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A COMPARISON BETWEEN THE EFFECT OF LINOLEIC ACID AND CONJUGATED LINOLEIC ACID ON BOVINE OOCYTE DEVELOPMENTAL COMPETENCE

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ABSTRACT

Optimization of in vitro oocyte maturation conditions is crucial to maximize the number and quality of the transferable embryos. A demand for optimised chemically defined, serum-free medium for this purpose is increasing. Polyunsaturated fatty acids (PUFAs) have been shown to influence oocyte competence. Many ruminant diets are rich in the omega 6 Linoleic acid (LA) which has detrimental effect on oocyte developmental competence *in vitro* in the absence of antioxidants. In ruminants, biohydrogenation of fatty acids changes the structure of a high proportion of dietary LA into conjugated LA (CLA). The aim of this study was to compare the effect of LA and CLA supplementation to serum-free maturation media on the oocyte maturation rate and subsequent embryo development. The result revealed that the cumulus cell expansion and oocyte nuclear maturation to MII stage were inhibited by LA supplementation. This was reflected in subsequent development where higher proportion of embryos produced from the LA-treated oocytes were blocked at 2–cell stage and resulted in lower cleavage and blastocyst rates. The quality of the blastocyst produced in the LA group was similar to that of the control. In contrast, CLA did not affect cumulus cell expansion, nuclear maturation or the subsequent cleavage and blastocyst rates, however, blastocysts produced form CLA-treated oocytes had more total cell numbers due to increased trophectoderm (TE) cells. In conclusion, in serum free conditions, CLA supplementation resulted better quality embryos when compared to LA.

Keywords: Linoleic acid, Conjugated, IVF, embryos, Blastocysts.

INTRODUCTION

Oocyte maturation is a crucial step that determines the developmental competence and quality of the embryos produced. Although it is easy to induce in vitro oocyte maturation (IVM) simply by removing the oocyte from the ovarian follicle, it is important to optimise the IVM environment to maximise yield of transferable embryos and their quality. When oocytes matured in vivo are fertilized and cultured in vitro, they results in twice as much blastocysts as those matured in vitro, suggesting that the current IVM protocols are still deficient of some crucial factors that are normally provided by the follicular environment.

Undefined serum supplementation was successfully used to achieve high maturation rate and maximize developmental rate to the blastocyst stage (Khurana and Niemann, 2000). Embryo cleavage, developmental rate and quality were reduced when serum was avoided during IVM (Korhonen *et al.*, 2010). Moreover, a larger proportion of apoptotic cells was observed in embryos produced in serum-free media (Korhonen *et al.*, 2010). However, the use of serum has several disadvantages (see Abe and Hoshi, 2003 for review) including variation in the content/effect from batch to batch (Younis *et al.*, 1989), the possible interference with other molecules under examination during research studies, and risk of viral contamination which escapes the routine 0.2µm syringe filter sterilization. The presence of serum in maturation media significantly decreased expression of mRNAs encoding FSH receptor, connexin 43, COX-2, and EP2 and EP3 receptors, which may have long-term consequences for embryonic development (Calder *et al.*, 2005). Serum is also suspected of contributing to the large offspring syndrome (Holm *et al.*, 1996). Therefore, chemically defined substitutes are needed to replace serum in maturation and embryo cultures.

There is growing evidence that polyunsaturated fatty acids (PUFAs) can influence reproduction in many ways including their direct effect on oocyte competence. Cumulus oocyte complexes (COCs) contain a great proportion of saturated fatty acids (45-87% of total FAs), monounsaturated FAs (11-34%) and PUFAs (2-21%) (Adamiak *et al.*, 2005). The quality of an oocyte is partially attributed to its fatty acid composition (Kim *et al.*, 2001).
Many diets routinely used in feeding ruminants are rich in the omega 6 Linoleic acid (LA; 18:2 n-6) like whole sunflower seed, sunflower oil, whole soybean, and whole cotton seed. Feeding LA-rich diets result in increased LA concentration in plasma, which is subsequently reflected in the fatty acid profile of the follicular fluid. The LA is the most predominant polyunsaturated fatty acid in the follicular fluid and is also found in the fatty acid composition of COCs (McEvoy *et al.*, 2000). However, under serum-free conditions, LA supplementation to maturation media in vitro was shown to induce molecular changes and oxidative stress that were associated with retarded oocyte maturation and lower development to blastocyst stage (Marei *et al.*, 2010, Marei *et al.*, 2012). In these studies the quality of the embryos produced was not assessed.

