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OOCYTE MATURATION AND EMBRYO DEVELOPMENT IN SHEEP: EFFECT OF BIOMOLECULAR POLYUNSATURATED FATTY ACIDS AND HYALURONAN

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Abstract

The prepubertal sheep ovaries have a large number of small follicles containing oocytes with a capability of developing to upper stages of nuclear maturation, cleaving subsequent post insemination and finally being a blastocyst, that can be transferred to surrogate ewes for progressing of animal breeding programs. Whereas, it has shown that oocytes from prepubertal animals have low competence to develop to higher stages. Accordingly, in this study we have tried to do experiments to improve this deficiency by using Alpha linolenic acid (ALA) and Linoleic Acid (LA). Also, we have tried to improve survival of sheep embryos cultured in vitro using Hyaluronan (HA).

Polyunsaturated fatty acids (PUFAs) have shown to have beneficial effects on oocyte maturation and embryo development in vivo and in vitro conditions. Also, PUFAs constitute the major portion of the fatty acid content of the follicular fluid in small and large follicles. ALA and LA from PUFAs used in the present study in 2 separate experiments (experiment 1 for ALA and experiment 2 for LA). To our knowledge there are no previous reports using ALA and LA in prepubertal sheep oocytes in vitro. We used in three concentrations (50, 100 and 200µM) of ALA and LA in maturation media. Subsequently, we evaluated parameters such as cumulus cell expansion, nuclear maturation, secretion of prostaglandins (PGE2 and PGF2α) and steroids (E2 and P4), two pro-nuclei, polyspermy, asynchrony, cleavage, blastocyst rate and embryo quality via counting total cell number and apoptotic cells.

In experiment 1, oocytes nuclear maturation and the number of fully expanded cumulus cells were reduced in 200 µM ALA treatment compared to other groups ($P \leq 0.05$). Supplementation with ALA increased both PGE2 and PGF2α concentration in the spent media ($P \leq 0.05$). No differences were observed in blastocyst development among control (12.2%) and 50, 100 and 200 µM ALA groups (6.9, 11.5 and 14.0%, respectively). However, total cells (46.50±5.85, 67.94±6.71, 45.20±6.37, and 59.80±5.51, respectively, $P \leq 0.05$) and apoptotic cell number (6.45±0.89, 2.48±0.81, 4.02±1.15, and 3.67±1.15, respectively, $P \leq 0.05$) were significantly improved. After IVM, E2 concentration was lower and P4 concentration was higher in ALA groups compared to control ($P \leq 0.05$). In conclusion, these results showed that ALA affects prepubertal sheep embryo quality associated with alteration of releasing reproductive hormones.

Abstract

In experiment 2, no changes were observed in the number of oocytes achieving MII nuclear maturation (91.8, 91.6, 87.9, 93.1 and 93.1, respectively). Production of PGE2 and PGF2α increased in all LA concentrations compared to control (P≤0.05). The ratio of PGE2/PGF2α was not altered. LA at 50µM significantly improved the rate of 2 pro-nuclear (2PN) compared to control (57.89 vs. 45.45, respectively, P≤0.05). There were no differences in cleaved embryos and blastocyst rates. However, embryo quality was improved by 50µM of LA with increase in total cell numbers compared to control (63.88±4.54 vs. 53.35±3.64, P≤0.05, respectively). There was no difference in apoptotic cell numbers. Also, production of E2 decreased significantly while there were not differences in P4 production and the rate of E2/P4 ratio. In conclusion, LA supplementation to prepubertal sheep oocytes in IVM media negatively altered the fully expanded cumulus cells significantly without inhibition of MII nuclear stage percentage of oocytes. The results from the present study provide evidence in increased number of zygotes with normal 2PN and also showed beneficial effects of low level LA on embryo quality of blastocysts at 8 day of post insemination in serum free media.

HA is a polysaccharide with long polymer chains of sugars and has found in the extracellular matrix and intercellular matrix of animal tissues. HA is found enormously as a plentiful Glycosaminoglycan (GAG) in the female reproductive tract like uterus, oviduct and follicular fluids. Furthermore, it has been illustrated that HA plays a role in introducing a delay of death in the oocyte with preventing of oocyte from fragmentation in porcine. Also HA improves *in vitro* produced bovine embryos to develop to the blastocyst stage. It has also been indicated that embryo cryo-survival improvement is due to the HA added to cryopreservation medium

