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Effects of Adding Natural Honey to Semen Extender on Ram Epididymal Sperms Quality

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ABSTRACT

Numerous studies have indicated that male infertility is often associated with poor semen quality. The present study investigated the use of natural honey as an antioxidant and nutrient additive to semen extender to evaluate its effect on sperm parameters. A total of 16 fresh testes from sexually mature rams were collected for the investigation and immediately transported to the reproductive technology laboratory at the College of Veterinary Medicine, Baghdad University. For the first group, sperm was collected and diluted with an egg yolk extender mixed with 10% natural honey**,** while the second group consisted of epididymal sperm diluted with 0.9% normal saline. Epididymal fluid was collected and evaluated for both groups. The results showed significant differences in mean individual motility between the two groups after 48 and 72 hours, as determined by the comparison of proportions. Egg yolk plus honey diluent was significantly more effective than normal saline diluent in preserving sperm cell viability after 48 and 72 hours. The same finding applied to progressive motility; the egg yolk plus honey diluent was significantly more efficient than the normal saline diluent for the time frame after 48 and 72 hours, respectively. In conclusion, the findings demonstrated that the egg yolk extender supplemented with 10% honey was more effective in preserving ram sperm motility over time than normal saline. The addition of honey to the egg yolk extender improved the motility, the live-dead ratio, and the viability of the liquid storage of ram epididymal fluid. Furthermore, egg yolk plays a crucial role in protecting sperm from the detrimental effects of low temperatures.

Keywords: Epididymal sperm, Honey, Ram, Semen extender, Sperm motility

INTRODUCTION

Honey may greatly raise the quality of semen in various bull breeds (Chung et al., 2019). A study by El-Sheshtawy et al. (2016) on Arab stallions in Egypt has revealed that supplementation of semen with honey not only improves its motility and morphology but also protects the sperm against free radical-induced DNA breakage and cryoprotectant damage. Another study indicated that a 15% concentration of pure honey in cryoprotectant media could maintain the quality of infertile semen (El-Sheshtawy et al., 2016). In another study, combining honey with a cryoprotectant medium increased the fertility of rabbits and enhanced the quality of their semen during conservation (Chung et al., 2019). Additionally, studies on Jersey bulls suggested that Bioxcell without additives was more effective than a 1% honey concentration for cryopreserving bull semen (Chung et al., 2019; Gulov and Laskin, 2021).

Honey provides a safe, efficient, and natural alternative for enhancing semen quality and prolonging its conservation in various sheep species. Farmers and breeders choose honey due to its natural qualities (Cheepa et al., 2022). Because of its ability to support oxidative stability, protect sperm from cryoprotectant damage, and prevent DNA breakage caused by free radicals, it also presents a viable strategy for increasing the fertility of infertile semen and fostering spermatogenesis (Kotze et al., 2024). The addition of honey to semen samples, particularly in artificial insemination and cryopreservation, requires careful consideration to maintain sperm quality and viability. Research has explored various methods and additives to optimize semen handling and storage, focusing on factors such as sperm motility, concentration, and the effects of cryoprotectants. These studies provide valuable insights into the challenges and potential solutions for enhancing the reproductive success of honey (Tsvetkov et al., 2024).

The studies have shown epididymal epithelium, the lining of the epididymis, plays a significant role in the processing of sperm (Rodriguez-Martinez et al., 1990). Studies have also been conducted to understand the impact of various factors on sperm maturation in the epididymis of rams (Wegener et al., 2014; Martínez-Fresneda et al., 2019). For instance, research on the human epididymis has focused on the consequences of vasectomy on the epididymal transcriptome, which is the complete set of RNA transcripts produced by the epididymis (Martin-DeLeon, 2006).

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Additionally, there is research on how environmental elements affect sperm health, such as the impacts of biphenyl A on the antioxidant system of rat epididymis sperm (Chitra et al., 2003). In summary, epididymal sperm are crucial for sperm maturation and fertility, and their health and function can be influenced by various factors (Collins, 2003).

