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Development Potential of Morphologically Selected Egyptian Baladi Goat Oocytes Under Different Culture Media

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Abstract

OOCYTE QUALITY and culture conditions affect *in vitro* embryo development. Thus, this study examined how oocyte quality and culture media affect goat embryonic development. In the first experiment, zygotes were cultured in basic (G1), synthetic oviduct fluid (SOF; G2), and GT-L™ (G3) media. Additionally, embryonic development was monitored for seven days. Morphologically, the second experiment split 730 cumulous oocyte complexes (COCs;6 repetitions) into (G1) good-quality and (G2) low-quality groups. COCs matured and fertilized *in vitro*, and the best medium from the first experiment was used to produce presumptive zygotes *in vitro*. The nuclear maturation rate was assessed by Hoechst staining. Two investigations examined embryonic development by measuring cleavage, morula, and blastocyst formation. Data from this study indicated that the embryonic development rate (cleavage and blastocyst rates) was higher in the group cultured with G-TL™ (50.56 and 42.83%, respectively) than in basic (5.96 and 2.64%, respectively) and SOF media (23.1 and 12.25% respectively). In the second experiment, the nuclear maturation rate (extrusion of first polar body (non-invasive assessment; morphology) and Metaphase II % (invasive assessment by Hoechst staining) was significantly increased ($P<0.05$) in good (29.79 and 46.67%) compared to bad COCs (9.33 and 13.33%). A higher proportion ($P\leq0.01$) of oocytes with diffuse mitochondria distribution (increased level of mitochondrial aggregation around the nucleus (central) indicates oocyte maturation) was observed in good (66.67 %) than low-quality COCs (6.64%). In conclusion, selecting good quality oocytes and culturing presumptive zygotes in G-TL™ medium improved goat *in vitro* embryo production (IVP).

Keywords: Goats, oocyte quality, IVM, IVF, IVC, SOF medium; G-TL™.

Introduction

Goats are termed "poor man's cows" because they produce meat, milk, and leather items. Their rubbish may be composted and used as fertilizer [1]. Assisted reproductive technologies (ART) are applied in many animal species to accelerate genetic improvement. Due to their short interval, prolificacy, and cost-effective upbringing, small ruminant species like sheep and goats receive greater attention than large species regarding the application of ART [2]. In addition, production of embryos *in vitro* (IVP) is practical tool in getting large number of embryos from elite female [3]. Taken into consideration, the culture media must contain the chemical and physiological components needed for mammalian cell viability *in vitro* [4,5]. Numerous factors, including the quality of COCs and the surrounding culture, have an impact on the success rate of IVP [6,7]. Therefore, extensive research has discovered the optimum *in vitro* maturation (IVM) and culture parameters to create high-quality embryos that maintain pregnancy at a high rate after transfer [5,8].

IVM is the first step in the IVP procedure, which involves cumulus bulk expansion, nuclear advancement, and cytoplasmic maturation [9,10]. IVM requires an optimum culture environment and

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high-quality COCs [11]. Indeed, The selection of exceptional and high-quality oocytes containing cumulus cells (Cs) improved the developmental competency of embryo development [12,13]. Thus, since the 19th century, embryologists have assessed oocyte quality subjectively by light microscopic inspection [14]. Morphological evaluation is still among noninvasive tools. Before IVM, cumulus expansion morphology was often used as a selection criterion [14,15].

During IVM, Cs provide messenger molecules, energy substrates, and nutrients that support oocyte competence [15,16]. However, Growing cytoplasmic reactive oxygen species (ROS) significantly impede oocyte maturation and embryo development; the degree of this effect is dependent in part on the type of medium employed [17, 18, 19, 20]. Noteworthy, the culture medium used during preimplantation embryos requires the inclusion of growth stimulants, antioxidants, and nutrients, which support development and enhance embryo quality [21,22,23].

Many commercial culture mediums for human embryo cultivation exist. Although numerous commercially available media were evaluated in mouse models, few studies analyzed them during goat preimplantative stages of development of an embryo [24,25]. Thus, the goal of this study is to assess how the quality of the oocytes and the type of culture medium affect the rate and caliber of development of Egyptian Baladi goat embryos created *in vitro*.

