Original Paper

Efficacy of Leptin and Linoleic Acid on Nuclear Succession and Early Embryos of Sheep Oocytes

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Article Details: Received: 2023-12-09 | Accepted: 2024-03-21 | Available online: 2024-06-31

https://doi.org/10.15414/afz.2024.27.02.110-117

Hormones and fatty acids play a crucial role in enhancing the yield and quality of *in vitro* produced embryos. The present study aimed to assess the effectiveness of leptin and linoleic acid in increasing the *in vitro* maturation rate of ovine oocytes and the resulting competence of embryos. Two experiments were conducted. The first experiment aimed to investigate the nuclear status of oocytes at three maturation intervals (9, 21, and 28 hours), while the second experiment aimed to examine the cleavage rates and quality of the resulting embryos. In both experiments, the oocytes were matured in six maturation treatments, primarily consisting of TCM-199 supplemented with varying concentrations of leptin and linoleic acid. The control group, without any addition, was designated as T0, while increasing concentrations of leptin and linoleic acid were added to four treatments: T1 (50 nM.ml^{-1} and 10 µM, respectively), T2 (75 nM.ml^{-1} and 100 µM, respectively), T3 (100 nM.ml^{-1} and 200 µM, respectively), and T4 (150 nM.ml^{-1} and 1,000 µM, respectively). In the last treatment (T5), only 15% fetal calf serum was added. In comparison with the control (T0) and T5 treatments, matured oocytes in the T3 treatment achieved the highest rates of M-II (88.46% at 28 hours; p <0.001), cleavage (81.27%; p = 0.001), 2–8 cells (22.55%; p = 0.001), morula (11.27%; p = 0.04), blastocyst (66.18%; p = 0.001), and Type I embryo (47.55%; p = 0.02). In conclusion, the maturation of sheep oocytes in a combination of leptin (100 nM.ml^{-1}) and linoleic acid (200 µM) through TCM-199 resulted in the highest rates of maturation and embryonic cleavage of excellent quality.

Keywords: embryos, leptin, linoleic acid, oocytes, sheep

1 Introduction

Artificial insemination, embryo collection by superovulation, embryo transfer (ET), *in vitro* embryo production (IVEP), and cryopreservation are among the most important reproductive techniques commonly used in the context of farm animals (Stroebech et al., 2015; Ferré et al., 2020). These assisted reproductive technologies (ARTs) have played a crucial role in preserving distinct genetic material and enhancing the productivity of individuals. The development of these technologies relies on a series of procedures aimed at achieving a high yield of embryos of excellent quality. The IVEP technology has significantly evolved since the 1980s, particularly due to advancements in the cryopreservation of embryos. The combination of IVEP and cryopreservation technologies holds great genetic and economic importance. Studies have shown that supplementing the oocyte maturation media with various additives contributes to increasing the rates of embryos with high vitality and good quality. Research conducted about 30 years ago focused on the morphological and functional characteristics of embryos to understand the reasons for differences in quality. It was found that a high intracellular lipid content is directly related to the sensitivity of embryos to cold (Amstislavsky et al., 2019). The conditions of *in vitro*-produced embryo development play a crucial role in the accumulation of lipids, particularly with the addition of serum or essential fatty acids. The addition of fatty acids, such as long-chain unsaturated fatty acids, has been found to impact blastocyst formation and the quality of embryos

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(Aardema et al., 2013). Leptin, known as the "obesity hormone" or "fat hormone," is secreted mainly by white adipose tissue and plays a role in regulating fat storage and controlling the number of calories consumed and burned in the body (Farr et al., 2015; Sheykhani et al., 2016). Linoleic acid, an essential polyunsaturated fatty acid, is converted into unsaturated fatty acids like eicosatetraenoic acid (EPA) and docosahexaenoic acid (DHA) in the body (Gramlich et al., 2015; Chen et al., 2015). The IVEP technology faces the challenge of obtaining embryos characterized by high yield and good quality, especially since a high percentage of *in vitro* produced embryos are cryopreserved, leading to the deterioration of their vital characteristics due to the accumulation of high levels of lipids in their cells. Given the results of previous studies, recent research aims to explore the feasibility of replacing fetal calf serum (FCS) with elements such as leptin and linoleic acid to obtain high-quality embryos that can withstand the effects of cold shock and increase pregnancy rates. This study seeks to investigate the feasibility of using linoleic acid and leptin in the maturation media of sheep oocytes and to track the rates and quality of the resulting embryos compared to the results of FCS.

