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

A. S. AbdElkhalek¹², N. Ghanem³*, M. G. Soliman¹, N. A. A. 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

Received: September 26, 2023; Accepted: March 12, 2024

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).
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, Gnosier®, 500 IU) and 20 IU/mL human chorionic gonadotropin (hCG) hormone (chorionom®, 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 prewarmed 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 \(1 \times 10^6\) 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 \(1 \times 10^6\) 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 µL 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 µL 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 Fluovert FU; Leica Microsystems, Wetzlar, Germany) was used to examine a total of 370 culture zygotes (6 replicates) from both the two treatments 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 ± 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±0.88% respectively) were significantly higher (\(P<0.01\)) than the frozen-thawed sperm (G2) group (44.83±2.01 and 48.33±2.26%, respectively). In the same trend, the mean of sperm cell concentrations of the G1 (158.83±2.97*10^6/mL suspension) was higher (\(P<0.01\)) than the G2 group (144±6.93*10^6/mL suspension). The mean of dead sperm in G1 (8.66±0.88 %) was lower (\(P<0.01\)) than in G2 (51.6±2.26 %). On the other hand, the mean abnormal sperm morphology in G1 was 25.33±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 could be responsible for the difference between the two groups. Spermatozoa 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 lysolcithin in the seminal plasma. When they interact with the phosphocaseinate component of skimmed milk-based extenders, they create...
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 (p<0.01) higher 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>
<thead>
<tr>
<th>Parameter</th>
<th>G1 Mean± S.E</th>
<th>G2 Mean± S.E</th>
<th>P value of t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm count (10^6/ml suspension)</td>
<td>158.83±2.97</td>
<td>144±6.93</td>
<td>0.028273*</td>
</tr>
<tr>
<td>Sperm motility (%)</td>
<td>88.68±1.12</td>
<td>44.83±2.01</td>
<td>P &lt;0.00001**</td>
</tr>
<tr>
<td>Viability</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Live sperm (%)</td>
<td>91.33±0.88</td>
<td>48.33±2.26</td>
<td>P &lt;0.00001**</td>
</tr>
<tr>
<td>Dead sperm (%)</td>
<td>8.66±0.88</td>
<td>51.67±2.26</td>
<td>P &lt;0.00001**</td>
</tr>
<tr>
<td>Abnormal sperm morphology (%)</td>
<td>25.33±1.89</td>
<td>27.5±2.35</td>
<td>0.181355</td>
</tr>
</tbody>
</table>

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.
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 (Ananthathmakula 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>
<thead>
<tr>
<th>Group</th>
<th>No. of culture oocytes</th>
<th>No. of fertilized oocytes</th>
<th>zygote formation rate Mean ±SE</th>
<th>Chi-square value (P value)</th>
<th>P value of t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>185</td>
<td>79</td>
<td>42.74±1.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.02&lt;sup&gt;(0.0002**)&lt;/sup&gt;</td>
<td>P&lt;0.0001**</td>
</tr>
<tr>
<td>G2</td>
<td>185</td>
<td>45</td>
<td>24.35±1.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data recorded as mean ± 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.
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.

**ACKNOWLEDGMENTS**

This project was supported financially by the Science and Technology Development Fund (STDF), Egypt, Grant No.44513. Also, thanks to Prof. Dr. Ali Saber for assisting in the statistical analysis of this work. Ms. Amira Salem AbdElkhalek completed every laboratory task, data analysis, and manuscript writing. The work plan was created, by Dr. Nehal Ali Abu Naga, Dr. Khalid Ahmed El Bahrawy and Dr. Nasser Ghanem. Dr. Nasser Ghanem assisted with laboratory work, manuscript writing and revision. Dr. Maha Ghazi Soliman edited the material and assisted in creating the work plan. Dr. Khalid Ahmed El Bahrawy assisted with work environments, work plan formulation, and manuscript revision. Dr. Ahmed Mohamed Kamel contributed to creating the work plan, laboratory work, and manuscript revision. Dr. Hesham Attia Shedeed assisted in the laboratory work and collecting of biological samples.

**REFERENCES**


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

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of culture oocytes</th>
<th>No. of morula oocytes</th>
<th>Morula rate formation Mean ±SE</th>
<th>Chi-square value (P value)</th>
<th>P value of t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>185</td>
<td>66</td>
<td>35.68±1.73</td>
<td>19.39</td>
<td>P&lt;0.0001**</td>
</tr>
<tr>
<td>G2</td>
<td>185</td>
<td>29</td>
<td>15.67±0.95</td>
<td>(P&lt;0.0001**)</td>
<td>P&lt;0.0001**</td>
</tr>
</tbody>
</table>

Data recorded as mean± 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.
Table 5. Effect of sperm state on blastocysts rate formation(%) in Egyptian goats'

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of culture oocytes</th>
<th>No. of blastocysts oocytes</th>
<th>Blastocysts (%) Mean ±SE</th>
<th>Chi-square value (P value)</th>
<th>P value of t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>185</td>
<td>62</td>
<td>33.55±2.06</td>
<td>26.11</td>
<td>P&lt;0.0001**</td>
</tr>
<tr>
<td>G2</td>
<td>185</td>
<td>21</td>
<td>11.4±1.46</td>
<td></td>
<td>P&lt;0.0001**</td>
</tr>
</tbody>
</table>

Data recorded as mean± 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.


Wieczorek, J., J. Koseniuk, M. Skrzyszowska