Material and Methods

Ethics approval

The ethical committee approved all the experimental protocols at the Institutional Animal Care and Use Committee at Cairo University (CU-IACUC). The given identification code was CuIIF6523.

Experimental design

This research was conducted in two experiments (Fig.1). The first was to test the goat embryo development rate *in vitro* under three different culture media. The trial has three groups: TCM-199 (G1), SOF (G2), and G-TL™ (G3). The second experiment examined how the oocyte grade affected goat embryo development in the optimal medium from the first experiment. Therefore, two experimental groups were used: Good (G1) (homogenous cytoplasm with more than 3 cumulus cell layers) and low quality COCs (G2).

Production of embryos in vitro

All of the media, chemicals, and other ingredients came from Sigma-Aldrich in Germany. A regular visit was done to the slaughterhouse to get Egyptian Baladi goat ovaries. The abattoir was located in Cairo, Egypt, and the collected goat ovaries had unknown reproductive history and were transferred to the lab within 10-15 minutes after death in physiological saline. In total, 920 high-quality COCs from ovaries were employed to recover and mature oocytes in vitro, according to our recent publication [26] and the procedure of Maksura et al. [27]. Epididymal sperm was used for IVF of mature oocytes. According to AbdElkhalek et al.[28], sperm from mature male goats was collected from the caudal epididymis and subjected to in vitro culture. The fertilized oocytes were cultivated in vitro for seven days under conditions of 38.5 degrees Celsius, 5% carbon dioxide tension, and maximum humidity (100%). The first experiment employed TCM-199 (G1), SOF (G2), and G-TLTM (G3); the second experiment used just G-TLTM. Refill TCM-199 and SOF media was performed every 48 hours. Due to its single-step nature, the G-TLTM medium only needs to be replaced once.

Procedures for mitochondria staining

All samples (oocytes and embryos) were washed twice with PVP-PBS and then fixed with 4% paraformaldehyde. Nuclear staining, lipid cytoplasmic content, and mitochondrial activity were measured according to Ghanem et al. [29] using Hoechst 33342®, Mito-tracker green® and Nile red probes. The fluorescent images were examined under a fluorescence microscope (VE-146YT, USA).

Statistical analysis

Experiment data was analyzed using SAS Enterprise Guide 4. The information is shown as mean ±SE, and the significant level was expressed with superscripts in the same column for statistical comparison. Value differences with distinct letter superscripts (a,b, and c) are statistically significant ($P \leq 0.01$). Chi-square was used to evaluate percentage-based data. To find group differences, the data were converted to an angle and then subjected to a one-way ANOVA.

Results

Experiment 1. Types of *in vitro* culture media impact on goat embryo development.

In vitro development of goat embryos

Zygotes cultivated in G-TL^{TM} medium (G3) had a greater cleavage rate ($p<0.01$) than those cultured in SOF (G2) and TCM-199 (G1), with values of 50.56%, 23.16%, and 5.96% as shown in Table 1. The G3 group showed a greater rate of morula and blastocyst development ($p<0.01$) compared to other groups (Table 1). The morula

formation rates for G1, G2, and G3 were 3.05%, 15.07%, and 49.53%. Additionally, Table 1 shows blastocyst formation rates for the same groups: 2.64%, 12.25%, and 42.83%.

Immunofluorescence staining results

The immunofluorescence labeling of mitochondrial intensity indicated that G1 had the lowest ($p<0.01$) lipid intensity and total number of embryonic cells, whereas G3 had the highest value (59.25, 18.73, and 32.63 vs. 106.1, 45.33, and 62.86, respectively) as shown Table 2 and Figure 2. In contrast, the G2 group results for the same parameters were 83.69, 28.93, and 43.13, respectively.

Experiment 2 :Effect of quality goats' oocytes

In vitro maturation

G1 oocytes (78.63 ±3.00%) showed a greater cytoplasmic maturation rate ($p<0.01$) compared to G2 oocytes (53.82 ±1.24%) as indicated by cumulus expansion. The nuclear maturation rate was calculated using initial polar body extrusion, which showed the same trend. Table 3 revealed that G1 had a greater maturation rate (29.79 ±2.19%) than G2 (9.33 ±1.29%) ($P<0.01$).