HA numerically increased blastocyst percentage at 7-day (33±5.7, 32±6.0, 35±5.5; P≥0.05) and survival rates 48 h after culture in serum free media (63±17.1, 83±15.2, 58±14.2; P≥0.05) as compared to the respective controls (25±5.2, 38±17.1). It increased the total cell (TC) number (83.6±4.6, 100.7±3.8, 97.2±3.7, 105.0±3.9; P<0.05) and trophectoderm cells (TE) (58.4±3.8, 74.2±3.2, 75.6±3.3, 80.1±3.4; P<0.05) at 7-day embryos. Survived embryos had higher TC (63.2±3.7, 130.8±3.6, 113.9±5.2, 149.8±5.4; P<0.05), TE (42.9±3.0, 96.7±3.1, 85.2±4.5, 111.9±4.7; P<0.05) and ICM (20.3±2.2, 32.9±1.8, 27.7±2.6, 36.5±2.7; P<0.05). The results indicate that HA improves the embryo development and viability even quality which might have implication for improving embryo transfer.

RESUMEN

La calidad o competencia del oocito se define por su capacidad para ser madurado, fecundado y dar lugar a una gestación normal y a un parto con un nacimiento sano. .La producción in vitro de embriones (PIVE)s posibilita la obtención de un gran número de embriones de una hembra seleccionada. Utilizar oocitos de hembras prepúberes para la PIVE permite acortar el intervalo generacional y de esta forma intensificar la selección genética en los programas de mejora animal. Sin embargo los oocitos de hembras prepúberes son obtenidos de folículos pequeños y son oocitos de menos calidad para formar embriones in vitro. En estudios anteriores en nuestro laboratorio observamos que la calidad del oocito está mas relacionada con el tamaño del folículo del cual provienen que de la edad de la hembra. Así observamos el mismo porcentaje de embriones producidos in vitro en oocitos de cabras prepúberes (30 a 45 días de edad) obtenidos de folículos mayores de 3mm que de oocitos de cabras adultas. LA conclusión a la que se llega es que la calidad y competencia del oocito está estrechamente relacionada con el desarrollo folicular y la composición del liquido folicular del cual proviene. En vacuno se han realizado varios estudios en los que se relaciona la fertilidad de las vacas con su dieta rica en grasas no saturadas. También los grasos poli insaturados son los ácidos grasos que se encuentran en mayor concentración en el líquido folicular. Entre estos ácidos grasos los que más atención y estudios han conseguido son el Acido Linoleico (AL) y el acido Linolenico (ALA) de las familias omega 6 y omega 3, respectivamente. Ambos ácidos han demostrado su efecto sobre la calidad de los oocitos in vivo e in vitro. Las hembras prepúberes tiene una composición corporal pobre en grasa y presumiblemente también su liquido folicular tenga baja concentración en ácidos AL y ALA. Así uno de los objetivos de este trabajo de tesis ha sido estudiar el efecto de la adicción de diferentes concentraciones de AL y ALA en los medios de maduración in vitro de oocitos de corderas sobre su desarrollo embrionario in vitro.

El Acido Hialurónico (AH) es un polisacárido con largas cadenas de azucares que se encuentra en la matriz extracelular e intracelular de los tejidos animales. El AH se encuentra como un glicosaminoglicano (GAG) en el tracto reproductivo de las hembras: úteros, oviducto y liquido folicular. Además se ha demostrado que el AH juega un papel importante retrasando la muerte por fragmentación de los oocitos porcinos. En vacuno, la adición del AH ha mejorado la producción in vitro de blastocistos y añadido

en los medios de crioconservación también ha mejorado la supervivencia de los embriones crioconservados. El siguiente objetivo de este trabajo es añadir AH en los medios de cultivo embrionario con el objetivo de incrementar el desarrollo hasta blastocisto de los cigotos de corderas producidos in vitro y su resistencia a la crioconservación.

Con estos objetivos se han realizado 3 estudios: 1) Efecto del Acido Linolenico (ALA) sobre la maduración del oocito y su desarrollo embrionario en oocitos de oveja prepúber.2) Efecto del Acido Linoleico (AL) sobre la maduración del oocito y su desarrollo embrionario en oocitos de ovejas prepúberes y 3) Efecto del Acido Hialuronico de alto peso molecular sobre el desarrollo a blastocisto y su resistencia a la crioconservación.

