

Evaluation of epididymal and frozen sperm to produce goat embryos through *in vitro* fertilization

A. S. AbdElkhalek^{1,2}, N. Ghanem^{3*}, M. G. Soliman¹, N. A. Abu El Naga¹, A. M. Kamel²,
H. A. Shedeed², and K. A. El Bahrawy²

¹Zoology and Entomology Department, Faculty of Science, Al-Azhar University (Girls) Cairo, Egypt.

²Animal and Poultry Production Division, Desert Research Center, 11753, Cairo, Egypt.

³Department of Animal Production, Faculty of Agriculture, Cairo University, Giza, Egypt.

*Corresponding E-mail: nassergo@agr.cu.edu.eg

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ABSTRACT

Preservation of the livestock genetic makeup could be performed by application of *in vitro* fertilization (IVF) using spermatozoa collected from cauda epididymis. Therefore, this study assessed the development of goat embryos following IVF of spermatozoa collected from cauda epididymis compared to those kept frozen. Oocytes were *in vitro* cultured in a maturation medium for 24 hours at a temperature of 38.5°C, 5% CO₂, and 95% humidity. Following maturation, the oocytes (n = 370) were *in vitro* fertilized by fresh epididymal sperm (G1) and G2 frozen-thawed sperm, and the Fert-TALP medium was used as the IVF medium. In addition, *in vitro* developed embryos were cultured in GT-L medium. The fertilization rate, percentage of morula, and blastocytes (p<0.01) were significantly higher in oocytes inseminated with epididymal than in frozen sperm. In conclusion, *in vitro* embryo production of goat oocytes may be successfully performed using epididymal spermatozoa for IVF.

Keywords: Blastocysts, Epididymal sperms frozen-thawed sperm, Goat, IVF.

INTRODUCTION

Numerous applications of biotechnology seem to have apparent advantages to *in vitro* reproduction. The oocytes, or early embryos, are recovered *in vivo* using laborious, costly procedures. *In vitro* procedures can quickly and cheaply produce many oocytes for use in various *in vitro* reproductive biotechnology research projects since they employ easily accessible slaughterhouse ovaries. A potentially effective method of producing embryos *in vitro* using sexed semen is possible (Vettical, 2016).

By using IVF, progenies can be created from

both alive and deceased animals. Through the use of IVF procedures, endangered species, and uncommon cattle breeds can be preserved (Mastromonaco and Songsasen, 2020). The testicles and ovaries taken from dead or slain animals can be used to create *in vitro* embryos at a fair price. The caudal epididymal spermatozoa from dead animals or animals that have suffered substantial damage is essential to reproduce and conserve animal specimens with high genetic qualities. Additionally, if epididymal sperm are collected within 24 to 48 hours of death, the gamete is still viable for fertilization (Jebur, 2022). The status of semen (fresh or frozen)

plays an important role in the rate of success of IVF in cattle (Zidan *et al.*, 2022). However, others found that either fresh or frozen-thawed semen did not have an effect on the embryo production of prepubertal goat oocytes fertilized by intracytoplasmic sperm injection (ICSI), despite the differences in viability, acrosomal status, and sperm capacitation (Menéndez-Blanco *et al.*, 2020).

Promoting sperm capacitation and inducing the acrosome response *in vitro* is essential for thriving in vitro fertilization (Soria-Tiedemann *et al.*, 2022). Before developing the potential to fertilize, and in the female reproductive tract, mammalian spermatozoa undergo several physiological changes. The capacity for capacitation of ejaculated and epididymal spermatozoa varies. Ejaculate and ruminant seminal plasma both contain elements that might positively or negatively affect the spermatozoa's ability to fertilize an egg (Jebur, 2022). The current study aimed to determine the impact of using frozen and epididymal spermatozoa during IVF on the subsequent development of in vitro matured oocytes in Egyptian goats.

MATERIALS AND METHODS

Experimental Site

The current work was carried out at Embryology Manipulation Unit (EMU), Department of Animal and Poultry Physiology, Division of Animal and Poultry Production, Desert Research Center (DRC), Cairo, Egypt.

Ethical Endorsement

The conduct of this study adhered to the code of ethics and animal rights standards of the Desert Research Center, Ministry of Agriculture, Egypt. In addition, this work attempted to comply as closely as possible with the laws and standards outlined in the European Union directive for protecting experimental animals (2010/63/EU).

Chemicals and Media

Unless otherwise stated, the authors of this study purchased the chemicals that were used in this experiment from Sigma-Aldrich in the United States.