The epididymis is responsible for the transportation, maturation, and storage of sperm (James et al., 2020). During transit through the epididymis, spermatozoa undergo changes in the luminal environment of each epididymis region, acquiring motility and fertilization capabilities (James et al., 2020). The epididymis is comprised of four anatomical regions, including the initial segment, caput, corpus, and cauda. The epithelial cells lining the epididymis lumen play a crucial role in establishing a unique luminal environment, providing a protective barrier, and performing the sectorial transport of ions, nutrients, solutes, proteins, and water. Additionally, the epididymis prevents autoimmune responses against spermatozoa and protects against ascending and blood pathogens through interactions with immune cells (Breton et al., 2019). The epididymis also synthesizes and secretes proteins that are important for sperm maturation (Fouchécourt et al., 2000). Overall, the epididymis facilitates the maturation, protection, selection, and storage of spermatozoa in the male reproductive system (Wong et al., 2000).

Epididymal fluid is a crucial component of the male reproductive system. Produced by the epididymis, a tube-like structure that transports and matures sperm, the composition of epididymis fluid is essential for sperm survival and function (Nasreen et al., 2020). The studies have shown that the epididymis epithelium, the lining of the epididymis, plays a significant role in forming the fluid microenvironment (Wong et al., 2000).

A study on stallions aimed to expand the understanding of epididymal protein composition and its dynamic changes (Fouchécourt et al., 2000). Other studies have examined the acid-base status of epididymal fluid, such as research on boars that reported on the *in vitro* acid-base status and *in vivo* pH of epididymal fluid (Rodriguez-Martinez et al., 1990; Zuo et al., 2011). The use of natural honey as a semen extender or diluent is advantageous due to its natural acidity, which does not negatively affect sperm motility or health (Banday et al., 2017). Moreover, honey possesses unique antibacterial properties that benefit semen quality (Banday et al., 2017; Nasreen et al., 2020). The main objectives of the present study were to evaluate the physical and microscopical properties of epididymal sperm from ram testes after slaughter.

MATERIALS AND METHODS

Ram testicles were cut shortly after the animal was slaughtered, placed in a cooled box containing ice and maintained at 4°C, and transported to the laboratory within 1–2 hours as fresh semen without extenders. The study involved the collection of 16 fresh testes from sexually mature rams post-slaughter. The samples were immediately transported to the Laboratory of Reproductive Technology, Department of Surgery and Obstetrics, College of Veterinary Medicine, Baghdad University. The study was conducted from December 2023 to February 2024. The samples were divided into two groups, each consisting of 8 tests. In the first group, sperm were collected and diluted with an egg yolk extender mixed with 10% natural honey (Kanz Al-tabeaa, natural clover honey made in Iraq). The second group consisted of epididymal fluid diluted solely with 0.9% normal saline following sperm collection.

Procedure

The addition of honey to the semen extender was performed prior to sperm collection. A 15% honey solution was added slowly to the semen extender while mixing to ensure an optimum mixture of the semen extender and honey. All testes were transported from the slaughterhouse to the laboratory under cooled conditions. Each testis was cleaned with soap and water and disinfected with 0.5% Povidone Iodine. All the layers that covered the testis were removed to reach the epididymis. A total of 5 ml of diluent was injected into the head, body, and head of the epididymis to facilitate mixing with epididymal sperm. Since the epididymis lacked sufficient fluid, an injection was necessary to aid sperm evacuation and collection. A longitudinal incision was made in the epididymis using a surgical blade and scissors. The area was then rinsed with an additional 5 ml of diluent to maximize the recovery of epididymal sperm (Figures 1 and 2). The epididymal fluid was then collected and evaluated. This procedure was repeated for both groups (the egg yolk plus honey group and the normal saline group). Semen evaluation was conducted at 24, 48, and 72 hours after cooling in a refrigerator set at 8°C.

Sperm's evaluation

Individual motility

Epididymal fluid was used to measure sperm motility. A single drop of freshly collected epididymal fluid was placed on a spotlessly heated slide before being covered with a cover slide. A light microscope (Noval, China) with a 40X magnification was used to score each semen's motility individually (Vilakazi and Webb, 2004).

Sperm's concentration

Hemacytometers, made of specially built slides with two counting chambers and two dilution pipettes, were used to concentrate sperm. The process was carried out by adding 0.1 ml of epididymal fluid to 19.9 ml of coloring solution in a test tube with a dilution rate of 1:20. One drop was then placed on the hemocytometer's slide chamber, and the calculation was done using the following formula (Vilakazi and Webb, 2004):

Number sperm/ml = number of sperm in 0.1 mm^3×10×dilution rate ×1000.