Digestion and absorption of dietary PUFAs in ruminants is a complex process. Majority of dietary fats usually undergo modification in rumen before absorption. Bacteria of the ruminal flora release fatty acids from their glycerol backbone and unsaturated fatty acids may have some of their double bonds reduced (saturated) in a process called biohydrogenation (Wu *et al.*, 1991). Biohydrogenation of LA was found to be ~68% (Mattos and Palmquist, 1977). This process results in synthesis of conjugated LA (CLA). In CLA conjugated double bonds in either cis or trans configuration are present in various positions to give different geometric and positional isomers which has different structural and biochemical characteristics from LA (Serini *et al.*, 2009). The most abundant forms are cis-9, trans-11 and trans-10, cis-12 CLA. Although these isoforms are present in the follicular fluid at very low concentrations (Castañeda-Gutiérrez *et al.*, 2007), in vitro supplementation

with trans-10, cis-12 CLA have been shown to improve bovine oocyte competence and development into higher quality embryos (Lapa *et al.*, 2011). However this effect was in the presence of 10% serum. There is scant information about the effect of CLA in serum-free media on oocyte development.

The aim of this study was to compare the effect of LA and CLA supplementation to serum-free maturation media on the oocyte maturation rate and subsequent embryo development. And to assess the quality of the embryos produced.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents were purchased from Sigma Chemical Co. (Poole, UK) unless otherwise stated.

Collection of Oocytes

Bovine ovaries were collected from a local abattoir, transported to the laboratory in PBS at 37°C within 2 h after slaughter, and washed with fresh phosphate buffer saline (PBS) immediately after arrival. The COCs were aspirated from antral follicles 2-8 mm in diameter with a 19-gauge needle mounted on a 10 ml syringe. Grade 1 COCs were selected under a stereomicroscope and washed two times in TCM-199 supplemented with 20 mM HEPES and 0.4% (w/v) BSA.

In Vitro Maturation

Selected COCs were washed twice and cultured in four-well dishes (NUNC; Thermo Fisher Scientific, Loughborough, United Kingdom) using 500 µl of maturation medium as described in (Fouladi-Nashta and Campbell, 2006). The COCs were incubated for 24 h at 38.5°C under 5% CO_2 in humidified air. Linoleic acid (LA) and *trans*-10, *cis*-12 conjugated linoleic acid (CLA) dissolved in DMSO (100 mM stock solution) were added to the oocyte maturation media (at final concentration 100 µM) in the presence of 0.6%w/v fatty acid-free bovine serum albumin (BSA) as a carrier. DMSO was also added to the control group. A total of 445 COCs were used for assessment of cumulus expansion and nuclear maturation in four independent repeats while a total of 246 COCs were used for assessment of further embryo development and blastocyst quality in three independent repeats.

Assessment of Cumulus Cell Expansion

The degree of cumulus expansion was assessed under a stereomicroscope after 24 h of maturation subjectively as not expanded, partially expanded (the outer layers of cells were loosened), or fully expanded (all cumulus cells were loosened) (Marei *et al.*, 2009).

Oocyte Staining and Determination of the Stage of Nuclear Maturation

To assess the stage of nuclear maturation at the end of the maturation time, oocytes were completely denuded of cumulus cells by repeated pipetting in TCM-199 containing Hyaluronidase (300 U/ml). Denuded oocytes were then washed twice and positioned on a grease-free slide and overlaid with a cover slip supported by four droplets of

Vaseline: paraffin mixture (40:1). Slides were placed in acetic acid: methanol fixative (1:3 [v/v]) for at least 24 h. Oocytes were then stained with aceto-orcein (1% orcein [w/v] in 45% acetic acid [v/v]) and examined under phase-contrast microscopy (Leica, Milton Keynes, United Kingdom). The stage of nuclear maturation was determined according to the morphology of the nuclear material, as described by Marei *et al*. (2009).