En el Experimento 1, se añadió al medio de Maduración in vitro (MIV) de oocitos de corderas concentraciones 50, 100 y 200 µM de Acido Linolenico (ALA) y se estudió su efecto sobre la maduración nuclear, la expansión del cumulus, la secreción de prostaglandinas E2 y F2α, estradiol, progesterona y el porcentaje de oocitos que alcanzaban el estadio de blastocistos. Estos resultados se compararon con un grupo control que no llevaba ALA. Los resultados de este experimento demostraron que a concentraciones 200 µM la maduración del oocito y la expansión del cumulus disminuían comparado con los otros grupos ($P \leq 0.05$). La adición de ALA incrementaba la secreción de ambas prostaglandinas analizadas después de las 24 h de maduración de los oocitos ($P \leq 0.05$). No se observaron diferencias en el porcentaje de blastocistos obtenidos entre el grupo control (12.2%) y los grupos de 50, 100 y 200 µM ALA (6.9, 11.5 y 14.0%, respectivamente). Sin embargo si que hubo diferencias en el total de células por blastocisto (46.50±5.85, 67.94±6.71, 45.20±6.37, y 59.80±5.51, respectivamente, $P \leq 0.05$) y en el total de células apoptoticas por blastocisto (6.45±0.89, 2.48±0.81, 4.02±1.15, y 3.67±1.15, respectivamente, $P \leq 0.05$). Después de la MIV la concentración de E2 fue mas baja y la de P4 mas alta en los grupos con ALA que en el grupo control ($P \leq 0.05$). En conclusión, estos resultados indican que la adición de ALA al medio de MIV han afectado la calidad de los blastocistos producidos y alterado la liberación de hormonas reproductivas

En el experimento 2, se añadió al medio de MIV concentraciones de 50, 100 and 200 µM de Acido Linoleico (LA) y se estudiaron los mismos parámetros que en el

experimento 1. Los resultados de este experimento no demostraron ningún cambio en el número de oocitos que alcanzaban la metafase II. La producción de PGE2 y PGF2α incrementó en todos los grupos de AL comparados con el control (P≤0.05). La relación PGE2/PGF2α tampoco fue alterada. Después de la FIV el grupo con 50 µM de AL presentaba un porcentaje de cigotos correctamente fertilizados (2PN) superior al control (57.89 vs. 45.45, respectivamente, P≤0.05). No se observaron diferencias en el porcentaje de blastocistos obtenidos. Sin embargo la calidad de los blastocistos fue superior en el grupo de 50 µM con un número total de células por blastocisto superior al control (63.88±4.54 vs. 53.35±3.64, P≤0.05, respectivamente). No hubo diferencias en el número de células apoptoticas. La producción de E2 fue menor en los grupos AL y no hubo diferencias en la producción de P4. En conclusión, la adición de LA no ha afectado la maduración nuclear ni la producción de blastocistos, sin embargo dosis bajas de LA han incrementado el porcentaje de cigotos normales con 2 pronucleos y la calidad de los blastocistos evaluada por el número total de células.

En el experimento 3, concentraciones de 0.25, 0.5 y 1 mg/ml de Acido Hialuronico (AH) fue añadido al medio de cultivo de los embriones producidos en in vitro con oocitos de corderas. El medio de cultivo se preparó sin suero. Los embriones de 2-4 células se cultivaron durante 7 días. Los resultados que se obtuvieron fueron que el porcentaje de blastocistos obtenidos con las distintas concentraciones de AH (33±5.7%, 32±6.0%, 35±5.5%, respectivamente) y el porcentaje de supervivencia embrionaria después de la vitrificación (63±17.1%, 83±15.2%, 58±14.2%; respectivamente) fue estadísticamente superior (P≥0.05) al grupo control (25±5.2%, 38±17.1%, respectivamente). Además, la adicción de AH aumentó el número total de células por blastocisto (83.6±4.6, 100.7±3.8, 97.2±3.7, 105.0±3.9; respectivamente, P<0.05) y el número de células de trofoectodermo ((58.4±3.8, 74.2±3.2, 75.6±3.3, 80.1±3.4; respectivamente, P<0.05). Los embriones que sobrevivieron a la vitrificación también presentaron mayor número de células totales (63.2±3.7, 130.8±3.6, 113.9±5.2, 149.8±5.4; P<0.05), del trofoectodermo (42.9±3.0, 96.7±3.1, 85.2±4.5, 111.9±4.7; P<0.05) y del botón embrionario (20.3±2.2, 32.9±1.8, 27.7±2.6, 36.5±2.7; P<0.05) los blastocistos cultivados con AH que los del grupo control.

En conclusión, con oocitos de corderas de 3 meses de edad, aproximadamente, la utilización de AL, ALA en el medio de MIV y de AH en el medio de cultivo no ha incrementado el porcentaje de blastocistos obtenidos. Sin embargo, los ácidos grasos

Resumen

LA y ALA han mejorado la calidad de los embriones aumentando su número de células y el AH ha mejorado la calidad de los blastocistos y mejorado su resistencia a la vitrificación.