Immunofluorescence staining of matured goat oocytes

The levels of mitochondrial and lipid intensity were significantly greater ($P<0.01$) in G1 (92.57 and 32.77) compared to G2 (52.64 and 22.14), respectively, as shown in Table 4 and Figure 3. In addition, significant differences ($P\leq0.01$) were observed in the distribution of functional mitochondria throughout the cytoplasm of goat oocytes that were matured in vitro, as indicated in Table 5. The G2 group had a significantly higher number of oocytes with peripheral mitochondria than the G1 group, with an increase of 80% ($P\leq0.01$). The G1 group had only 13.33% of oocytes with peripheral mitochondria. G1 exhibits a 20% increase in semi-peripheral active mitochondria compared to G2, which only has a 13.33% presence of such mitochondria. Table 5 shows that the G1 group had a substantially more significant percentage (66.67%) of oocytes with distributed mitochondria compared to the G2 group (6.64%) ($P\leq0.01$).

The percentage of goat oocytes in metaphase I and II was significantly greater ($P<0.01$) in G1 (33.33% and 46.67%) compared to G2 (13.33% and 13.33%), as observed through nuclear chromatin staining (Table 6/Figure 4). In the G2 group, a significantly higher proportion of oocytes (40% vs. 6.67%) persisted at the germinal vesicle (GV) stage compared to the G1 group ($P<0.01$).

IVF results

The G1 group of high-quality oocytes exhibited a higher rate of zygote formation ($P<0.01$) due to second polar body extrusion (23.8% vs. 8.12%, respectively) after *in vitro* fertilization (Table 7 and Figure 5). In the cleavage stage, the G1 group had more cleaved embryos (19.98±1.21 vs. 8.12±0.75%, $p<0.01$) than the G2 group. At the morula stage, the G1 group generated more morulae ($p<0.01$) than the G2 group (17.79±0.9 and 5.94±0.96%, respectively). The G1 group had a considerable ($p<0.01$) increase in blastocyst numbers compared to the G2 group (16.21±0.72 vs. 4.32 ± 0.68 %).

Immunofluorescence staining of embryos affected by oocyte quality

As shown in Table 8 and Figure 6, the G1 group had higher mitochondrial intensity, lipid intensity, and total cell number ($P\leq0.05$) compared to the G2 group (98.97±0.64, 38.06± 0.54 and 55.9±0.78 vs. 57.85±1.02, 17.22±1.25, and 42.07±0.5, respectively).

Discussion

The quality of the oocyte is a crucial element that significantly affects embryo development [30]. Data from this investigation revealed that high-quality COCs had a higher cleavage rate than low-quality COCs. Moreover, the prospective fertilized eggs in the low-quality eggs barely reached 8-16 cell stages. In contrast, COCs of good quality proceeded to morulae and blastocyst stages at higher rates than low quality counterparts, confirming improved developmental ability. In support of the data of this investigation, the appearance of PB1 was linked with increased porcine embryonic development *in vitro* [31].

Morphological assessment of COCs affects embryonic development *in vitro* until blastocyst formation [32]. Oocytes recovered from Pre-pubertal ovaries are of poor quality [33]. However, Kątska-Książkiewicz *et al.* [34] found that selecting adult goat COCs with BCB (brilliant cresyl blue) did not improve developmental competence following the IVF procedure. This may highlight the difference between morphological selection compared to BCB evaluation of COCs.

In addition, the score of cumulus expansion is used as a criterion for oocyte maturation evaluation [35]. However, it was indicated that the expansion of Cs is not related to embryonic development [36,37]. The mitochondria in mature oocytes are typically evenly distributed throughout the cytoplasm, ensuring uniform distribution of mitochondria throughout zygote cells following fertilization. Research has demonstrated a positive correlation

between a greater concentration and arrangement of functional mitochondria and accelerated nuclear maturation [38].

Indeed, morphological selection is mainly judged based on the intensity of Cs. Studies in mice [39], rats [40], cattle [41], and pigs [42]have shown the importance of Cs in enhancing oocyte quality to support early embryonic development. These studies suggest that removing Cs before IVM or IVF impairs fertilization and embryo development. Mammalian embryos' early growth depends on the intrinsic COC quality, which is regulated by the environment of the embryo culture [43,44]. Our study's findings supported this concept by demonstrating that, in comparison to the SOF (G2) and the TCM-199 media groups (G1), the commercial G-TL medium (G3) increased goat embryo cleavage, morula formation, and blastocyst development. These results could be due to the manual preparation of SOF and TCM-199 media in the laboratory, which also involves supplying these two media every 48 hours.