Sperm morphology

Eosin and Nigrosin were the chemical staining agents used to determine the viability of the sperm (dead and living sperm). While the backdrop was dyed with Nigrosin to make the unstained sperm visible, eosin was able to penetrate through the membranes of the non-living cells (Al-Dahabi, 2010). A drop of the solution was added to a drop of fresh semen situated on a heated microscope slide after 1% eosin and 5% nigrosine had dissolved in 2.9% sodium citrate dehydrated buffer (Kushwaha, 2019).

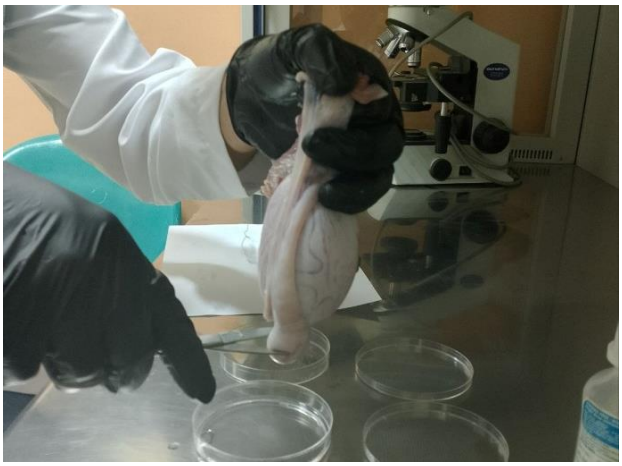

Figure 1. The longitudinal incision in the ram epididymis

Figure 2. Evacuation of ram epididymal fluid using 10 ml of extender containing 15% honey

Statistical analysis

The data were analyzed statistically using a two-way ANOVA. Significant differences ($p < 0.05$) between means were determined using the Least Significant Differences (LSD) post hoc test. Additionally, the Chi-square test was employed to identify any significant variations in proportions.

RESULTS AND DISCUSSIONS

The examination of sperm morphology was conducted to determine any primary and secondary abnormalities. After staining a drop of fresh semen with eosin and nigrosine, the semen slide field exhibited an acceptable standard for normal morphology (70%). Figures 3 and 4 illustrate the sperm morphology, indicating normal sperm structure (head and tail).

Table 1 presents the mean individual motility of sperm in the first group (egg yolk plus 10% honey). The individual motility and progressive motility were found to be 70% and 54%, respectively, at day 0. After 24 hours, these values decreased to 47% and 47%, respectively. By 48 hours, the individual and progressive motility further declined to 37% and 43%, respectively. On day three (72 hours), the individual and progressive motility reached their lowest values of 27% and 38%, respectively.

Table 2 presents the mean individual motility of sperm in the second group (normal saline). At day 0, the individual motility and progressive motility were recorded as 67% and 53%, respectively. Individual and progressive motility values started to decrease with time; after 24 hours, they were 43% and 37%, respectively, while the values of individual and progressive motility after 48 hours reached 20% and 23%, respectively. Finally, after 72 hours, the individual and progressive motility were found to be 12% and 13%, respectively.

Tables 3, 4, and 5 present the physiological characteristics of ram epididymal sperm diluted with egg yolk diluent plus 15% honey compared to normal saline. At day 0, the individual motility scores of ram sperm diluted with egg yolk plus honey and normal saline were 27% and 12%, respectively. On day three, the progressive motility percentages of ram sperm diluted with egg yolk plus honey and normal saline were 38% and 13%, respectively. The maintenance of sperm motility was considerably better with egg yolk plus honey diluent compared to the normal saline diluent.

Statistically significant differences were observed in the mean individual motility between the two groups after 48 hours and 72 hours, as demonstrated by the comparison of proportions. Egg yolk plus honey diluent at 15% was significantly ($p < 0.05$) more effective than the normal saline diluent in preserving sperm cell viability after 48 hours ($p < 0.05$) and after 72 hours ($p < 0.05$). The same trend was observed for progressive motility, where the egg yolk plus honey diluent of 15% was significantly more efficient than the normal saline diluent.

