy in sperm preservation (Table 4).

The results showed a gradual improvement in individual sperm motility after incorporation of the *Silybum marianum* aqueous extract which could be related to the plant's rich composition of bioactive molecules like a silymarin content, a flavonoid complex comprising silybin, silychristin and silydianin[23]. These molecules are well known for their ability to neutralize free radicals, stabilize cell membranes and preserve mitochondrial integrity, thus helping to maintain metabolic activity so it is a powerful antioxidant [24] that protects cells against oxidative stress by stabilizing cell membranes and reducing lipid peroxidation [14].This protection is essential for maintaining sperm motility and preserving their structural integrity. At various concentrations, Silybum marianum shows an ability to maintain relatively stable sperm individual motility during the preservation at 5°C, offering a significant advantage for long-term cryoconservation [25] which is in accordance with several studies that have reported the beneficial effects of silymarin on sperm cryopreservation in various animal species [26],[27], Therefore, Silybum marianum appears to be a promising compound for the creation of enriched diluents for the conservation of animal semen.

Table 4: Effect of *in vitro* incorporation of aqueous extract of *Silybum marianum* on individual ram sperm motility? during chilled semen storage

Treatments	Storage period (h)			
	6	**24**	**48**	**72**
Control	52.8 ^{a}± 1.4	39.6^{a} ± 1.3	32.8^{a}± 2.1	14.2 ± 0.7
2 g/L	56.4^{b} ± 2.5	48.2^{b} ± 1.7	46.4^{b}± 1.4	23.7 ± 1.7
5 g/L	67.3^{b} ± 1.9	55.3^{b}± 1.4	43.5^{b}± 0.8	33.3 ± 2.1
10 g/L	70^{b} ± 1.9	57.8^{ab}± 1.4	41^{a} ± 0.5	34.8 ± 1.2
***p* value**	0.001	0.016	0.005	0.15

Values are means ± SEM semen fraction in each group, (n=48). Means with different superscripts (a and b) in the same column differ from each other (P<0.05).

These findings highlight the potential benefits of plant extracts in preserving semen quality and align with previous research suggesting that antioxidants can improve sperm motility by reducing oxidative stress and protecting spermatozoa from damage [9], he antioxidant potential of aqueous extracts of *Urtica dioica, Scabiosa atropurpurea, and Silybum marianum* in maintaining ram sperm motility during refrigeration, with protective effects primarily attributed to their richness in polyphenols, flavonoids, and phenolic acids known to neutralize reactive oxygen species (ROS) and stabilize cellular structures, particularly the sperm membrane and mitochondria (19).t is well established that cold storage induces oxidative stress through the accumulation of ROS, which damage sperm membranes via lipid peroxidation, impair mitochondrial function, and ultimately lead to the loss of motility and viability [18] [9] especially considering the high content of polyunsaturated fatty acids (PUFAs) in the sperm membrane. We hypotheses that the antioxidant compounds in the aqueous extracts, used in our study, contribute by scavenging ROS such as superoxide anions and hydroxyl radicals, chelating transition metals like iron and copper that catalyze ROS formation, enhancing endogenous antioxidant enzyme systems including superoxide dismutase, catalase, and glutathione peroxidase, and by stabilizing the sperm membrane to maintain structural integrity. Furthermore, the dose-dependent effects observed underscore the importance of precise optimization, as moderate doses of *Urtica dioica* (2–5 g/L) and *Silybum marianum* (5–10 g/L) significantly enhanced motility preservation, likely due to effective antioxidant capacity without disturbing physiological redox signaling. In contrast, *Scabiosa atropurpurea* showed its maximal effect at the lowest tested dose (1 g/L), with higher concentrations reducing sperm viability, a paradox consistent with the biphasic dose-response phenomenon described in antioxidant pharmacology [22]. Indeed excessive antioxidants may exhibit pro-oxidant behavior, interfere with redox-regulated pathways, or disrupt mitochondrial electron transport, leading to reduced ATP production. The variation in dose-response profiles can also be attributed to the distinct phytochemical compositions of the extracts, as *Urtica dioica* contains quercetin and caffeic acid that improve mitochondrial activity [17]; [19], *Scabiosa atropurpurea* is rich in anthocyanins, chlorogenic acids, and flavonols with potent but dose-sensitive ROS-scavenging ability [21]; [11], and *Silybum*

marianum owes its efficacy to silymarin, a complex of silybin, silychristin, and silydianin known to stabilize mitochondria and preserve metabolic function [24]; [25].

Thus, aqueous extracts derived from medicinal plants such as *Urtica dioica*, *Scabiosa atropurpurea*, and *Silybum marianum* have been shown to exert significant protective effects on semen quality, particularly by preserving individual sperm motility during storage provided their concentrations are carefully optimized to prevent redox imbalance and highlight the potential for developing standardized or combined formulations for improved sperm viability during storage.

CONCLUSION

The strategic and optimized application of these natural extracts in semen preservation protocols could offer a valuable alternative to synthetic antioxidants, aligning with increasing demand for eco-friendly and sustainable practices in animal reproduction. Moreover, beyond preserving motility, these extracts may contribute positively to overall reproductive health, potentially improving fertilization outcomes. However, despite these promising findings, additional studies are required to fully elucidate the molecular and cellular mechanisms underlying their protective effects. Further research should focus on optimizing extract preparation methods, dosages, and combinations to enhance their bioavailability and synergistic action. Evaluations of long-term safety and efficacy in various animal species will also be critical to validate their practical applications in reproductive biotechnology.

Additional Declaration

Author Contributions

Hela Derbali: Formal analysis, methodology, investigation, original draft and writing. Samia Ben Saïd: Conceptualization, methodology, investigation, writing and editing, Supervision. Wijden Niama: Methodology and Mokhtar Mahouachi: Revision and Funding.

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Responsible Artificial Intelligence Statement

No artificial intelligence support was received in any part of this study.

Conflicts of Interest

The authors declare that there are no conflicts of interest related to the publication of this study.

Ethics Approval

This study does not require ethics committee approval as it does not involve any direct application on human or animal subjects.

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