In contrast, well-adjusted G-TL media is a commercially available medium that is ready to use without further steps. Additionally, G-TL is a commercial medium that was made to be more similar to the composition of the reproductive tract in order to support cleaving embryos until blastocyst formation [45]. These discoveries corroborate the concept that the suitable culture medium will provide the embryos with the required nutrients, which subsequently enhance their development [46]. Moreover, minimizing the duration of external manipulation in the incubator can potentially improve the efficiency of blastocyst formation. Embryos nearby create autocrine/paracrine supportive factors.

Maintaining stable culture conditions can enhance the concentration of these substances and increase the chances of embryo survival [47]. The one-step culture system has a minor impact on pH and temperature, potentially creating more favorable conditions for the development of embryos [48]. In addition, it was found that commercial media reduced the variation of a laboratory-made medium from one batch to another [21]. However, despite the disturbance to the incubation conditions, it is considered that changing the media every 48 hours has beneficial effects as it prevents the accumulation of hazardous chemicals [49, 21]. These outcomes are consistent with those of Mohd-Fazirul *et al*. [50], who examined three commercial media in mice embryos developing from the zygote to the blastocyst stage: basic medium M16, human tubal fluid (HTF), and potassium (K+) simplex optimized medium (KSOM). They found no appreciable differences between the three tested media. According to reports by López-Pelayo *et al*. [51] and Stimpfel *et al*. [52], the culture system utilized during IVC can significantly impact pre-implantation stage embryo development. The success of IVP depends on the viability of the embryos during IVC. From fertilization until the blastocyst stage, significant developmental events take place, and the selection of culture mediums supports these activities.

Conclusion

Based on the study findings, selecting good-quality oocytes and culturing presumptive zygotes in commercial medium (G-TL^{TM}) improved goat IVP.

Acknowledgments

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Authors' Contributions

Dr. Amira Salem AbdElkhalek fulfilled all laboratory assignments, conducted data analysis, and wrote the manuscript. Dr. Nehal Ali Abu ElNaga devised the work plan. Dr. Nasser Ghanem supported conducting laboratory experiments and preparing and revising the manuscript. Dr. Maha Ghazi Soliman revised the content and aided in developing the project schedule. Dr. Khalid Ahmed El Bahrawy provided support in the areas of work settings, work plan development, and manuscript editing. Dr. Ahmed Mohamed Kamel played a role in developing the work strategy, conducting laboratory experiments, and revising the text. Dr. Sarah Ali Ghanem Althubyani and Dr. Fatma El-Saeed El-Demerdash assisted in conducting laboratory experiments and gathering biological specimens.

Funding statement

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Declaration of Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical of approval

The ethical committee approved all the experimental protocols at the Institutional Animal Care and Use Committee at Cairo University (CU-IACUC). The given identification code was CuIIF6523.

TABLE 1. Different in vitro culture mediums affect goat embryo development

Groups	Total COCs (N)	No. of replicates	Cleavage rate Mean ±SE	Morula rate Mean ±SE	Blastocyst rate Mean ±SE
G1	301	6	5.96±1.04 [c] (18/301)	3.05±0.52 [c] (9/301)	2.64±0.38 [c] (8/301)
G2	312	6	23.16± 1.04 [b] (72/312)	15.07± 0.93 [b] (47/312)	12.25± 1.08 [b] (38/312)
G3	308	6	50.56± 1.56 [a] (155/308)	49.53± 1.42 [a] (152/308)	42.83± 0.94 [a] (136/308)

G1 is group 1 (medium TCM-199); G2 is group 2 (medium SOF); and G3 is group 3 (medium G-TLTM).