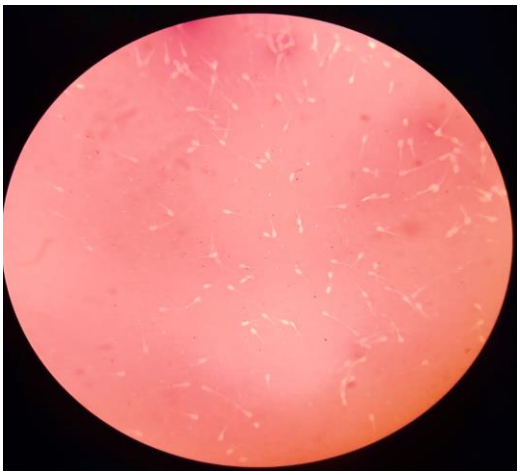

Figure 3. Sperm cells of ram epididymal fluid under the light microscope (40X). This image demonstrates ram sperm progressive motility stained with an eosin-nigrosine.

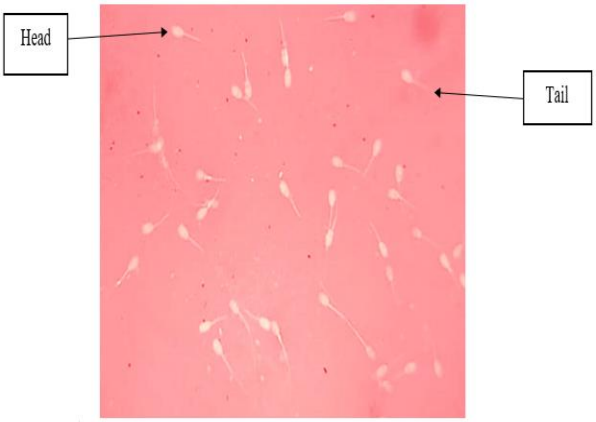

Figure 4. Sperm cells of ram epididymal fluid under the light microscope. A normal feature of ram epididymal sperms (head and tail) stained with eosin-nigrosine at (40X)

Table 1. Mean of individual motility of epididymal sperm from rams, diluted with egg yolk plus 15% honey

Period	Individual motility (%)	Progressive motility (%)
Day 0	70[a]	53[b]
24 hours	47[a]	47[a]
48 hours	37[a]	43[b]
72 hours	27[a]	38[b]

[a, b] Different superscript letters in the same row are significantly different at $p < 0.05$.

Table 2. Mean individual motility of ram epididymal sperm diluted with normal saline

Period	Individual motility (%)	Progressive motility (%)
Day 0	67[a]	53[b]
24 hours	43[a]	37[a]
48 hours	20[a]	23[a]
72 hours	12[a]	13[a]

[a, b] Different superscript letters in the same row are significantly different at $p < 0.05$.

Table 3. Sperm evaluation of ram epididymal sperm diluted with egg yolk diluent plus 15% honey in comparison with normal saline

Day 0 / Characters	Egg yolk plus honey	Normal saline
Individual motility	70%	67%
Progressive motility	53%	53%
Sperm concentration (ml)	1.36 × 106	1.36 × 106
Sperms anomalies	8%	9%

Table 4. A comparison of proportions was used to compare the two groups- egg yolk with 15% honey and normal saline- in terms of motility, individual motility, and progressive motility of ram sperm

Period	E+H In motility	NS In motility	P-value	E+H Pr motility	NS Pr motility	P-value
Day 0	70%	67%	0.648	53%	53%	0.1000
After 24 h	47%	43%	0.570	47%	37%	0.1530
After 48 h	37%	20%	0.007*	43%	23%	0.0027*
After 72 h	27%	12%	0.007*	38%	13%	0.0001*

NS: Normal saline diluent, E+H: Egg yolk + honey. In motility: Individual motility, Pr motility: Progressive motility

Table 5. Sperm evaluation of ram epididymal sperm diluted with egg yolk diluent plus 15% honey in comparison with normal saline