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CHAPTER 1:

GENERAL BACKGROUND

1. General background

Nowadays, for improvement of reproductive functions, several assisted reproductive technologies (ART) such as multiple ovulation and embryo transfer (MOET), in vitro embryo production (IVEP), sperm cryopreservation and embryo freezing have been utilized and are available. Embryo production and even freezing of embryos produced in vitro provide also useful tools for controlling and manipulating of domestic mammalian reproduction [1-2]. IVEP has exhibited to be important in sheep as it has shown possibilities of producing embryos even in non breeding season via in vitro techniques; and also shortening of generation interval [3]. Furthermore, it has thought that using IVEP techniques can overcome some of in vivo limitations such as low efficiency and low number of embryos achieved by MOET method. Embryos produced in vitro still have lower quality compared to those grown in vivo [4]. In fact, IVEP domestic mammalian embryos have lower cell numbers and smaller inner cell mass (ICM) and also the ratio of ICM to trophectoderm compared to those derived in vivo [5-6]. Several literatures have reported higher fragmentations in blastomeres in bovine [7], higher DNA fragmentation by labeling the terminal end of nucleic acids in pigs [8], chromosomal aberrations in porcine [9-10] and hydrogen peroxide levels in mouse in vitro produced embryos compared to in vivo [11]. Also, despite the improvements carried out in producing embryos in vitro the pregnancy rate is lower and early embryonic death is still reporting by researchers after embryo transfer. As 40% early embryonic death has reported at days 8 and 17 of pregnancy [12] that also it has indicated that one of the possible reasons can be due to the quality of oocytes [13].

Assisted reproductive technologies have being utilized for improving genetic of domestic mammalian animals and also in prevention of endangered species and or breeds. However, most of the techniques have used for cattle and there is some in small mammalians such as sheep and goat. IVEP provides a cheap and easy available source of low cost embryos for research projects. The use of prepubertal females as a oocyte source for IVEP has shown a considerable potential in accelerating the genetic gain due to reducing of generation interval [14]. However, lower developmental competence has reported in oocytes recovered from juvenile animals compared to adult females when

the oocytes have matured in vitro in domestic animals such as goat [15-16], sheep [17-18], bovine [19].

It has shown that reduction in reaching to blastocyst stage in prepubertal females is mainly related to the lower competence of oocytes derived from these young animals and this competence has defined as a capability to progress of nuclear maturation, cleaving and developing to the blastocyst stage and finally inducing pregnancy and giving birth in good and healthy condition [20].

In the laboratory embryos routinely produce and progress to blastocyst stage via using techniques including in vitro maturation of derived oocytes from sliced ovaries, and subsequently separating motile sperms and doing in vitro fertilization of matured oocytes and finally following in vitro culture of presumptive zygotes up to the blastocyst stage that can be transferred to recipients females or cryopreserved for future usages. Although, IVEP still is not so efficient due to the low pregnancy and survival rates. Embryos can be stored if there is a limitation in the number of recipients, and cryopreservation is a technique that can be useful to reserve them until the required recipients be available. Also, if the production of specific breed needs to be postponed for future, cryopreservation techniques are essential and beneficial [1, 21-22]. Cryopreservation of bovine semen and embryos has made the great possibilities and progresses recently, but little progress has however been occurred in sheep [23]. Normally two techniques are being used for embryo cryopreservation, the conventional slow freezing and the vitrification techniques. Conventional slow freezing has been reported to be the preferable technique, especially for embryos derived from both in vitro and in vivo [24]. The slow freezing technique because of some limitations such as being costly requires expensive equipments, physical damage to the embryos because of formation of crystals and the long time processing [25-28]. Whereas, vitrification has reported as a cheaper method compared to conventional slow freezing. Vitrification has lead to have minimum cell injuries while formation of crystals, however, damages can be still happen due to cryoprotectants toxicity [27-30]. Cryopreservation without forming damaging ice crystals is the foremost advantage of vitrification over slow freezing. We thought improving embryo quality might be helpful to increase the survival rate.