In Vitro Maturation (IVM)

Goat ovaries were obtained from a nearby abattoir to the laboratory in a normal saline solution of 0.9% NaCl within an hour at 35-37 °C (Wieczorek *et al.*, 2020). Cumulus-oocyte complexes (COCs) were recovered by slicing the ovarian surface in phosphate-buffered saline (PBS). We selected COCs with three or more dense cumulus cell layers and homogenous cytoplasm (Grade A+B). In the current study, the COCs were washed using TCM-Hepes medium supplemented with 10% fetal bovine serum (FBS). According to previous publications (Wieczorek *et al.*, 2020), COCs were cultured in groups of 25-30 in 100 µL drops of IVM medium (TCM-Hepes-free medium) supplemented with 50 µg/mL gentamycin, 10% FBS, 10 ng/mL epidermal growth factor (EGF), 1 µg/mL estradiol (E2), 0.25 mg/ml Na⁺ pyruvate, and 20 IU/mL pregnant mare serum gonadotropin (PMSG, Gonaser®, 500 IU) and 20 IU/mL human chorionic gonadotropin (hCG) hormone (choriomon®, 5000 IU). Mineral oil covered the media drops containing COCs for 24 hours at 38.5 °C in an atmosphere with 5% CO₂ and 95% humidity (Jose *et al.*, 2021).

Sperm Preparation of Epididymal Sperm

The IVF procedure was carried out according to Jebur (2022) with a few modifications. The sperm of adult male goats were recovered from the caudal epididymis directly into Tyrode's albumin lactate pyruvate (TALP). It is an electrolyte solution often used as a medium to induce capacitation in sperm, consisting of 100 mM NaCl, 3.1 mM KCl, 25 mM NaHCO₃, 0.29 mM NaH₂PO₄, 21.6 mM Na lactate, 2.0 mM CaCl₂, 1.5 mM MgCl₂, 10 mM HEPES and 10 mg/L phenol red. This TALP medium was supplemented with 0.6% BSA, 1 mM sodium pyruvate, and 50 µg/mL gentamicin (Ashour *et al.*, 2020). Sperm suspension was spun at 500 xg for 1 minute at room temperature. The spermatozoa pellet was incubated for 60 min at 38.5°C before fertilization in a 10 ml capacitation medium. The supernatant was carefully obtained and centrifuged. After the centrifugation, the pellet was resuspended again in a suitable amount of pre-warmed fertilization medium (Fert. TALP medium consisted of 100 mM NaCl, 3.1 mM KCl, 25 mM NaHCO₃, 0.29 mM NaH₂PO₄, 21.6 mM Na lactate, 2.0 mM CaCl₂ and 1.5 mM MgCl₂). Fi-

nally, the sperm concentration was adjusted to 1×10^6 sperm cells/mL (Jebur, 2022), as determined by the sperm concentration measured through the hemocytometer slide.

Sperm Preparation of Frozen Semen

Goat's frozen semen was obtained from the Animal Reproductive Research Institute - El Haram, Ministry of Agriculture, Giza, Egypt. The frozen semen in 0.25 mL straws was transported in liquid nitrogen to the laboratory. Although the concentration and characteristics of the frozen semen were known, a quick examination was conducted to evaluate the post-thawing characteristics before using it in the IVF process. Two 0.25 mL straws were thawed in a water bath at 37 °C for 40 seconds. The content of these straws was washed using sperm-TALP medium by centrifugation twice at 300 xg for 1 minute at room temperature. Then the sperm pellet was resuspended in 1 mL of fert-TALP medium. The concentration and viability were examined in a very short time. After that, the concentration of fertilization drop was adjusted to 1×10^6 sperm cells/mL containing 15 – 20 matured oocytes (Olivares *et al.*, 2015).

In vitro Fertilization

The *in vitro* matured COCs were first washed twice with the washing medium followed by washing three times with the fertilization medium. After this, the mature COCs were co-cultured with the prepared epididymal (G1) and frozen-thawed sperm (G2) in a four-well plate that contained 400 μ L sperm suspension in fertilization medium covered with 400 mL mineral oil per well at 38.5°C, 5% CO₂ and 95% humidity for 18 hrs (Ashour *et al.*, 2020).

In vitro Embryo Culture (IVC)

Presumptive zygotes were washed in pre-warmed ready-to-use commercial IVC medium (GT-L Vitrolife®; Gothenburg, Sweden) and then transferred into 100 μ L drops of GT-L medium covered with embryo-tested mineral oil in groups of 6–10 zygotes. Zygotes were cultured at 38.5 °C, 5% CO₂ and 95% humidity in air for nine days. An inverted microscope (Leitz Fluovolt FU; Leica Microsystems, Wetzlar, Germany) was used to examine a total of 370 culture zygotes (6 replicates) from both the two treat-

ments for cleavage at 48 hours post-IVF and embryo development (blastocyst formation) at day 7 post IVF (Hajian *et al.*, 2022).