Characters	Day 0		P-value	After 72 h		P-value
	Egg yolk + honeybee	Normal saline		Egg yolk + honeybee	Normal saline	
Individual motility	70%	67%	0.648	27%	12%	0.0076*
Progressive motility	53%	53%	1.000	38%	13%	0.0076*
Sperm concentration (ml)	1.36 ×106	1.36 × 107	-	-	-	-
Sperm anomalies	8%	9%	0.800	-	-	-

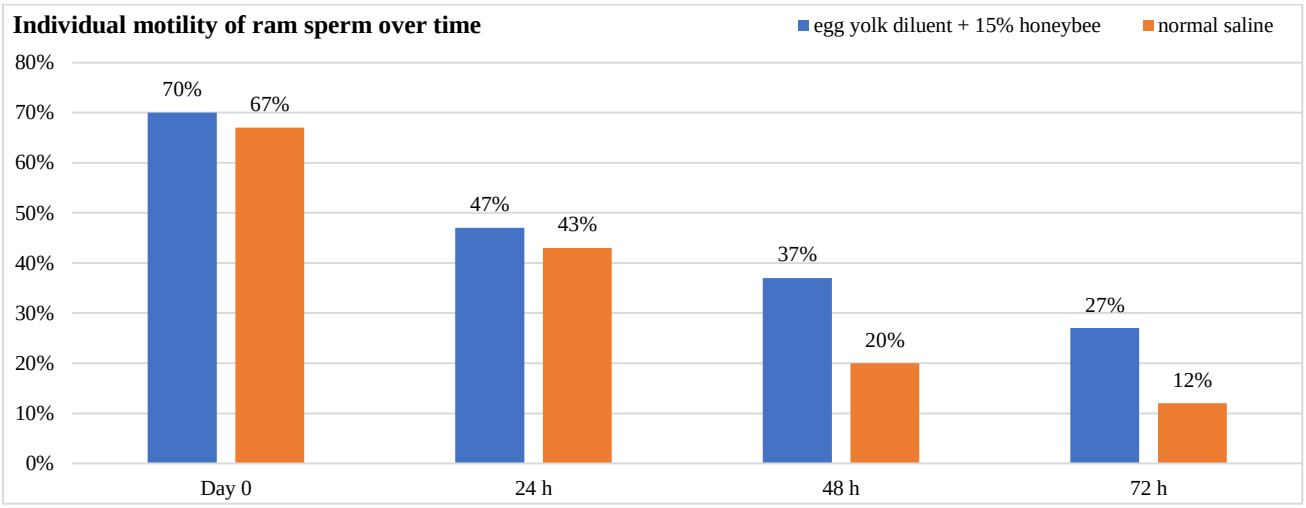

Figure 4. The individual motility of ram sperm diluted with egg yolk plus 15% honey and normal saline diluents