It has been shown that supplementation of diets with fats in lactating dairy cattle has increased blastocyst rate and has improved blastocyst quality [31]. Polyunsaturated fatty acids (PUFAs) have described to have an important role in many physiological performances in reproductive system [32]. In other word fatty acids have an important role in determining developmental competence of oocytes and finally reaching to higher stages of embryo development. The long chain PUFAs are synthesized in the body from the short chain omega 3 alpha linolenic acid (ALA) and omega 6 linoleic acid through a number of steps involving desaturation and elongation. ALA and LA cannot be synthesized in the body of animals [33]. It has been shown that the concentrations of ALA and LA in diet are influenced metabolism in reproductive tissues in bovine [32]. The long chain PUFAs eicosapentaenoic acids and arachidonic acid are the precursors for eicosanoids including prostaglandins, prostacyclins, thromboxanes and leukotrienes [34-35]. The removal of two double bonds from arachidonic acid by prostaglandin H synthase leaves two double bonds and leads to the formation of series-2 eicosanoids that are inflammatory, while the removal of two double bonds from eicosapentaenoic acid leads to the formation of series-3 eicosanoids that are less inflammatory. Prostaglandin-2α plays an important role in several aspects of reproduction, including ovulation, estrous, embryo survival and parturition [34, 36-37]. Omega 3 and 6 PUFAs have shown to be associated with a number of factors with the synthesis and metabolism of important reproductive hormones such as the steroid hormones (progesterone and estradiol). It has been shown that diets high in omega 3 are related with lower plasma cholesterol concentrations which may lead to reduced hormone synthesis, as cholesterol is a precursor for both progesterone and estradiol.

The communication between the oocyte and the granulosa cells surrounding is crucial for the acquisition of oocyte competence [38]. One way is characterized by the secretion of several growth factors such as Glycosaminoglycan (GAG), which play an important role in proliferation and differentiation of a variety of cell types [39]. Among the Glycosaminoglycan, hyaluronan is a high molecular weight polysaccharide found in the extracellular matrix of most animal tissues and is one of the most abundant GAGs in the uterine, oviductal and follicular fluid [40]. During the process of ovulation, cumulus cells secret hyaluronan that actively participates in processes of cytoskeletol modification, gap junction losses that accompany cumulus expansion in cumulus oocyte complexes and oocyte meiotic progression [41]. The expansion of cumulus cells may be positively

correlated to the ovulation, fertilization and subsequently zygote development [42]. It has been shown that addition of hyaluronan to the culture medium supports the development of 1 and 2 cell porcine embryos [43] as well as improves in vitro bovine embryo development to the blastocyst stage [44].

CHAPTER 2:

LITERATURE REVIEW

2. Literature Review

2.1. Current situation of the In vitro embryo production in sheep

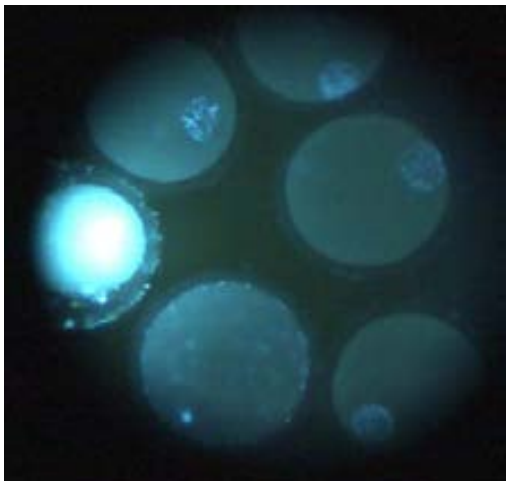
A more easily available and cheaper source of matured oocytes, zygotes, and embryos is needed for research projects, and this can be a justification of growing interest *in vitro* production (IVP) systems since the birth of several lambs and kids 15 years ago. *In vitro* embryo production in small ruminants has introduced to be used as a source of low cost embryos in basic studies on developmental fields such as biology and physiology and even for commercial usages [45]. Furthermore, in vitro embryo production (IVEP) has been described to be used in rescuing the endangered animals [46]. The method of IVEP involves three main steps: maturation of oocytes retrieved from antral follicles, fertilization of matured oocytes by frozen-thawed semen and, culture of presumptive zygotes for 7 to 8 days to be formed blastocysts that can be usable to cryopreservation or transferable to recipients (reviewed by [47]).