Statistical Analysis

The data obtained in the current experiment were analyzed statistically by SAS Enterprise Guide 4 2008. A chi-square test and t-test were performed (after the angular transformation of the data), The analyzed data were expressed as mean \pm standard error (SE). Comparisons were significantly highly different if $P < 0.01$.

RESULTS AND DISCUSSION

Semen Analysis

In Table 1 and Figure 1, the mean percentage of sperm motility and live sperm in the fresh epididymal sperm (G1) (88.68 ± 1.12 and $91.33 \pm 0.88\%$ respectively) were significantly higher ($P < 0.01$) than the frozen-thawed sperm (G2) group (44.83 ± 2.01 and $48.33 \pm 2.26\%$, respectively). In the same trend, the mean of sperm cell concentrations of the G1 ($158.83 \pm 2.97 \times 10^6$ /mL suspension) was higher ($P < 0.01$) than the G2 group ($144 \pm 6.93 \times 10^6$ /mL suspension). The mean of dead sperm in G1 ($8.66 \pm 0.88\%$) was lower ($P < 0.01$) than in G2 ($51.67 \pm 2.26\%$). On the other hand, the mean abnormal sperm morphology in G1 was $25.33 \pm 1.89\%$, which was not significantly different ($P > 0.01$) when compared to G2 (27.5 ± 2.35). To the best of our knowledge, this is the first study to compare the impact of two sources of goat spermatozoa (frozen and epididymal) on subsequent development following *in vitro* maturation of Egyptian goat oocytes. In this study, the fresh epididymal sperm in the G1 group was superior in physical quality characteristics such as sperm motility and live sperm compared to the frozen-thawed sperm (G2) group. This could be attributed to that ruminant freshly collected epididymal semen contained seminal plasma at ejaculation that had elements that tend to enhance the spermatozoa's viability and fertilization ability (Peris-Frau *et al.*, 2020). However, when spermatozoa are cryopreserved in semen extenders this leads to harmful effects on their quality. The enzymes phospholipases A2 break down egg yolk lecithin in fatty acids and lysolecithin in the seminal plasma. When they interact with the phosphocaseinate component of skimmed milk-based extenders, they create

Table 1. Semen analysis of fresh epididymal and frozen-thawed sperms

Parameter	G1	G1	P value of t test
	Mean± S.E	Mean± S.E	
Sperm count (10 ⁶ /ml suspension)	158.83±2.97 ^a	144±6.93 ^b	0.028273*
Sperm motility (%)	88.68 ±1.12 ^a	44.83±2.01 ^b	P <0 .00001**
Viability	Live sperm (%)	48.33±2.26 ^b	P <0 .00001**
	Dead sperm (%)	8.66±0.88 ^b	51.67±2.2 ^a
Abnormal sperm morphology (%)	25.33±1.89 ^a	27.5±2.35 ^a	0.181355

Data recorded as mean±standard error (SE); a and b: superscripts to be compared statistical analysis. Values with different letter superscripts are **P value < 0.01 means highly significant. G1: fresh epididymal sperm; G2: frozen-thawed sperm.