2 Material and methods

2.1 Animals and oocytes collection

To obtain the ovaries, multiple visits were conducted to the governmental slaughterhouses in the city of Aleppo, Syria during the reproductive season. Ewes of the Awassi breed (n = 457) weighing between 30–40 kg and aged 2–5 years were utilized in the study after confirming their disease-free status based on veterinary health reports. Immediately after slaughter and evisceration, the ovaries (n = 914) were excised using a blade and placed in a thermos containing physiological saline solution at a temperature of 39 °C. The ovaries were then transported to the Biotechnology Laboratory at the University of Aleppo within 30 to 40 minutes and immersed in a beaker containing tissue culture media-199 (TCM-199) at 39 °C. Follicles larger than 2 mm in diameter were selected to obtain oocytes. To ensure the retrieval of the maximum possible number of cumulus-oocyte complexes (COCs), the slicing method was employed on the ovary surface using a sharp blade. Throughout the slicing process, the ovaries were rinsed multiple times with TCM-199 and heparin (20 IU.ml^{-1}L). The washing solution containing the COCs was collected in graduated dishes measuring 10 × 10 cm. The COCs (n = 2,893) were isolated using a stereomicroscope (KS OSE 421, 20x/40x) and micropipette, and then placed in four-well tissue culture dishes containing the maturation medium. The collected COCs underwent morphological examination using a stereoscope and an inverted microscope (Nikon Eclipse TS100, inverted phase contrast, Nikon objectives), and fully grown oocytes surrounded by more than two layers of cumulus cells were selected for further processing (Aryan et al., 2022).

2.2 Experimental design

The current experimental study comprised two distinct experiments. The first experiment aimed to investigate the impact of leptin and linoleic acid on the progression and events of nuclear maturation across three intervals (9, 21, and 28 hours), encompassing four phases: germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase 1 (M-I), and metaphase 2 (M-II). The second experiment involved examining the influence of the aforementioned additions on cleavage, embryonic stage, and embryo quality rates. The experiments followed a one-factor random design (leptin and linoleic acid levels) for a set of traits, including rates of maturation, cleavage, embryonic stage, and embryo quality. Four maturation treatments containing TCM-199 with varying levels of leptin and linoleic acid were designated as T1, T2, T3, and T4, as outlined in Table 1, while T0 treatment served as the control group (without any additions), and T5 treatment involved the addition of 15% fetal calf serum (FCS).

Table 1 Levels of leptin and linoleic acid across the four treatments used in the study (per 100 mL TCM-199)

Treatment	Leptin (nM.mL^{-1})	Linoleic acid (μM)
T1	50	10
T2	75	100
T3	100	200
T4	150	1,000

2.3 In vitro embryo production (IVEP)

The IVEP (IVM, IVF, and IVC) processes were conducted in accordance with established methodologies outlined in previous literature, either partially or in their entirety. In brief, the collected cumulus-oocyte complexes (COCs) were transferred to four-well dishes, with 5–10 oocytes in each well, to which TCM-199 was added based on the requirements of each treatment. Subsequently, the COCs were placed in an incubator under conditions of 95% humidified air and 5% CO_2 at 38.5 °C for the specified duration in the two experiments (Hammood, 2020). Fresh semen was obtained from proven fertility Awassi rams using an electro ejaculator and processed by percoll gradient (90%). Subsequently,