In Vitro Fertilization and Embryo Culture

Experiments designed to assess the effects of LA and CLA in maturation medium on the developmental potential of oocytes, in vitro-matured oocytes were fertilized with frozen semen from a single bull as described previously by (Fouladi-Nashta and Campbell, 2006). Briefly, motile sperms were selected by swim-up for 45 min in calcium-free medium followed by centrifugation at x300 g at 20°C and resuspension of the pellet in fertilization medium (Tyrode's Albumin-Lactate- Pyruvate [TALP] media supplemented with 0.6% [w/v] fatty acid-free BSA, 1 µg/ml heparin, 50 ng/ml epinephrine, and 50 ng/ml hypotaurine). Groups of about 30 COCs were then washed once in fertilization medium and transferred into 400 µl of fertilization medium containing $1x10^6$ sperm/ml and were cultured for 18 h at 38.5°C in a humidified incubator of 5% CO_2 in air. Presumptive zygotes were then cultured in 500 µl of synthetic oviductal fluid (SOF) at 38.5ºC in a humidified incubator with 5% O_2, 5% CO_2, and 90% N_2. The culture was continued up to Day 8, and medium was changed every 2 days. Fetal calf serum 5% was only added to the embryo culture media of all groups starting from Day 3. The number of cleaved embryos (dividing to 4 cells or more) was recorded on Day 3 (fertilization was Day 0), and blastocysts were counted on Day 8.

Differential Staining of Blastocysts

Day 8 blastocysts were differentially stained for counting cells in the inner cell mass (ICM) and trophectoderm (TE) as previously described (Fouladi-Nashta *et al.*, 2005). Briefly, TE cells were permeablized by incubating the embryos in a 0.2% solution of Triton X-100 in SOF–BSA for 20 sec. Immediately after, embryos were washed and the TE cells were stained by incubating in 30 µg/ml propidium iodide (PI) for 10 min. Embryos were then washed and fixed in 4% paraformaldehyde containing 30 µg/ml bisbenzimide (Hoescht 33342). This allows fixation of embryos as well as staining for counting cells. Embryos were then washed and mounted in small droplets of anti-fading Vectashield mounting medium (Vector laboratories, Inc. Burlingame, A94010) and examined under a Leica epiflourescent microscope (Leica).

Statistical analysis:

In all the experiments, the data were from at least three independent repeats. Binominal data from cumulus expansion, oocyte nuclear maturation, cleavage and blastocyst rates were converted into percentages. All data were then analysed in SPSS v19 (SPSS Inc., Chicago, IL, USA) using generalised mixed model taking the different batches of ovaries into consideration as a random effect. If the main treatment effect was significant, Bonferroni post-hoc tests were performed. Differences were considered significant at 5% probability level.

RESULTS

Effect of LA and CLA on nuclear oocyte maturation

Supplementation of oocyte maturation media with CLA had no significant effect (P>0.05) on cumulus cell expansion (figure 1A) and the percentage of oocytes at different stages of nuclear maturation (figure 1B). In contrast, LA supplementation reduced the percentage of COCs exhibiting full cumulus cell expansion resulting in more COCs with partial or no expansion (P<0.05). LA supplementation also decreased the percentage of oocytes that reached MII stage at 24h of culture and increased oocytes at MI and TI stages compared to control and CLA groups (P<0.05).