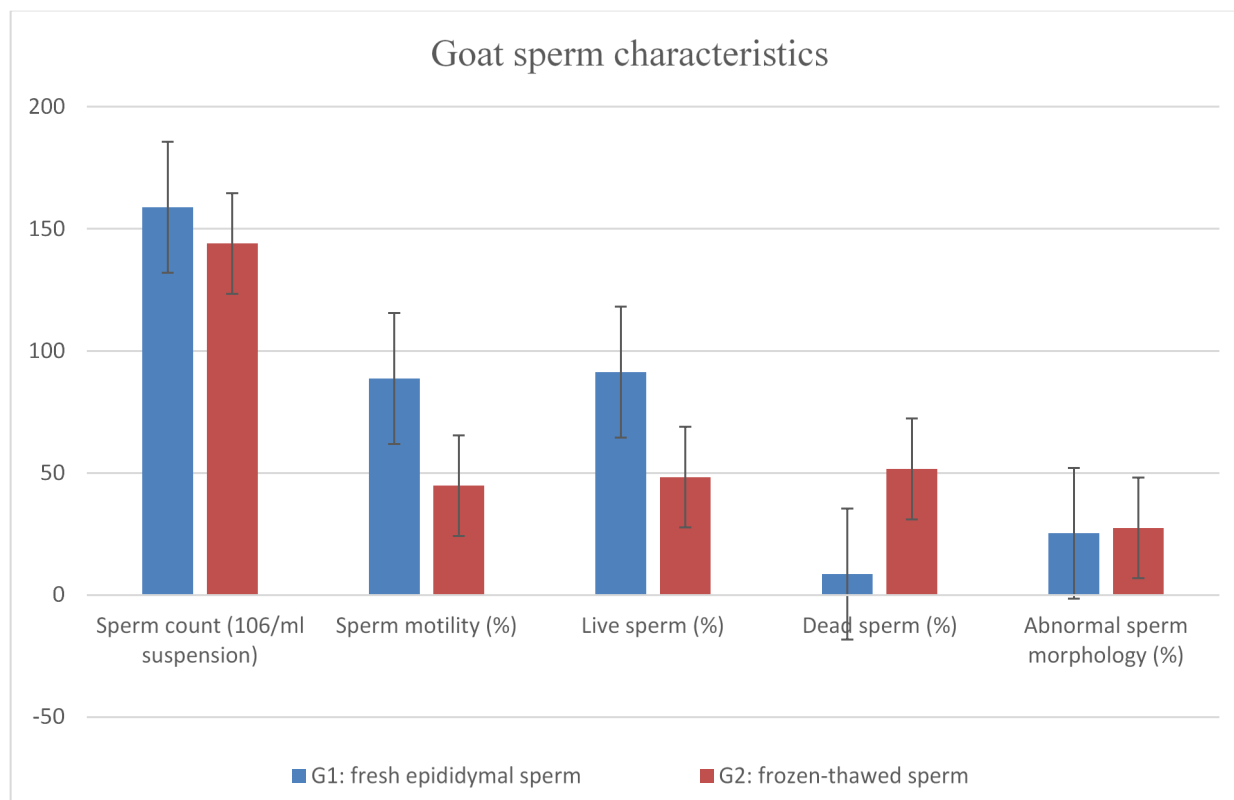


Figure 1. Semen analysis of preserved and epididymal sperms

harmful chemicals in spermatozoa (Sharma and Sood, 2020).

IVF and IVC Rates

After *in vitro* fertilization, the results indicated that there was a significant ($P < 0.01$) higher rate of zygote formation (according to the second polar body extrusion) in G1 than in G2 (42.74 ± 1.66 and 24.35 ± 1.42 %, respectively) as shown in Table 2 and Figure 2 and 4.

Following zygote *in vitro* culture, the number of cleaved zygotes was significantly higher ($p < 0.01$) in G1 than in G2 (38.45 ± 2.15 and 20.57 ± 1.16 %, respectively). This means that the source of sperm affects cleavage rates as shown in Table 3 and Figure 3 and 4. At the late

embryo development stages, the number of morula was significantly ($p < 0.01$) higher in G1 than in G2 (35.68 ± 1.73 and 15.67 ± 0.95 %, respectively). Therefore, the selection of the sperm source affects morula formation (Table 4 and Figure 3 and 4). In Table 5 and Figure 3 and 4, it was demonstrated that the number of blastocysts was significantly ($p < 0.01$) higher in G1 than it was in G2 (33.55 ± 2.06 and 11.4 ± 1.46 %, respectively).

The findings of this study revealed that the group of oocytes fertilized by fresh epididymal sperm (G1) had a higher rate of zygote formation (according to the second polar body extrusion) than the second group that fertilized by frozen-thawed sperm (G2). In addition, G1 had consid-

Table 2. Effect of the sperm state on zygote formation rate (%) in Egyptian goats'

Group	No. of culture oocytes	No. of fertilized oocytes	zygote formation rate	Chi-square value	P value of t-test
			Mean \pm SE	(P value)	
G1	185	79	42.74 \pm 1.66 ^a	14.02	P<0.0001**
G2	185	45	24.35 \pm 1.42 ^b	(0.0002**)	

Data recorded as mean \pm SE; a and b: superscripts to be compared statistical analysis. Values with different letter superscripts are **P value < 0.01 means highly significant. G1: fertilized oocyte by fresh epididymal sperm; G2: fertilized oocyte by frozen-thawed sperm.