0.2 ml of semen was added to 45% Percoll (1 : 1 90% Percoll with TL-Sperm/Sperm-Tyrode's Albumin Lactate Pyruvate liquid (SP-TALP)). The resulting gradient was centrifuged at 700 g for 18 minutes, following which the upper part of the liquid (sperm pellet) was separated and placed in SP-TALP medium supplemented with 100 µg.ml^{-1} heparin, and incubated for 70 minutes in the incubator at 39 °C. After determining the initial spermatozoa concentration, the final solution was diluted in the equilibrated SP-TALP to achieve a final concentration of $0.1 \cdot 10^6$ sperms in 4 µl. Subsequently, the oocytes were partially denuded of granulosa cells in TCM-199 supplemented with 300 IU.ml^{-1} hyaluronidase for 6 minutes. Each set of 10–15 oocytes was then transferred to a TALP medium, covered by mineral oil, and incubated for 20 hours. The resulting zygotes were denuded by successive pipetting and transferred to TCM-199 for further development (Mardenli et al., 2021). The stages of embryo development (2–8 cells, morula, and blastocyst) were assessed on the seventh day of culture. Any arrests in the studied stages were documented using a phase-contrast microscope with a magnification of 500x (Fathi and Elkarmoty, 2021).

2.4 Nuclear maturation assessment

The oocytes within each group and across the maturation intervals underwent denudation by vortexing and were subsequently washed twice with TCM-199 supplemented with 4% fetal calf serum. Following this, they were placed on slides in 20 µL microdroplets. A cover slip was then applied to the slides using a needle, and the fixation process was initiated using the acetic acid: ethanol (1:3 v/v) solution, which was passed from one side to the other three times, with excess solution removed using filter paper. The slides were left for a maximum of five minutes, after which they were washed with a final solution comprising acetic acid, distilled water, and glycerol at a ratio of 1 : 3 : 1. The stained oocytes were examined under a light microscope (500×) to identify the subsequent stages of nuclear maturation. During the microscopic examination, the four stages of nuclear division, namely GV (well-defined nuclear membrane and diffuse chromatin), GVBD (stained chromatin), M-I (mitotic plate), and M-II (stained chromatin with 1 or 2 polar bodies), were meticulously traced (Kaabi et al., 2020).

2.5 Embryo quality assessment

The assessment of embryo quality, based on the general appearance of the embryonic cells and the degree of fragmentation, involved the classification of embryos into three types, as outlined by Mardenli et al. (2020). These classifications are as follows: Type I (excellent), characterized by compact cells and complete absence of fragments; Type II (good), exhibiting a heterogeneous appearance with some fragments; and Type III (poor), displaying a heterogeneous appearance with numerous fragments.

2.6 Statistical analysis

The binomial data pertaining to nuclear maturation (GV, GVBD, M-I, and M-II stages), cleavage rates, embryonic stages (2–8 cell, morula, and blastocyst), degeneration rates, and embryo quality (Type I, Type II, and Type III) were expressed as percentages. These data underwent analysis using the Statistical Analysis System (SAS) package (SAS Institute Inc. NC 27513, USA (2017) through cross-tabulation with a Chi-Square test. Additionally, the Fisher exact test was employed to assess the significance among different rates. Notably, differences at $p < 0.05$ were deemed to be statistically significant.

3 Results and discussion

3.1 Succession of nuclear maturation

During the initial nine hours of maturation, the outcomes of fixation and staining (Table 2) revealed noteworthy variations among the treatment groups in attaining the M-I stage ($p = 0.03$). The rates exhibited a relative convergence in the treated groups with leptin and linoleic acid, differing significantly from the control group (T0). Notably, oocytes in the T3 treatment demonstrated the highest rate (38.00%), while the lowest value was recorded in the control group (12.50%). Conversely, no disparities were observed among oocyte groups across different treatments at the GVBD stage. Subsequently, after 21 hours of maturation, Table 3 indicated a relative increase in the rates of oocytes that completed M-I and M-II. Oocytes in the T3 treatment exhibited the highest rates in M-I (31.48%; $p = 0.001$) and M-II (37.03%), and the lowest rates in the GVBD phase (5.56%). However, no significant differences were noted among treatment groups for oocyte maturation. In the third maturation period (28 hours), following a similar trend, the oocytes in the T3 treatment continued to outperform those in the other treatments, including the T0 treatment (control group), with the M-II rate reaching 88.46% ($p = 0.001$). The data in Table 4 indicated high rates of oocytes reaching the M-II stage across the six treatments, ranging between 50.91% (T0 treatment) and 88.46% (T3 treatment). Additionally, significant differences ($p = 0.03$) in the rates of oocytes reaching the M-I stage among groups were observed, with the highest value for T0 and the lowest for T3. Furthermore, no significant differences were observed in the rates of arrested and degenerated oocytes across the groups in the six