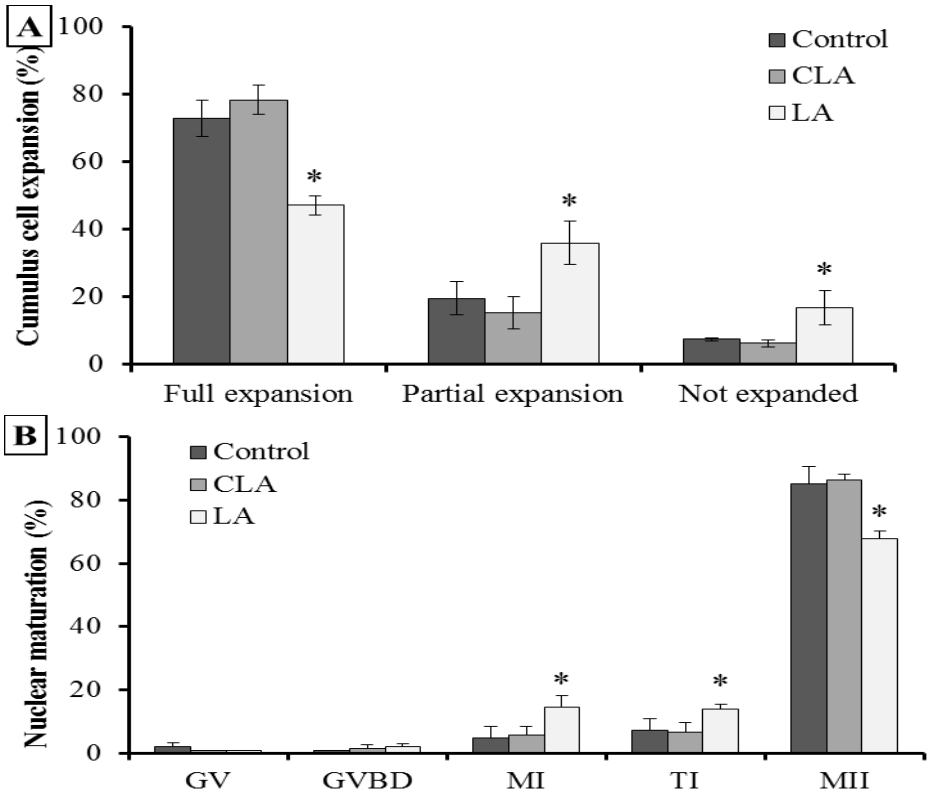

Figure 1: Effect of CLA and LA (100 µM) supplementation on (A) cumulus cell expansion and (B) nuclear maturation of bovine COCs at 24 h of culture. GV; Germinal vesicle, GVBD; GV breakdown, MI, TI; metaphase and telophase of first meiosis, MII, metaphase of second meiosis. Data are presented as mean ± SEM from four independent repeats. Bars with asterisks are significantly different from control at P<0.05.

Effect of CLA and LA on cleavage and blastocyst rates

Cumulus oocyte complexes (COCs) were treated with LA or CLA during maturation then fertilised and cultured in SOF embryo culture media. CLA supplementation did not have any significant effect on cleavage or blastocyst rates. In contrast, LA significantly increased the percentage of oocytes blocked at 2-cell stage after 48h and significantly reduced cleavage rate ($P<0.05$). The proportions of oocytes or embryos developing to blastocyst stage were also reduced in LA-treated group ($P<0.05$) (table 1).

Table 1: The effect of LA or CLA (100 µM) supplementation during oocyte maturation on cleavage and blastocyst rates. Data are presented as mean ± SEM from three independent repeats.

	Control	CLA	LA
Total number of fertilised oocytes	83	80	83
2-cell block at 48 h	10±3.3^{a}	10±3.1^{a}	17±5.9^{b}
Cleaved Embryos with 4 cells or more at 48h	78±3.3^{a}	78±3.3^{a}	71±1.7^{b}
Blastocyst/total oocytes at 8 day	29±2.7^{a}	24±3.5^{ab}	19±0.7^{b}
Blastocyst/cleaved embryos at day 8	37±1.9^{a}	31±4.1^{ab}	26±0.7^{b}
Hatched Blastocysts/ total blastocysts	5±1.0	4±1.9	2±1.5

Different superscripts (a, b) denote statistical difference at P<0.05.

Effect of CLA and LA on embryo quality

Although treatment of COCs during maturation with LA inhibited cleavage and blastocyst rates, this did not affect blastocyst quality since the number of ICM, TE and total number of cells in blastocysts produced from LA-treated oocytes were statistically similar to the blastocysts from the control group ($P>0.05$). In contrast, CLA supplementation significantly increased TE cells and total cell numbers in the blastocysts at day 8 ($P<0.05$) (table 2).

Table 2: The effect of LA or CLA (100 µM) supplementation during oocyte maturation on cleavage and blastocyst rates. Data are presented as mean ± SEM from three independent repeats.

	Control	CLA	LA
Total cell number ±SE	89.5±7.3^{a}	111±3.0^{b}	102±7.5^{ab}
No. ICM cells ±SE	23±2.3	29±4.7	22±3.2
No. TE cells ±SE	67±7.4^{a}	82±5.3^{b}	80±8.2^{ab}
Ratio ICM:TE±SE	0.4±0.06	0.4±0.07	0.3±0.07

Different superscripts (a, b) denote statistical difference at P<0.05.

DISCUSSION

The present study compared oocyte maturation, cumulus cell expansion and early embryo development of oocytes supplemented with or without 100 µM omega-6 linoleic acid (LA, 18:2) or its biohydrogenated isomer; *trans*-10, *cis*-12 conjugated linoleic acid (CLA). In cattle, the concentration of LA was reported to be 0.118 - 0.475 mg/ml (670 - 1629 µM)

in plasma and 0.02 - 0.2 mg/ml (71 - 710 µM) in follicular fluid depending on the FA content of the diet (Adamiak *et al.*, 2006). Therefore the LA concentration used in the present study is within the physiological range. CLA concentration was reported to be much lower than LA in the follicular fluid (Castañeda-Gutiérrez *et al.*, 2007) however at 100 µM CLA was effective in improving development into higher quality embryos (Lapa *et al.*, 2011).