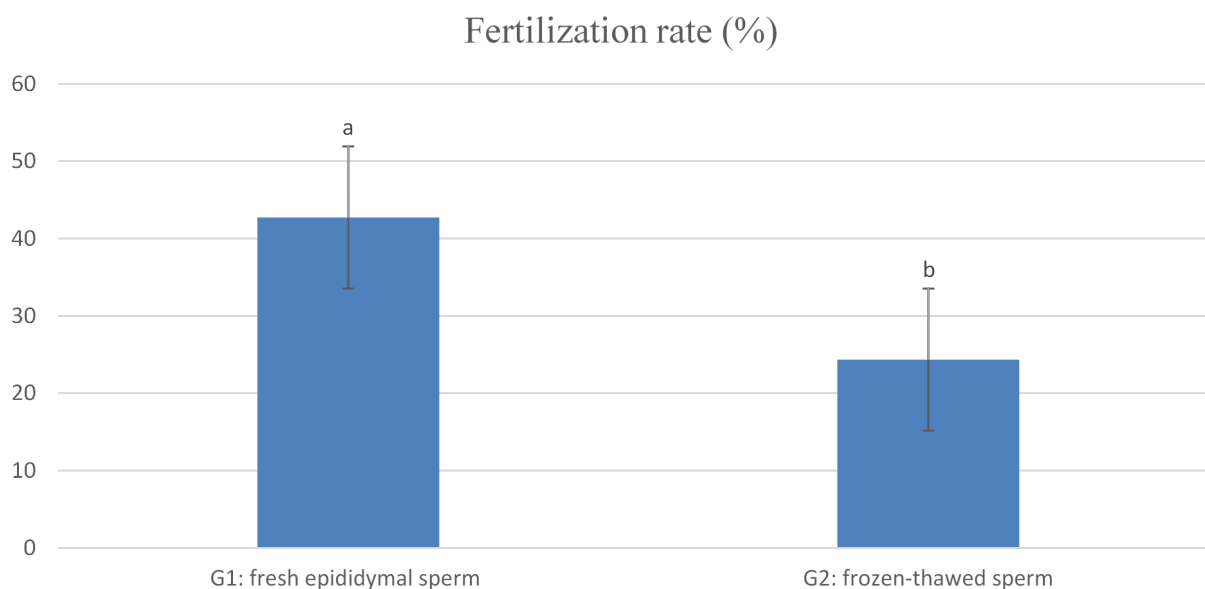


Figure 2. Effect of sperm state on IVF successful rate (%) in Egyptian goats'

erably more cleavage, morula formation, and blastocyst rates than G2.

The impact of seminal plasma on the ability of bull spermatozoa to fertilize in vitro has been the subject of numerous research (Indhu *et al.*, 2020). Bull spermatozoa's ability to fertilize oocytes in vitro was examined by Vertical (2016). Compared to the outcomes with freshly ejaculated semen, the in vitro embryo production efficiency with frozen-thawed epididymal spermatozoa was more significant for fertilization, cleavage rate, and blastocyst yield. Furthermore, compared to ejaculate sperm, the seminal plasma-deprived sperm demonstrated considerably higher rates of oocyte fertilization, cleavage, and development to the blastocyst stage

It is believed that the *in vitro* production of embryos had been improved by the absence of seminal plasma during capacitation. Therefore, inhibition of several acrosomal enzymes has been proposed to cause the seminal plasma's reversible antifertility impact. According to several

reports conducted on various species, including cattle, seminal plasma may contain elements that affect male fertility (Anamthathmakula and Winuthayanon, 2020). According to Bansal *et al.* (2023), these elements are generally thought to suppress sperm capacitation, the acrosome reaction, or acrosomal enzymes and eventually obstruct fertilization. On the other hand, numerous studies have shown the beneficial effects of seminal plasma in the capacitation of bull sperm, markedly increasing the number of heparin-binding sites (Contreras *et al.*, 2023).

In addition, mammalian spermatozoa are vulnerable to cryo-injury produced by cryopreservation procedures. Furthermore, the variables that cause cryo-injuries are challenging, and the process that leads to cryo-damage has not been fully explored till now, which has a direct impact on the quality of frozen-thawed spermatozoa (Khan *et al.*, 2021). Therefore, the low results of preimplantation development in the frozen group when compared to the epididymal group could be

Table 3. Influence of sperm state on the cleavage rate of in vitro fertilized oocytes in Egyptian goats

Group	No. of culture oocytes	No. of fertilized oocytes	cleavage rate Mean \pm SE	Chi-square value (P value)	P value of t-test
G1	185	71	38.45 ^a \pm 2.15	14.16 (0.0002**)	P<0.0001**
G2	185	38	20.57 ^b \pm 1.16		

Data recorded as mean \pm standard error (SE); a and b : superscripts to be compared statistically within the same column. Values with different letter superscripts are **P value < 0.01 means highly significant. G1: IVF by fresh epididymal sperm; G2: IVF by frozen-thawed sperm