Table 2 Rates of GBVD, M-I and M-II of ovine*in vitro*-matured oocytes for 9 hours in TCM-199 across different levels of leptin and linoleic acid

Maturation treatment	Incubated oocytes (No.)	Stage of nuclear maturation						Arrested oocytes at GV stage		Degenerated oocytes	
		GVBD		M-I		M-II					
		No.	%	No.	%	No.	%	No.	%	No.	%
T0	56	17	30.36	7	12.50^{c}	0	0	30	53.57	2	3.57
T1	52	13	25.00	8	15.38^{ab}	0	0	30	57.69	1	1.92
T2	53	10	18.87	15	28.30^{b}	0	0	27	50.94	1	1.89
T3	50	6	12.00	19	38.00^{a}	0	0	24	48.00	1	2.00
T4	54	11	20.37	14	25.93^{b}	0	0	29	53.70	0	0.00
T5	50	9	18.00	13	26.00^{b}	0	0	28	56.00	0	0.00
p			NS		0.03		–		NS		NS

a, b, c – differences among groups with different superscripts in the same column are statistically significant (P <0.05); T0 – control; T1 – supplementation with 50 nM.ml^{-1} leptin and 10 µM linoleic acid in TCM-199 culture media; T2 – supplementation with 75 nM.ml^{-1} leptin and 100 µM linoleic acid in TCM-199 culture media; T3 – supplementation with 100 nM.ml^{-1} leptin and 200 µM linoleic acid in TCM-199 culture media; T4 – supplementation with 150 nM.ml^{-1} leptin and 1,000 µM linoleic acid in TCM-199 culture media; T5 – supplementation only with 15% FCS in TCM-199

Table 3 Rates of GBVD, M-I and M-II of ovine *in vitro*-matured oocytes for 21 hours in TCM-199 across different levels of leptin and linoleic acid

Maturation treatment	Incubated oocytes (No.)	Stage of nuclear maturation						Arrested oocytes at GV stage		Degenerated oocytes	
		GVBD		M-I		M-II					
		No.	%	No.	%	No.	%	No.	%	No.	%
T0	50	12	24.00^{d}	7	14.00	3	6.00^{c}	25	50.00	3	6.00
T1	50	9	18.00^{d}	10	20.00	4	8.00^{c}	21	42.00	6	12.00
T2	52	7	13.46^{c}	14	26.92	13	25.00^{a}	12	23.08	6	11.54
T3	54	3	5.56^{a}	17	31.48	20	37.03^{a}	10	18.52	4	7.41
T4	50	4	8.00^{b}	14	28.00	8	16.00^{b}	17	34.00	7	14.00
T5	56	5	8.93^{b}	17	30.36	8	14.29^{b}	20	35.71	6	10.71
p			0.04		NS		0.001		0.007		NS

a, b, c, d – differences among groups with different superscripts in the same column are statistically significant (P <0.05); T0 – control; T1 – supplementation with 50 nM.ml^{-1} leptin and 10 µM linoleic acid in TCM-199 culture media; T2 – ssupplementation with 75 nM.ml^{-1} leptin and 100 µM linoleic acid in TCM-199 culture media; T3 – supplementation with 100 nM.ml^{-1} leptin and 200 µM linoleic acid in TCM-199 culture media; T4 – supplementation with 150 nM.ml^{-1} leptin and 1,000 µM linoleic acid in TCM-199 culture media; T5 – supplementation only with 15% FCS in TCM-199