In vivo studies have previously shown that feeding diets enriched with polyunsaturated fatty acids markedly increase the corresponding fatty acid content in the COCs (Zachut *et al.*, 2010). The percentage of LA; 18:2n-6 in the COCs was 54% higher in cows supplemented with sunflower vs. flaxseed and was 2.4 fold higher than the controls fed no fat supplement (Zachut *et al.*, 2010). Similarly, it has been recently proven that in vitro supplemented fatty acids like LA can be incorporated by the oocytes. The incorporated LA was metabolized by the oocytes mainly into major lipid classes, e.g., triacylglycerols and phospholipids (Carro *et al.*, 2013). Supplementation of the maturation medium with LA also increased triacylglycerol accumulation in cytoplasmic lipid droplets (Carro *et al.*, 2013). CLA was also shown to be incorporated by bovine (Lapa *et al.*, 2011) and porcine (Prates *et al.*, 2013a) COCs. Trans10,c12 CLA were capable of modifying the distribution and morphology of cytoplasmic lipid droplets during porcine oocyte maturation, thus reducing its lipid content in a time-dependent manner (Prates *et al.*, 2013b).

In the present study, LA (100µM) inhibited oocyte nuclear maturation and cumulus cell expansion at 24h of culture. These results are in accordance with our previous studies which revealed that LA at 100µM induced changes in the molecular events regulating oocyte maturation (Marei *et al.*, 2010) and altered mitochondrial distribution and activity (Marei *et al.*, 2012) leading to lower MII rate. Similarly supplementation of Linoleic acid 100 µM LA inhibited germinal vesicle breakdown, resulting in a higher percentage of oocytes arrested at the germinal state (43.5 vs. 3.0 in controls; $P < 0.05$) while lower concentrations (9 and 43 µM) did not affect the nuclear status of oocytes matured in vitro (Carro *et al.*, 2013). It has been recently reported that the inhibitory effects of LA at 100µM can be abrogated in the presence of antioxidants like glutathione peroxidase or vitamin E (Khalil *et al.*, 2013). In contrast, in the present study no antioxidants were used and we are showing that CLA does not inhibit oocyte nuclear maturation when supplemented in vitro.

Although oocyte nuclear maturation is a prerequisite for successful fertilization and embryo formation, not all in vitro matured oocytes can achieve this goal. This is dependent on the intrinsic quality of the oocyte. Addition of polyunsaturated fatty acids to oocyte matured media has been used as a chemically defined supplementation to improve intrinisic quality of the oocyte during maturation resulting in high cleavage and blastocyst rates and better embryo quality (Marei *et al.*, 2009). In the present study, supplementation of maturation media with LA resulted in higher 2 cell block and lower cleavage and blastocyst rates. This is in accordance with previous reports (Marei *et al.*, 2010). Interestingly, in the present study, blastocysts

formed from LA supplemented oocyte were similar in cell numbers to those in the control group.

Supplementation with t10,c12 CLA (100 µM) during maturation interfered on lipid metabolism improving bovine oocyte competence to develop into higher quality embryos assessed morphologically (Lapa *et al.*, 2011). CLA was also reported to improve Cryosurvival of bovine blastocysts as compared to the control when added to the embryo culture medium (Pereira *et al.*, 2007). This effect was in the presence of 10% serum. In the present study, in the absence of serum, supplementation with CLA did not affect cleavage and development of the fertilized oocytes to blastocyst stage, however, CLA treated COCs resulted in blastocyst with higher total and TE cell numbers indicating an improved embryo quality. Suggesting that transfer of these embryos may improve pregnancy rates. These results are in accordance with a previous multi-study analysis which showed that diatery supplementation with CLA resulted in 26% increase in probability of cows becoming pregnant and 34 d decrease in median time to conception (de Veth *et al.*, 2009). Our results suggest that the improvement in reproductive performance observed in cows supplemented with dietary t10, c12 CLA may be mediated by a direct effect of CLA on oocytes.