TABLE 2. Mitochondrial and lipid fluorescent intensity and total cell number of goats' embryo

Groups	No. of mature oocytes	Mitochondrial intensity Mean ±SE	Lipid intensity Mean ±SE	Total cell number Mean ±SE
G1	80	59.25±0.82[c]	18.73±0.3[c]	32.63±0.32[c]
G2	80	83.69±0.84[b]	28.93± 0.24[b]	43.13±0.61[b]
G3	80	106.1±0.83[a]	45.33±0.67[a]	62.86±0.52[a]

G1 is group 1 (medium TCM-199); G2 is group 2 (medium SOF); and G3 is group 3 (medium G-TL^{TM}).

TABLE 3. Showing oocyte quality on mature goat oocytes

Groups	No. replicates	Total COCs (n)	Cumulus expansion rate Mean ±SE	1st polar body extrusion rate Mean ±SE
G1	6	364	78.63±3.00 [a] (286/364)	29.79±2.19 [a] (108/364)
G2	6	364	53.82 ±1.24 [b] (196/364)	9.33 ±1.29 [b] (34/364)

Good-quality group 1 oocytes (G1), low-quality group 2 oocytes (G2), and COCs: Oocyte Complexes Cumulus.

TABLE 4. The mitochondrial fluorescent intensity of mature goat oocytes

Groups	No. of mature oocytes (MII)	Mitochondrial intensity Mean ±SE	Lipid intensity Mean ±SE
G1	150	92.57±1.57[a]	32.77±1.49[a]
G2	150	52.64±2.2[b]	22.14±1.26[b]

Good-quality group 1 oocytes (G1), low-quality group 2 oocytes (G2), and COCs: Oocyte Complexes Cumulus.MII: oocytes at metaphase II stage.

TABLE 5. Mitochondrial distribution of mature goat oocytes

Parameters	G1		G2		P value of Chi-square test
	N	%	N	%	
Diffused	100	66.67	10	6.64	0.0007**
Periphery	20	13.33	120	80	0.0003**
Semi-periphery	30	20	20	13.33	0.634
Total No.	150		150		

G1: group 1 good-quality oocyte and G2: group 2 low-quality oocyte.

TABLE 6. Nuclear chromatin evaluation of mature goat oocytes

Parameters	G1		G2		P value of Chi-square test
	N	%	N	%	
GV	10	6.67	60	40	0.03*
GVBD	20	13.33	50	33.34	0. 195
MI	50	33.33	20	13.33	0. 195
MII	70	46.67	20	13.33	0.04*
Total No.	**150**		**150**		

G1 and G2 denote group 1 and low-quality oocytes, respectively; GV stands for germinal vesicle, GVBD for germinal vesicle breakdown, MI stands for metaphase I, and MII for metaphase II.

TABLE 7. Effect of oocyte quality on in vitro development of goats' oocytes

Groups	No. of oocytes	No. of replicates	Fertilization rate Mean ±SE	Cleavage rate Mean ±SE	Morula rate Mean ±SE	Blastocysts rate Mean ±SE
G1	370	6	23.8±1.39 [a] (88/370)	19.98±1.21[a] (74/370)	17.79±0.9[a] (66/370)	16.2±0.72[a] (60/370)
G2	370	6	8.12± 0.75[b] (30/370)	8.12± 0.75[b] (30/370)	5.94± 0.96[b] (22/370)	4.32±0.68[b] (16/370)

Group 1 oocytes are of high quality, whereas group 2 oocytes are of low quality.

TABLE 8. Immunofluorescence staining results of *in vitro*-produced embryos affected by oocyte quality

Groups	No. of mature oocytes (MII)	Mitochondrial intensity Mean ±SE	Lipid intensity Mean ±SE	Total cell number Mean ±SE
G1	100	98.97±0.64[a]	38.06± 0.54[a]	55.9±0.78[a]
G2	100	57.85±1.02[b]	17.22±1.25[b]	42.07±0.5[b]

Group 1 oocytes are of high quality, whereas group 2 oocytes are of low quality.