Table 4 Rates of GBVD, M-I and M-II of ovine *in vitro*-matured oocytes for 28 hours in TCM-199 across different levels of leptin and linoleic acid

Maturation treatment	Incubated oocytes (No.)	Stage of nuclear maturation						Arrested oocytes at GV stage		Degenerated oocytes	
		GVBD		M-I		M-II					
		No.	%	No.	%	No.	%	No.	%	No.	%
T0	55	7	12.73	17	30.91d	28	50.91c	2	3.64	1	1.82
T1	53	6	11.32	12	22.64c	30	56.60c	2	3.77	3	5.66
T2	50	6	12.00	9	18.00c	35	70.00b	0	0.00	0	0.00
T3	52	2	3.85	4	7.69a	46	88.46a	0	0.00	0	0.00
T4	57	4	7.02	8	14.04b	41	71.93b	3	5.26	1	1.75
T5	54	5	9.26	7	12.96b	41	75.93b	1	1.85	0	0.00
p			NS		0.03		0.001		NS		NS

a, b, c, d – differences among groups with different superscripts in the same column are statistically significant (P <0.05); T0 – control; T1 – supplementation with 50 nM.ml^{-1} leptin and 10 µM linoleic acid in TCM-199 culture media; T2 – supplementation with 75 nM.ml^{-1} leptin and 100 µM linoleic acid in TCM-199 culture media; T3 – supplementation with 100nM.ml^{-1} leptin and 200 µM linoleic acid in TCM-199 culture media; T4 – supplementation with 150 nM.ml^{-1} leptin and 1,000 µM linoleic acid in TCM-199 culture media; T5 – supplementation only with 15% FCS in TCM-199

treatments (Tables 2, 3, and 4), except for the matured oocytes in the T3 treatment at the 21-hour maturation interval, which achieved the lowest arrest rate (18.52%; $p = 0.007$) compared to the other groups.

3.2 IVC and embryo quality

The results of experiment II revealed significant differences in cleavage ($p = 0.001$), 2–8 cell ($p = 0.001$), morula ($p = 0.04$), and blastocyst ($p = 0.001$) rates among the groups (Table 5). Notably, a consistent linear relationship was observed in the cleavage stage, wherein the cleavage rates exhibited a numerical increase with the rise in leptin and linoleic acid concentrations across the different treatments until reaching stability in the T3 treatment (81.27%; the highest value). Upon reviewing the cleavage stages, it was evident that the matured oocytes in the T3 treatment maintained the lowest rates of arrest at the 2–8 cell and morula stages, while demonstrating the highest rates in the blastocyst stage. Conversely, it was noted that the rates of arrested embryos at the 2–8 cell and morula stages escalated, reaching the highest value in the control group (44.90% and 27.21%, respectively), followed by the T1 treatment (37.50% and 24.34%, respectively) and the T2 treatment (30.59% and 18.82%, respectively).

The rates of ovine embryo types resulting from the various maturation treatments are presented in Table 6. The T3 treatment exhibited the highest Type I rate (47.55%) compared to the other treatments ($p = 0.02$). Additionally, a significant difference ($p = 0.001$) was noted in the rates of Type II embryos among the various treatments, with the T5 treatment achieving the highest rate (44.44%) compared to the other treatments. Conversely, non-significant differences were observed among treatment groups in the rates of Type III embryos, with values ranging between 22.22% (T5 treatment) and 31.86% (T3 treatment), demonstrating a notable convergence in the values.