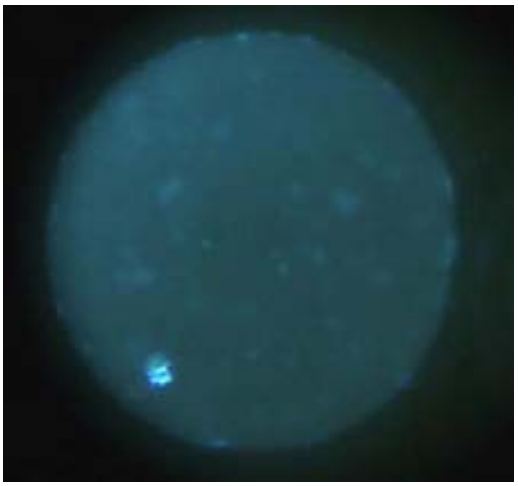
2.1.1. In vitro maturation of oocytes

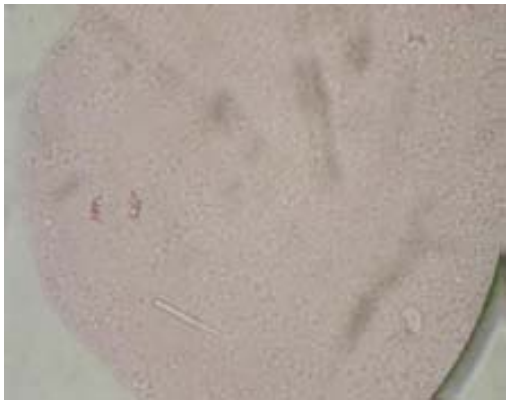
In vitro maturation (IVM) of prepubertal sheep oocytes in small ruminants have low level of efficiency compared to those produced from adults (reviewed by [47]). Previous experiments also have reported a lack of developmental competence up to the blastocyst stage in species like goats, a percentage of blastocysts of 10% was obtained by Izquierdo et al. [48] using oocytes from 2 month old females obtained in a abattoir. It has been shown that follicle size might be related to oocyte development in most species indicating a specific size of follicle for initiating the molecular cascade of normal nuclear and cytoplasmic maturation in prepubertal and adults [21, 49-50]. Oocytes retrieved from bovine follicles greater than 6 mm in diameter have better blastocyst production compared to follicles with 2-6 mm in diameter; substantially follicles smaller than 2 mm give fertilization but no capable to cleave beyond the 8 cell stage [51]. In goat has been reported that follicles around 2-3 mm have 6% blastocyst, follicles with 3-5 mm have 12% and follicles bigger than 5 mm higher percentage of blastocyst rate around 26% [52]. Recently, Romaguera et al. [16] have reported the rate of blastocyst for follicle smaller than 3 mm in goat was 5.45% compared to bigger than 3 mm in diameter

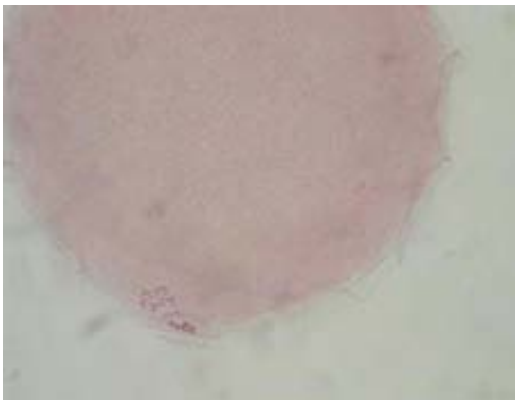
20.83%. Catala et al. [18] have reported differences in low quality of prepubertal sheep oocytes reaching to blastocysts almost 13%. A positive correlation has also been reported between size of follicles and the oocytes reaching Metaphase II after in vitro maturation [53-54]. It has already been described that the best option to obtain improvements on in vitro embryo production is to select the highest quality oocytes. For this, the size of oocytes has become an important criteria to select them because oocyte diameter has a direct and positive relationship with their competence to develop up to blastocyst. It has been shown that oocytes with 100 µm significantly produce less blastocysts (30%) in cattle compared to oocytes bigger than 110 µm (60%) [55]. Also, it has been reported that oocytes in diameter of smaller than 126µm have blastocysts rate for zero percentage, 127-144µm have 1%, 145-162µm have 7.3% and finally oocytes with greater than 163µm have blastocysts rate around 10.4% in buffalo [56]. Hammami et al. [15] in goats have shown that oocytes smaller than 125µm have significantly lower embryo developmental competence compared to oocytes bigger than 125µm (10% vs. 20%). Accordingly, small oocytes come from small follicles (less than 3mm) and it has been shown to have a reduced development competence in vitro that it might be due to lack of pre-maturation events that should occur during the final follicular growth phase [57]. Mammalian oocytes are surrounded by a compact cumulus cell layers that have called cumulus oocyte complexes (COCs). These cumulus cells respond to gonadotrophins during culture in maturation media and have known to secret different substances which interfere in cytoplasmic maturation [58]. In pigs, the male pronuclear formation has been shown to be depending to presence or absence of cumulus cells during oocyte maturation [59]. Also, this phenomenon has noticed in bovine oocyte maturation *in vitro* [60]. An oocyte that has not completed cytoplasmic maturation will not be able to have normal embryo developmental processes [61]. As cytoplasmic completion is important any deficiencies will cause failure of sperm penetration and decondensation, no capability to make normal male pronuclei, failure in blocking the zona pellucid to polyspermy, failure to produce early cleavage, failure to reach or survive during the transition time from maternal to embryonic genomic expression and finally cause to fail developing of embryos at later preimplantation and post implantation stages [62].