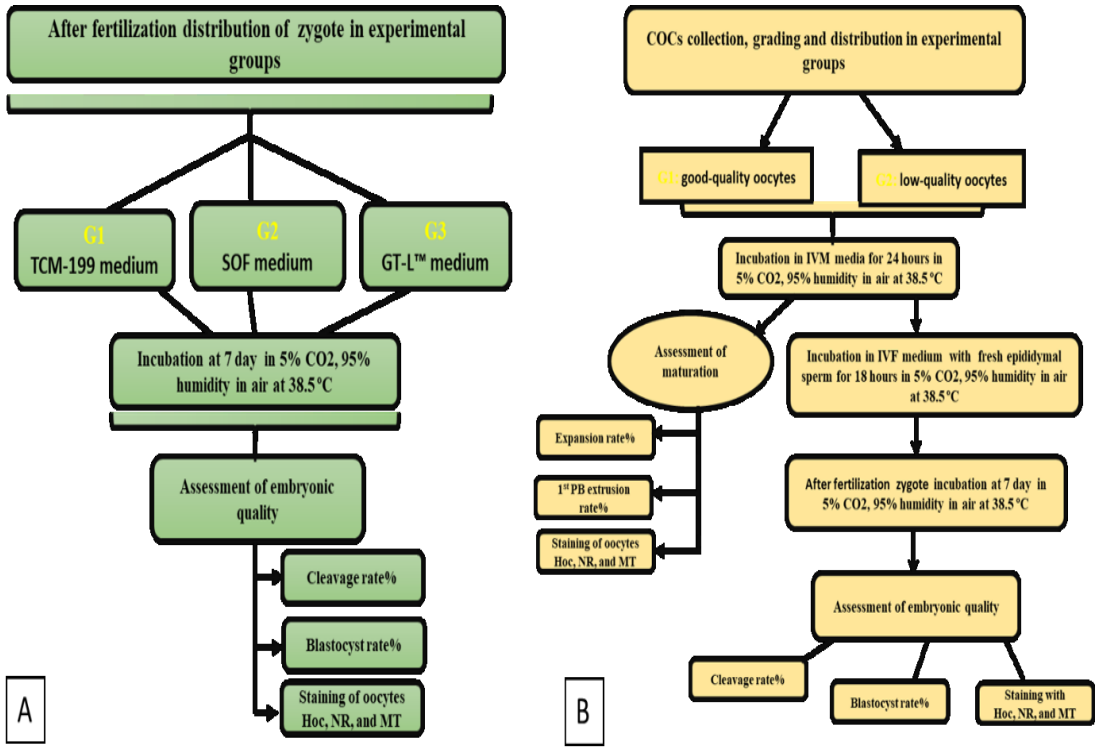

Fig.1. Experimental design; A: the first experiment and B: the second experiment.

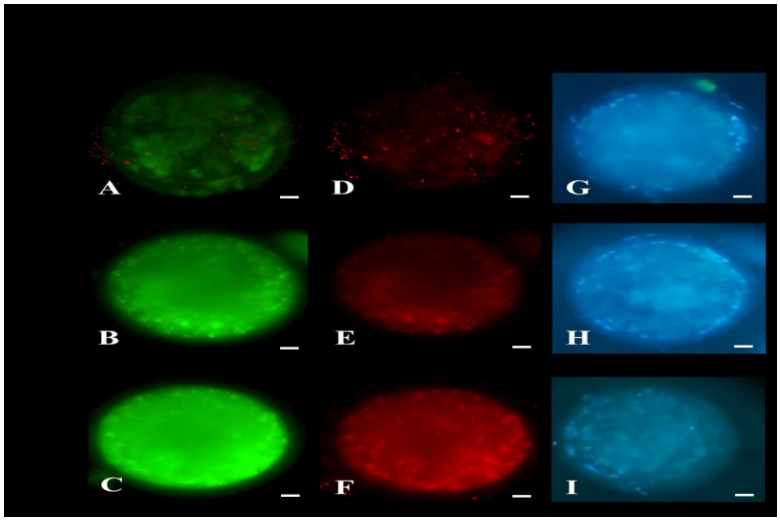

Fig. 2. Fluorescent goat embryo: A, B, and C show embryos stained with Mito-Tracker-Green to illustrate mitochondrial intensity. D, E, and F show embryos stained with Nile Red to show lipid intensity. For total embryonic cell count, G, H, and I stained embryos with Hoechst 33342®. Group 1 (TCM-199 medium), Group 2 (SOF medium), and Group 3 (G-TL™ medium).