The vitality and quality of *in vitro* produced embryos play a pivotal role in the success of techniques following the IVEP technology, such as cryopreservation, embryo transfer, and splitting. Recent studies have underscored the active role of hormones and unsaturated fatty acids in the quality of *in vitro* produced embryos. In the first experiment of this study, a nearly linear increase was observed in the successive stages of nuclear maturation across the time intervals of maturation and in response to the various treatments applied in our study (Tables 2, 3, and 4), culminating in a peak in the oocytes matured in TCM-199 supplemented with 100 nM.ml^{-1} leptin and 1,000 µM linoleic acid (T3 treatment). These results can be attributed to a combination of factors related to both additives. Leptin, through its effect on the enzyme phosphodiesterase of 3B (PDE3B) and the promoting factor (MPF), activates mitogen-activated protein kinase (MAPK) to induce a set of reactions resulting in GVBD initiation, chromosomal condensation, and spindle formation. Fatty acids, particularly linoleic acid, play a critical role in the growth of ovarian follicles, cumulus cells, and the developmental potential of oocytes during the cytoplasmic and nuclear maturation stages, regulating a molecular mechanism that ultimately supports oocytes in reaching metaphase II. The values of various maturation stages of oocytes for different treatments recorded in the current study are notably higher than the results obtained by Silva (2018) and Satrio et al. (2020). The results of the second experiment demonstrated that the maturation medium in T3 treatment significantly enhanced the development

Table 5 Rates of cleavage, 2–8 cell, morula and blastocyst stages of ovine *in vitro*-matured oocytes in TCM-199 across different levels of leptin and linoleic acid

Maturation treatment	Incubated oocytes (No.)	Cleaved oocytes		Embryonic stage					
				arrested at 2–8 cell		arrested at morula		blastocyst	
		No.	%	No.	%	No.	%	No.	%
T0	240	147	61.25^{b}	66	44.90^{c}	40	27.21^{c}	41	27.89^{d}
T1	245	152	62.04^{b}	57	37.50^{c}	37	24.34^{b}	58	38.16^{c}
T2	238	170	71.42^{ab}	52	30.59^{b}	32	18.82^{b}	86	50.59^{b}
T3	251	204	81.27^{a}	46	22.55^{a}	23	11.27^{a}	135	66.18^{a}
T4	238	191	80.25^{a}	57	29.84^{b}	44	23.04^{b}	90	47.12^{b}
T5	242	189	78.09^{a}	58	30.69^{b}	41	21.69^{b}	90	47.62^{b}
p			0.001		0.001		0.04		0.001

a, b, c, d – differences among groups with different superscripts in the same column are statistically significant ($P < 0.05$); T0 – control; T1 – supplementation with 50 nM.ml^{-1} leptin and 10 µM linoleic acid in TCM-199 culture media; T2 – supplementation with 75 nM.ml^{-1} leptin and 100 µM linoleic acid in TCM-199 culture media; T3 – supplementation with 100nM.ml^{-1} leptin and 200 µM linoleic acid in TCM-199 culture media; T4 – supplementation with 150 nM.ml^{-1} leptin and 1,000 µM linoleic acid in TCM-199 culture media; T5 – supplementation only with 15% FCS in TCM-199

Table 6 Rates of Type I, Type II and Type III of ovine *in vitro* – produced embryos in TCM-199 across different levels of leptin and linoleic acid

Maturation treatment	Cleaved oocytes	Embryo type					
		type I				type III	
	No.	No.	%	No.	%	No.	%
T0	147	50	34.01b	60	40.82c	37	25.17
T1	152	53	34.87b	60	39.47c	39	25.66
T2	170	61	35.88b	67	39.41c	42	24.71
T3	204	97	47.55a	42	20.59a	65	31.86
T4	191	63	32.98b	83	43.46b	45	23.56
T5	189	63	33.33b	84	44.44b	42	22.22
p	0.02	0.001	NS				

a, b, c – differences among groups with different superscripts in the same column are statistically significant (P <0.05); T0 – control; T1 – supplementation with 50 nM.ml^{-1} leptin and 10 µM linoleic acid in TCM-199 culture media; T2 – supplementation with 75 nM.ml^{-1} leptin and 100 µM linoleic acid in TCM-199 culture media; T3 – supplementation with 100nM.ml^{-1} leptin and 200 µM linoleic acid in TCM-199 culture media; T4 – supplementation with 150 nM.ml^{-1} leptin and 1,000 µM linoleic acid in TCM-199 culture media; T5 – supplementation only with 15% FCS TCM-199