In vitro maturation of prepubertal sheep oocytes have improved by using components such as follicle stimulating hormone (FSH), luteinizing hormone (LH),









collected from the bottom of the 90% gradient and then diluted to a favourite concentration such as 1-2 million sperms per ml then used for fertilization [72]. Also, swim up carried out through incubating sperms under a modified synthetic oviduct fluid (SOF) for around 45 to 60 minutes then the supernatant contain motile sperms collected and centrifuged for 5 to 10 minutes. Finally the pellet collected at the bottom of falcon tube is diluted and used for fertilization [71]. 20h after co-incubation of motile sperms with matured oocytes presumptive zygotes can be checked for testing in vitro fertilization. These zygotes are classified as: normal fertilized oocytes or zygotes with 2 pronuclei (2PN) (Fig. 5), polyspermic (Fig. 6), asynchronous (Fig. 7) and sperm head with MII stage of nuclear division (Fig. 8). In the sheep, cleavage rates using ESS are normally between 50 to 70% with a 30 to 40% of oocytes developing into blastocysts. These percentages are different according to different experiments [66]. Catala et al. [17] using commercial Ovipure density gradient for collecting motile sperms from sheep frozen straws in 1×10^6 spermatozoa/ml in 50µL droplets of SOF medium supplemented with 20% of ESS had fertilization rate for 75.9% and 13.6% blastocyst in prepubertal sheep oocytes. In another study from Catala et al. [18] using commercial Ovipure density gradient for collecting motile sperms from fresh semen in 1×10^6 spermatozoa/ml in 50µL droplets of SOF medium supplemented with 10% of ESS had fertilization rate for 79% and 21% blastocyst in prepubertal sheep oocytes.

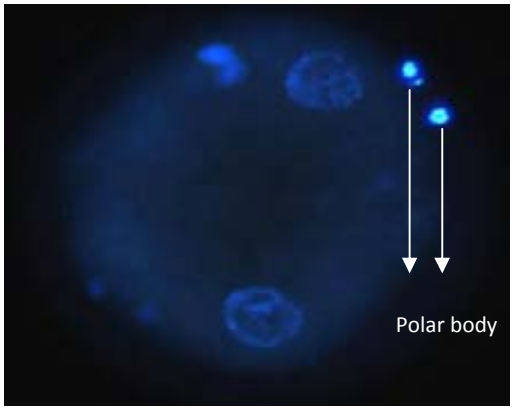


Fig. 5

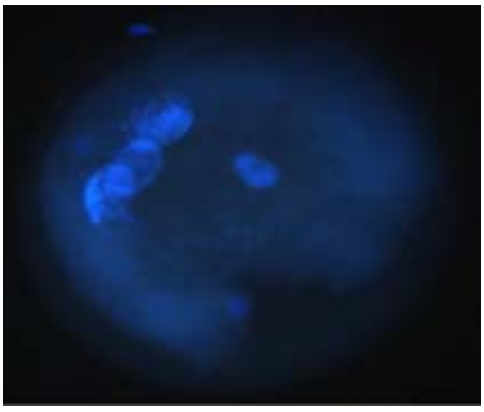


Fig. 6

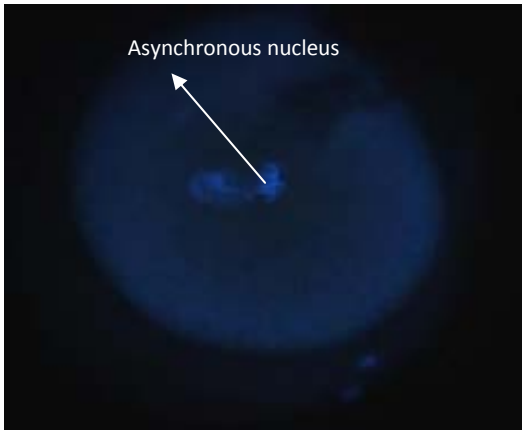


Fig. 7

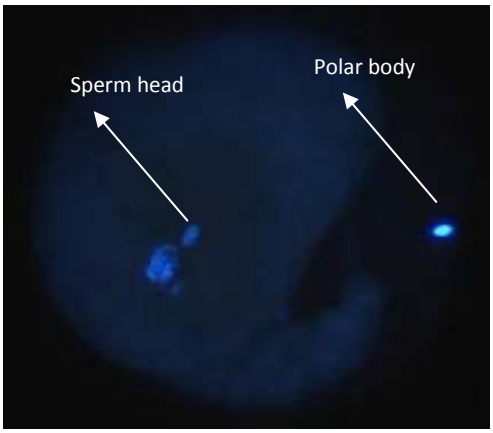


Fig. 8

Figs. 5, 6, 7, 8 stages of presumptive zygotes produced in vitro 20h after fertilization with frozen/thawed semen in prepubertal sheep