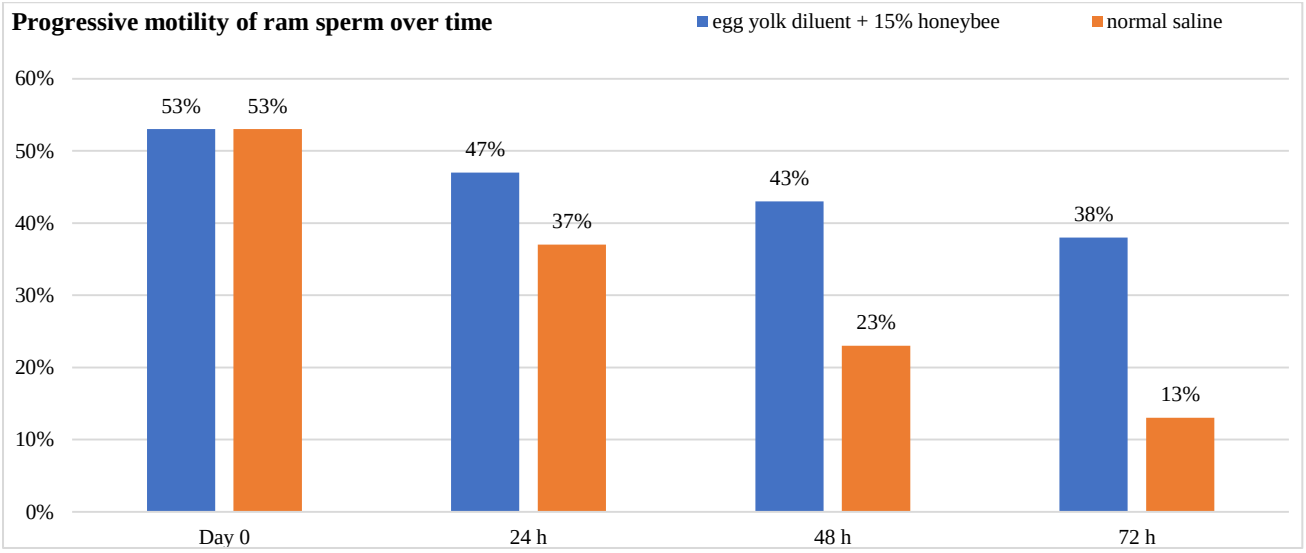

Figure 5. The progressive motility of ram sperm diluted with egg yolk plus 15% honey and normal saline diluents

It is widely recognized that several parameters, such as antibiotics, dilution components, semen storage techniques, frequency of semen collection, and dilution times influence the quality of semen (Liaqat et al., 2022). The natural habitat of sperm cells is replicated *in vitro* using semen dilution. It is feasible to decrease the velocity of sperm migration, block sperm metabolism, extend the period that spermatozoa survive, raise the amount of semen, and enhance spermatozoa consumption by adding an appropriate diluent, as supported as well by Van de Hoe et al. (2022). Honey possesses antibacterial and antioxidant properties due to the presence of flavonoids, which can scavenge free radicals and prevent consequent damage to DNA (Zhang et al., 2024). Studies have demonstrated that adding honey to an egg yolk extender enhances the viability, motility, and live-dead ratio of liquid-stored goat semen (Wong et al., 2000). Egg yolk plays a critical role in protecting sperm from the detrimental effects of low temperatures, as noted by Zhang et al. (2024). Lecithin and unsaturated fatty acids in the yolk may increase sperm metabolism, stabilize the cell membrane, promote the production of lipoproteins, and mitigate the effects of free radicals.

Furthermore, adding yolks can increase sperm's resistance to osmosis (Collins, 2003). Further research is needed to understand the mechanism of action and optimal dosage of supplementing honey products in semen extenders for different species (Hashem et al., 2021). Although studies speculate that adding egg yolk to the diluent may increase the danger of animal disease transmission, egg yolk is a frequently utilized protective ingredient in low-temperature semen preservation diluents (Vilakazi et al., 2004). The motility of individual and large sperm is seen in Figures 4 and 5. Compared to regular saline, the egg yolk plus 15% honey diluent was more effective in maintaining sperm motility over time.

Numerous stress types, including osmotic, biochemical, and thermal variables, can cause sperm destruction as a result of semen storage. Nonetheless, the normal saline solution consists of 9 grams of sodium chloride (NaCl) dissolved in water and lacks stabilizers, antioxidants, or antibacterial qualities (Vilakazi et al., 2004; Van de Hoek et al., 2022).

CONCLUSION

The findings of the current study regarding the efficiency of two dilution solutions in preserving ram sperm motility lead to the conclusion that the egg yolk plus 15% honey diluent is more efficient in preserving sperm motility over time than the normal saline diluent. The addition of honey to egg yolk extender mixed with 15% honey may improve the motility, live-dead ratio, and viability of the liquid storage of epididymal fluid in rams. The egg yolk was found to be essential in protecting sperm from low-temperature effects.

DECLARATIONS

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Authors' contributions

Baqer Jafar Hasan conducted the conceptualization, as well as writing the original draft, investigation, methodology, formal analysis, and visualization. Hayder Aabd-al-Kareem Hasan Al-Mutar contributed to the investigation, project administration, and funding acquisition. Jawed Kadhum and Taher handled the investigation, data curation, and funding acquisition. Baqer Jafar Hasan contributed to the supervision, validation, formal analysis, and writing of the review and editing. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that there is no conflict of interest.

Ethical considerations

All authors have diligently reviewed the manuscript for potential ethical issues, including plagiarism, research misconduct, data fabrication or falsification, and redundant publication.

Availability of data and materials

All data generated during this research are pertinent and have been included in the published article. For further information or inquiries, please contact the corresponding author.

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