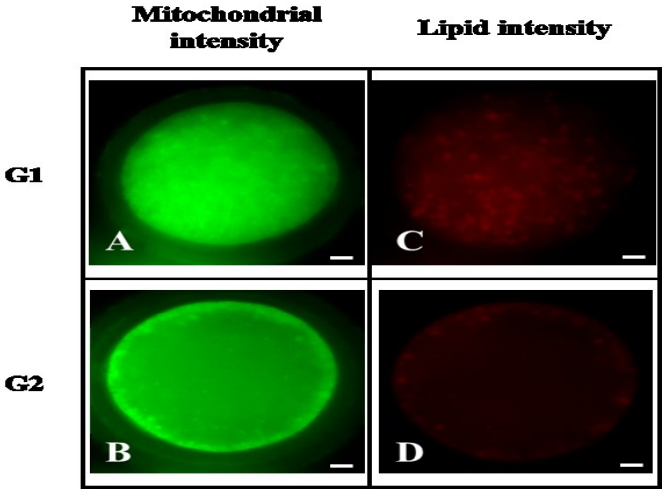

Fig. 3. A–B goat oocyte fluorescence pictures are shown in Fig.3. Mito-Tracker-Green stain depicts mitochondrial intensity and Nile (C–D). The red stain shows lipid drop intensity. Group 1 has good-quality oocytes, while Group 2 has low-quality ones.

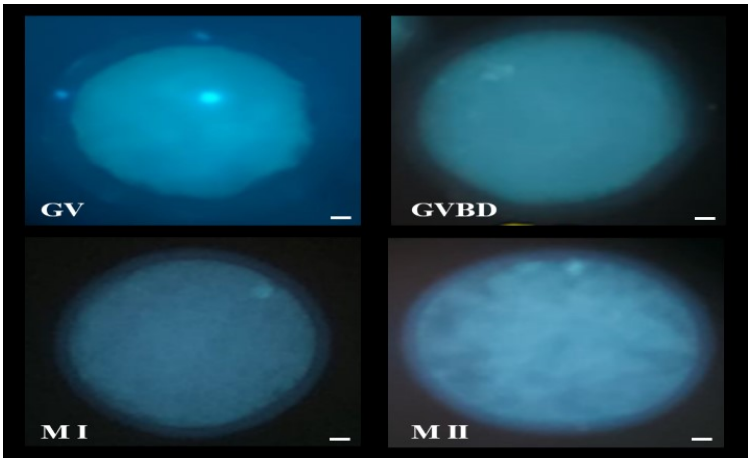

Fig. 4. Nuclear maturation status by Hoechst 33342® stain of goats' oocytes. GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I, and MII: metaphase II stage.

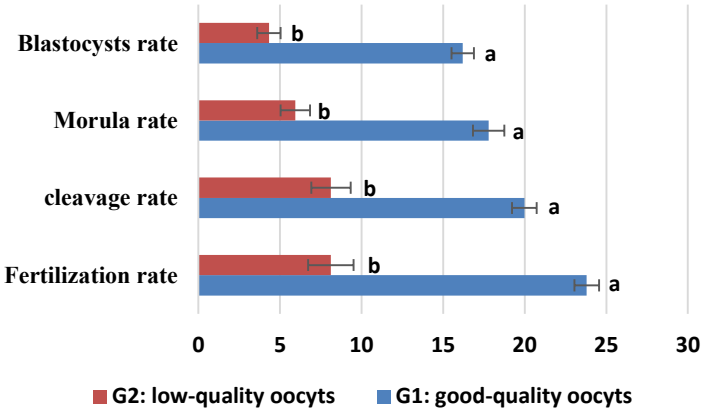

Fig. 5. Embryo devolvement of goats.