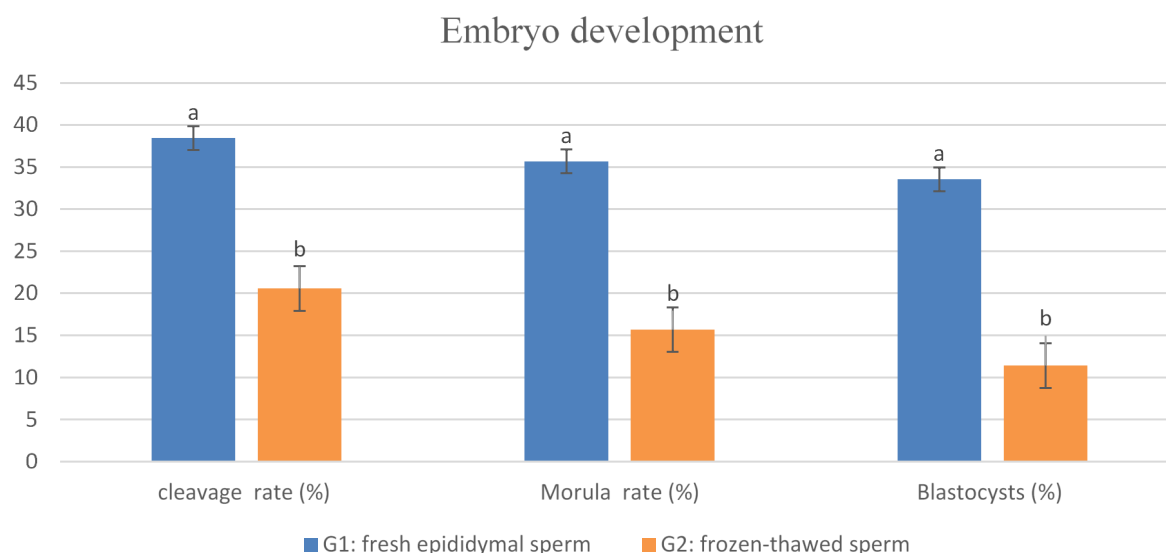


Figure 3. Effect of the sperm state on embryo development in Egyptian goats

attributed to cryo-damage stress of sperm cells.

CONCLUSION

The study's results suggest that epididymal spermatozoa recovered from abattoir slaughtered bucks could be used effectively as or even better than frozen-thawed semen to fertilize in vitro mature goat oocytes. This technique provides a suitable method for the IVP of embryos from wild animals, endangered species, and other animals in which the collecting of ejaculated semen is a challenging undertaking, in which cauda epididymal spermatozoa might be used.

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REFERENCES

- Anamthathmakula, P., and W. Winuthayanon. 2020. Mechanism of semen liquefaction and its potential for a novel non-hormonal contraception. *Biol Reprod.* 103(2): 411-426. <https://doi.org/10.1093/biolre/ioaa075>
- Ashour, G., A. El-Sayed, M. Khalifa and N. Ghanem. 2020. Effect of heat stress on developmental competence of in vitro matured oocytes of camelus dromedaries with different qualities. *World Vet. J.* 10: 658-664.

Table 4. Effect of sperm state on morula rate formation(%) in Egyptian goats'

Group	No. of culture oocytes	No. of morula oocytes	Morula rate formation Mean \pm SE	Chi-square value (P value)	P value of t-test
G1	185	66	35.68 ^a \pm 1.73	19.39	P<0.0001**
G2	185	29	15.67 ^b \pm 0.95	(P<0.0001**)	

Data recorded as mean \pm standard error (SE); a and b: superscripts to be compared statistically within the same column. Values with different letter superscripts are **P value < 0.01 means highly significant. G1: IVF by fresh epididymal sperm; G2: IVF by frozen-thawed sperm.