of a large percentage of oocytes into the cleavage stage compared to other treatments, particularly the T0 and T5 (Table 5). This significant effect of T3 treatment on the rates of cleavage and reaching the blastocyst stage can be explained by a set of hypotheses. The developmental potential of the oocytes plays a significant role in their ability to complete cytoplasmic and nuclear maturation. Leptin is believed to serve as an antioxidant against reactive oxygen species (ROS) through its long-term effect, similar to the role of cysteamine in reducing cellular damage caused by oxygen. Lipid metabolism has been found to play a critical role in the mechanisms of fertilization and subsequent embryo division. Studies have also highlighted the active role of linoleic acid in these processes. The results of the current study regarding the cleavage rates are consistent with most studies that included the study of leptin and linoleic acid individually. The study of Alshaheen et al. (2021) demonstrated that for oocytes matured in medium supplemented with 20 ng.ml^{-1} of leptin, the rates of expanded blastocyst increased to 94% (fresh) and 74% (post thawing), while the rates of hatching blastocyst at the same concentration reached 88% (fresh) and 90% (post thawing). In the *in vitro* fertilization stage, the fertilization rates of mature sheep oocytes at a leptin concentration of 10 ng.ml^{-1} reached 84%. At concentrations of 10, 100, and 1,000 µM of linoleic acid, the cleavage rates were 62.59, 50.00, and 58.55%, respectively, the morula rates were 11.56, 5.44, and 6.12%, respectively, while the blastocyst rates were 8.16, 5.44, and 10.20%, respectively. In a protocol containing 100 µM *trans*-10, *cis*-12 octadecadienoic acid (CLA), the cleavage rates of fresh embryos were 27.4% and 22.4% for frozen-thawed embryos, while the rates of Type I, Type II, and Type III embryos were 39.5, 5.2, and 57.1%, respectively, for fresh embryos and 19.2, 19.3, and 59.7%, respectively, for frozen-thawed embryos. The results of the supplementation of FCS (T5 treatment) seemed somewhat modest compared to the relevant studies. However, the synergistic role of leptin and linoleic acid in oocyte maturation showed encouraging results that deserve consideration. The troubles associated with the use of FCS, represented by the low survival rates after cooling, can potentially be mitigated by replacing the two previous elements, especially since these two elements have contributed to raising the rates of Type I and Type II embryos compared to those in T5 treatment (Table 6). In this context, it was found that *in vitro*-produced embryos store a high percentage of triglycerides and a low percentage of phospholipids, which gives weak resistance to embryo cryopreservation. The accumulation of fat droplets gives embryo cells an opaque appearance, prompting some theoretical schools to search for a genetic indicator related to the mechanism of glyceride storage in embryonic cells. On the other hand, it is believed that the interaction between linoleic acid and leptin in the nuclear maturation of ruminant oocytes and resulting embryos is complex and multifaceted. Linoleic acid has been shown to inhibit cumulus cell expansion and oocyte maturation, leading to a decrease in the percentage of oocytes at the metaphase II stage and a subsequent inhibition of early embryo development (Marei et al., 2010). On the other hand, leptin has been found to enhance nuclear maturation of oocytes and promote preimplantation embryo development through MEK1/2 signaling. Furthermore, linolenic acid, a type of polyunsaturated fatty acid, has been shown to increase the percentage of oocytes at the metaphase II stage and improve early embryo development (Ye et al., 2009). These findings suggest that linoleic acid and leptin may have opposing effects on oocyte maturation and embryo

development, with linolenic acid potentially mediating the effects of linoleic acid. However, the specific interactions between these factors in ruminants require further investigation. Unsaturated fatty acids and leptin were among the most important subjects of relevant studies.

4 Conclusion

The current study concluded that supplementation of culture media for maturation of sheep oocytes with 100 nM.ml^{-1} leptin and 200 µM linoleic acid would raise the rates of IVM and produce embryos characterized by abundant yield and good quality.

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