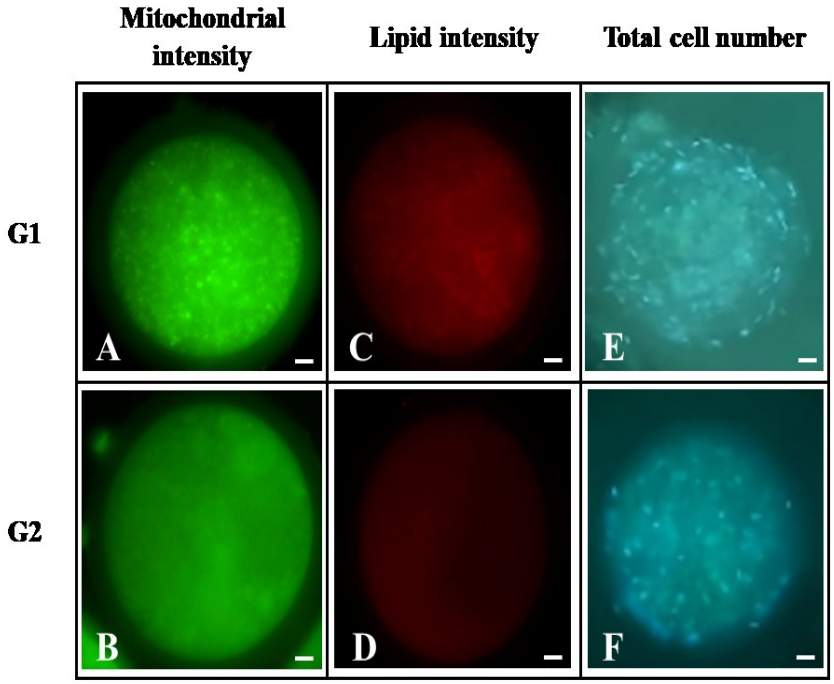

Fig. 6. Oocyte quality-affected goat embryo fluorescence pictures (A–B). The Mito-Tracker-Green stain shows the mitochondrial distribution, Nile Red shows lipid content, and Hoechst 33342® shows goat embryo cell count. Oocytes in groups 1 and 2 are high and low-quality, respectively.

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امكانات تطوير بويضات الماعز البلدي المصري المختارة مورفولوجيا تحت بيئات زراعية مختلفة

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الملخص

تؤثر جودة البويضة و وسائط إستزراع الأجنة على نمو الأجنة معمليا. وبالتالي، بحثت هذه الدراسة في كيفية تأثير جودة البويضة و وسائط إستزراع الأجنة علي معدل إنقسام الأجنة وتطويرها وذلك بعد مرحلة الإخصاب المعملي . في التجربة الأولى، تم نمو الزيجوت في وسط أساسي (G1) ، و وسط SOF (G1) ووسط جاهز G-TL™ (G3) بالإضافة إلى ذلك، تمت متابعة نمو الجنين لمدة سبعة أيام. من الناحية اخري بعد جمع البويضات من المبيض (طبقا للمورفولوجية البيويضة)، قسمت التجربة الثانية إلى مجموعتين: (G1) بويضات ذات نوعية جيدة و بويضات أخري ذات نوعية رديئة (G2) .ثم نضجت البويضات (كل مجموعة علي حدي) وخُصبت معمليا، وتم إستخدام أفضل وسط إستزراع من التجربة الأولى لإنتاج أجنة ماعز معمليا . وقد تم تقييم معدل النضج النووي عن طريق صبغة الهوكست. وسجلت الدراسة معدل التطور الجنيني (معدلات الإنقسام والكيسة الأريمية) أعلى بكثير ($P \leq 0.01$) في المجموعة المزروعة في وسط جاهز G-TL ™ (50.56 ± 1.56 و 42.83 ± 0.94٪ علي التوالي) مقارنة بالوسط الأساسي (5.96 ± 1.04 و 2.64 ± 0.38٪ علي التوالي) و وسائط SOF (23.16 ± 1.04 و 12.25 ± 1.08٪ علي التوالي).و كشفت الدراسة أن معدل النضج النووي (معدل ظهور الجسم القطبي الأول) كان مرتفعاً وزاد إحصائيا بشكل معنوي ($P \leq 0.01$) في بويضات ذات نوعية جيدة (29.79 ± 2.19٪) مقارنة مع بويضات ذات نوعية رديئة (9,33±1,29٪) .

وفي الختام، أدى اختيارالبيويضة ذات الجودة الجيدة وإستزراع الأجنة في وسط جاهز G-TL™ إلى تحسين إنتاج أجنة الماعز معمليا في مصر .

الكلمات الدالة : الماعز، جودة البويضة، نضوج البيويضة، زرع أجنة ،اخصاب معملي ،انتاج اجنة معمليا ، وسط G-TL™ ؛SOF.