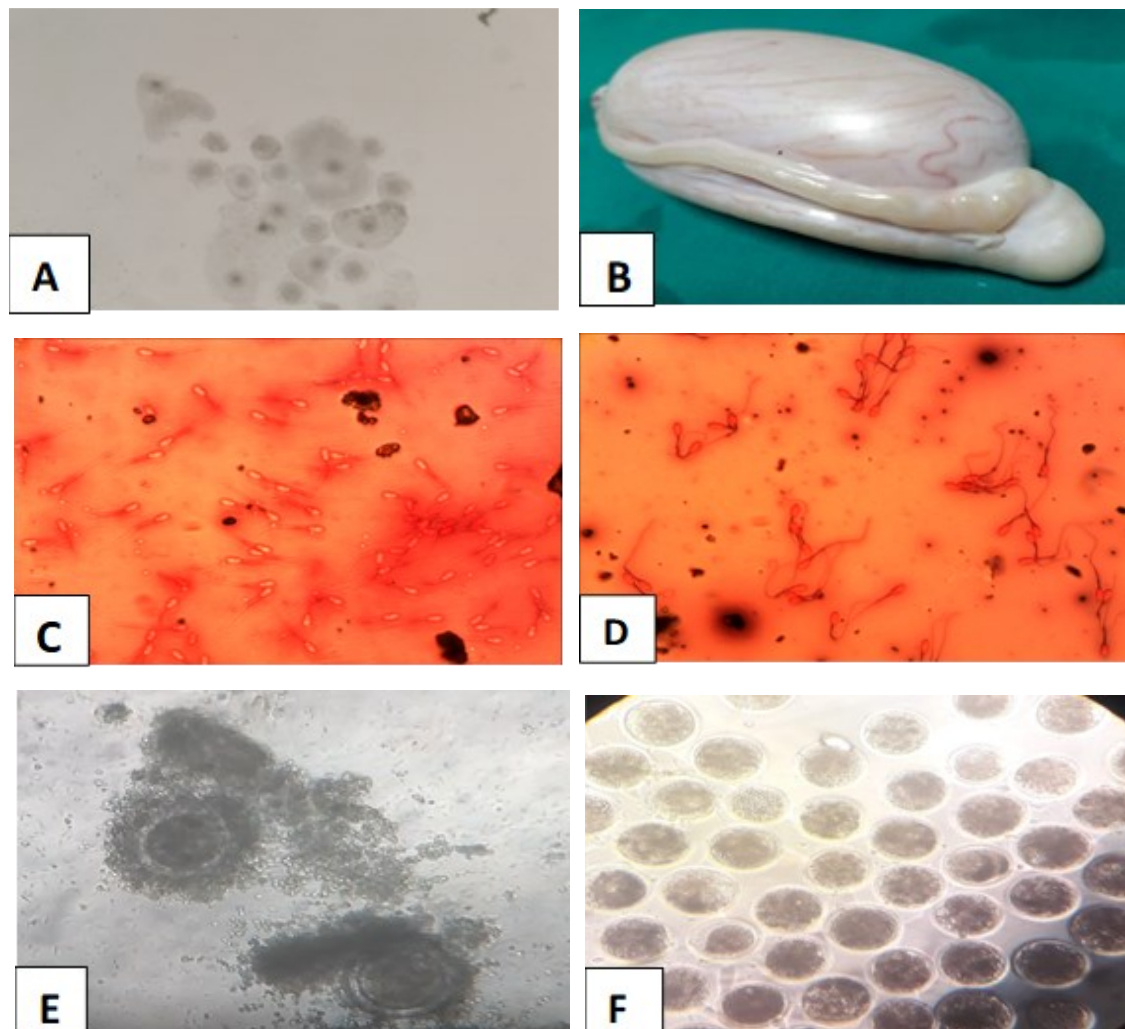


Figure 4. *In vitro* embryo production of goats. **A:** goats' oocytes after maturation; **B:** testes of Buk; **C:** life epididymal sperm ; **D:** dead epididymal sperm; **E:** oocytes incubated with sperm and **F:** different stages of the goat's embryo.

<https://doi.org/10.54203/scil.2020.wvj79>
 Bansal, K., G.B. Upender, M. Meena, M. Meel, and P. Kumar. 2023. Proteins and enzymes related to sperm motility, capacitation and acrosome reaction. *Int. J. Appl. Sci. Biotechnol.* 4(2): 166-171. <https://www.thepharmajournal.com/archives/2023/vol12issue7S/PartU/S-12-7-342-612>.
 Contreras, M. J., M.E. Arias, F. Fuentes, E.

Muñoz, N. Bernecic, S. Fair and R. Felmer. 2023. Cellular and molecular consequences of stallion sperm cryopreservation: Recent approaches to improve sperm survival. *J. Equine Vet. Sci.* 104499. <https://doi.org/10.1016/j.jjevs.2023.104499>
 Hajian, M.,F. Jafarpour, S.M. Aghamiri, S.R. Varnosfaderani, M.R. Andani and M.H. Nasr-Esfahani. 2022. The impact of two

Table 5. Effect of sperm state on blastocysts rate formation(%) in Egyptian goats'

Group	No. of culture oocytes	No. of blastocysts oocytes	Blastocysts (%) Mean \pm SE	Chi-square value (P value)	P value of t-test
G1	185	62	33.55 ^a \pm 2.06	26.11(P<0.0001**
G2	185	21	11.4 ^b \pm 1.46	P<0.0001**)	

Data recorded as mean \pm stander error (SE); a and b: superscripts to be compared statistically analysis. Values with different letter superscripts are **P value < 0.01 means highly significant. G1: IVF by fresh epididymal sperm, and G2: IVF by frozen-thawed sperm.