It is important to mention that CLA has been used in the field of cancer research to reduce tumor incidence. In vitro, CLA was shown to induce caspase-dependent apoptosis in colon SW480 tumor cells. CLA-treated cells displayed an increase in caspase 3 and caspase 9 activities, increased membrane annexin V levels and reduced expression of bcl-2 compared with untreated controls (Miller *et al.*, 2002). The t10, c12-CLA isomer was more biologically active compared with c9, t11-CLA (Miller *et al.*, 2002). There is some evidence in the literature suggesting a relation between early apoptosis (with positive Annexine-V and caspase activity) and improved developmental potential of oocytes. Bovine COCs with highest Annexin-V staining, highest Bax (which initiate apoptosis) and lowest Bcl2 (which prevent apoptosis) mRNA expression had highest nuclear maturation, cleavage, blastocyst, and hatching blastocyst rates compared to other groups of COCs that showed less Annexin-V staining (Li *et al.*, 2009). In addition, when mouse COCs were treated in vitro with the anticancer drug 5-azacytidine (5-AzaC), this increased levels of mRNA levels of Caspase3, Caspase8, and Caspase9 with the occurrence of early stage apoptosis. This was associated with a higher GVBD rate at 3 h and increased the PB1 extrusion rate at IVM 17–18 h as compared with the control oocytes. These data together suggest that the positive effects of c10, t12 CLA on embryo quality may be mediated by the induction of early apoptosis in maturing COCs. Further studies are required to confirm this hypothesis.

In conclusion, in serum free culture and in the absence of antioxidants, supplementation of CLA during oocyte maturation media did not affect oocyte maturation, cleavage and development to blastocyst stage but improved TE cell proliferation and increased total cell numbers. In contrast LA inhibited maturation and early embryo development but did not affect the quality of the blastocysts formed.

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مقارنة بين تأثير حمض اللينوليك وحمض اللينوليك المترافق على كفاءة تطور بويضات الأبقار

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يعتبر تحسين ظروف عملية الإنضاج المعملى للبويضات أمر بالغ الأهمية لزيادة معدلات إنتاج الأجنة القابلة للنقل. يزداد الطلب على تحسين البيئة المعروف تركيبها الكيميائي والخالية من السيرم. وجد أن الأحماض الدهنية عديدة اللا تشبع تؤثر على كفاءة تطور البويضات. تحتوى الكثير من علائق الأبقار على الأوميجا ٦ (حمض اللينوليك) وهو له تأثير ضار على كفاءة تطور البويضات معملياً فى غياب مضادات الأكسدة. الهدرجة الحيوية للأحماض الدهنية فى المجترات تغير من تركيب نسبه كبيرة من حمض اللينوليك إلى حمض اللينوليك المترافق. لذلك كان الهدف من هذه الدراسة هو مقارنة تأثير إضافة حمض اللينوليك وحمض اللينوليك المترافق إلى بيئة الإنضاج المعملى الخالية من السيرم ومن مضادات الأكسدة على معدل الإنضاج المعملى للبويضات وتطور الأجنة اللاحق. أظهرت النتائج أن إضافة حمض اللينوليك إلى بيئة الإنضاج ثبط كل من تمدد خلايا التراكم المبيضى و النضج النووي للبويضات (الطور الميتوزى الثانى). وانعكس ذلك على تطور الأجنة اللاحق حيث توقف تطور نسبة كبيرة من الأجنة الناتجة من البويضات المعاملة بحمض اللينوليك عند مرحلة الخليتين وكذلك انخفض معدل الأنشقاق ونسبة الأجنة التى وصلت لمرحلة العلقة (البلاستوسيست). كانت جودة البلاستوسيست المنتجة فى المجموعة المعاملة باللينوليك متقاربة مع المجموعة القياسية. على العكس من ذلك لم يؤثر إضافة حمض اللينوليك المترافق على كل من تمدد خلايا التراكم المبيضى، النضج النووى للبويضات، معدل الانشقاق ومعدل الأجنة التى وصلت لمرحلة البلاستوسيست. بينما كانت تحتوى البلاستوسيست المنتجة من البويضات المعاملة بحمض اللينوليك المترافق على عدد أكبر من الخلايا الكلى حيث يزيد فيها خلايا التروفوبلاست. نستخلص من هذا البحث أنه فى حالة استخدام بيئة خالية من السيرم فإن استخدام حمض اللينوليك المترافق أدى إلى إنتاج أجنة ذات جودة أعلى بالمقارنة بحمض اللينوليك.

الكلمات المفتاحية: حمض اللينوليك، حمض اللينوليك المترافق، الإخصاب المعملى، الأجنة، البلاستوسيست.

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