- embryo culture media, synthetic oviduct fluid and commercial BO, on pre-and post-implantation development of cloned Saanen goat embryos. *Int. J. Fertil. Steril.* 16(1): 23. doi: 10.22074/IJFS.2021.531302.1130
- Indhu, M.S., M. Ramamoorthy, S. Pandey, K. Mathesh, M. Mahawar, M. Sarkar, S.K. Ghosh, G.T. Sharma and S.K. Bhure. 2021. Improved quality and fertilizability of cryopreserved buffalo spermatozoa with the supplementation of methionine sulfoxide reductase A. *Andrology* 9(6):1943-1957. <https://doi.org/10.1111/andr.13080>
- Jebur, A. 2022. Effect of epididymal spermatozoa of local iraqi goat on in vitro fertilization and evolution of embryos. *Arch. Razi Inst.* 77(2): 641. doi: 10.22092/ARI.2022.357269.2008
- Jose, B., M.K. Tripathi, A. Kumar and P. Kumar. 2021. In-vitro embryo production in caprine using slaughterhouse ovaries. *Pharm. Innov. J.* 10(4S): 11-14. <https://www.thepharmajournal.com/archives/2021/vol10issue4S/PartA/S-10-3-32-850>.
- Khan I.M., Z. Cao, H. Liu, A. Khan, S.U. Rahman, M.Z. Khan, A. Sathanawongs and Y. Zhang. 2021. Impact of cryopreservation on spermatozoa freeze-thawed traits and relevance OMICS to assess sperm cryotolerance in farm animals. *Front Vet. Sci.* 8: 609180. doi: 10.3389/fvets.2021.609180.
- Mastromonaco, G.F. and N. Songsasen. 2020. Reproductive technologies for the conservation of wildlife and endangered species. n: Presicce GA, editor. *Reproductive technologies in animals*. Amsterdam: Academic Press; 2020. pp. 99-117. <https://doi.org/10.1016/B978-0-12-817107-3.00007-2>
- Menéndez-Blanco, I., S. Soto-Heras, M.G. Catalá, A.R. Piras, D. Izquierdo and M.T. Paramio. 2020. Effect of vitrification of in vitro matured prepubertal goat oocytes on embryonic development after parthenogenic activation and intracytoplasmic sperm injection. *Cryobiology* 93: 56-61. <https://doi.org/10.3390/ijms21082781>
- Olivares, C. C. S., da Fonseca, J. F., de Almeida Camargo, L. S., de Souza-Fabjan, J. M. G., Rodrigues, A. L. R., and Brandão, F. Z. 2015. Comparison of different methods of goat sperm selection and capacitation for optimization of assisted reproductive technologies. *Small Rumin. Res.* 127: 44-49. <https://doi.org/10.1016/j.smallrumres.2015.04.009>
- Peris-Frau, P., A.J. Soler, M. Iniesta-Cuerda, A. Martín-Maestro, I. Sánchez-Ajofrín, D.A. Medina-Chávez, M.R. Fernández-Santos, O. García-Álvarez, A. Maroto-Morales, V. Montoro and J.J. Garde. 2020. Sperm cryodamage in ruminants: understanding the molecular changes induced by the cryopreservation process to optimize sperm quality. *Int. J. Mol. Sci.* 21(8): 2781. <https://doi.org/10.3390/ijms21082781>
- Sharma, A. and P. Sood. 2020. Caprine semen cryopreservation and the factors affecting it: an overview. *Vet. Sci. Res. Rev.* 6(1): 46-57. <http://dx.doi.org/10.17582/journal.vsr/2020/6.1.46.57>
- Soria-Tiedemann, M., G. Michel, I. Urban, M. Aldrovandi, V.B. O'Donnell, S. Stehling, H. Kuhn and A. Borchert. 2022. Unbalanced expression of glutathione peroxidase 4 and arachidonate 15-lipoxygenase affects acrosome reaction and In Vitro fertilization. *Int. J. Mol. Sci.* 23(17): 9907. <https://doi.org/10.3390/ijms23179907>
- Vettical, B. S. 2016. In vitro fertilizability of oocytes using fresh, frozen and epididymal spermatozoa from crossbred bulls in the tropics. *Int. J. Appl. Sci. Biotechnol.* 4(2): 166-171. <https://api.semanticscholar.org/CorpusID:53630539>
- Wieczorek, J., J. Koseniuk, M. Skrzyszowska

and M. Cegła. 2020. L-OPU in goat and sheep different variants of the oocyte recovery method. *Animals*. 10(4): 658- 668. <https://doi.org/10.3390/ani10040658>
Zidan, G., T.M. EL-sherry, K.A. Amein, H.A. Daghash and G.B. Mahmoud. 2022. Effect

of season on oocytes and in vitro fertilization by fresh and frozen semen of cattle. *Assiut J. Agri. Sci.* 53(5): 1-12. DOI: 10.21608/ajas.2022.159419.1172