2.1.3. In vitro culture

SOF medium has used as a routine media for the culture of embryos that has established by Tervit et al. [73] supplemented with amino acids and bovine serum albumin or heat deactivated serum [74]. SOF media supplemented with 5-10% of FCS 2-3 days after post-insemination used to promote to higher viability after transfer to in vitro culture media (reviewed by [1]). Presumptive zygotes 20h of IVF washed at least three times via gentle pipetting in culture medium and then moved to the *in vitro* culture (IVC) media for recording embryo development in different stages in 48h after fertilization and at different stages of embryo development such as blastocyst, expanded blastocyst and hatched blastocyst (Fig. 9). The rate of bovine blastocysts cultured in SOF media using serum or without serum has been significantly increased around 40% vs. 20% [75]. Furthermore, the rate of blastocysts in prepubertal sheep has reported almost 13% using serum in all steps (IVM, IVF and IVC) of embryo production [17]. Prepubertal sheep produces 16% blastocyst that significantly is lower than adult that is around 34% [76]. Also, recently the rate of blastocysts on prepubertal sheep in our lab has reported to be around 13.6% for *in vitro* produced embryos and 13.1% for blastocysts produced from intra cytoplasmic sperm injection (ICSI) technique [17]. These results are comparable in

bovine and it has been reported that the blastocyst in adult bovine is 20% compared to calf that is 10% [77]. Accordingly, the results from different literatures showed that prepubertal in vitro embryo production techniques have not developed enough to support oocytes to be reached to blastocyst stage and for this reason we have aimed to try to make improvements in prepubertal sheep oocytes and sheep embryos.

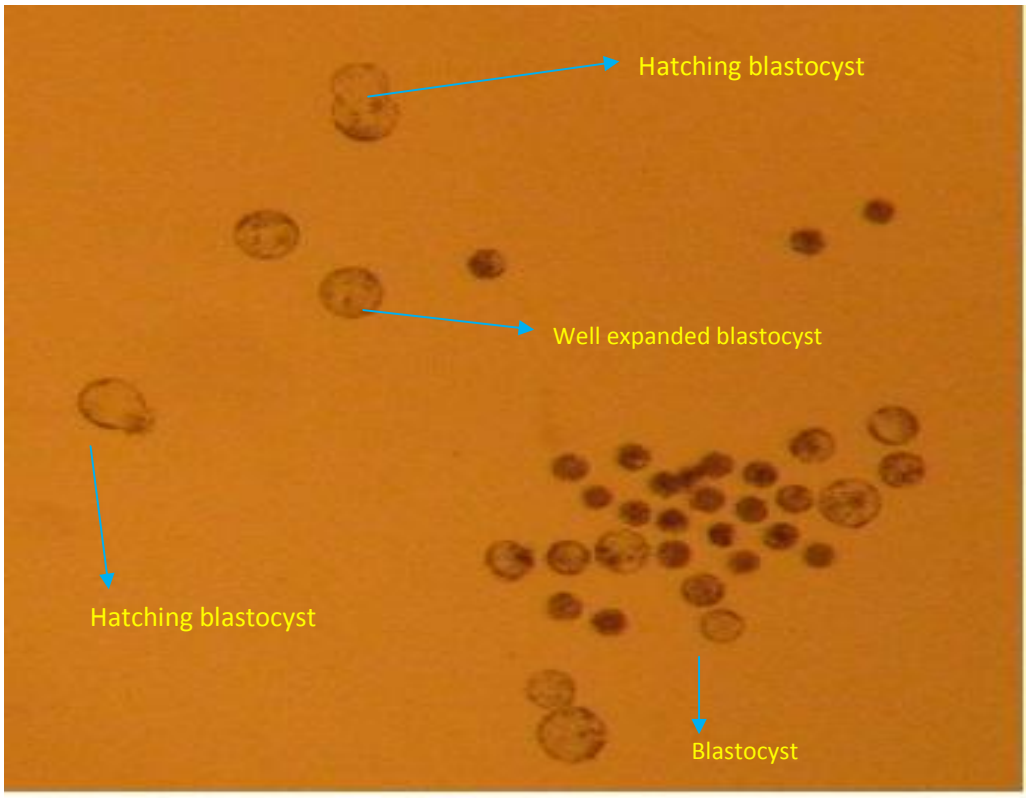


Fig. 9 Blastocysts in different stages produced *in vitro* in cow at day 8

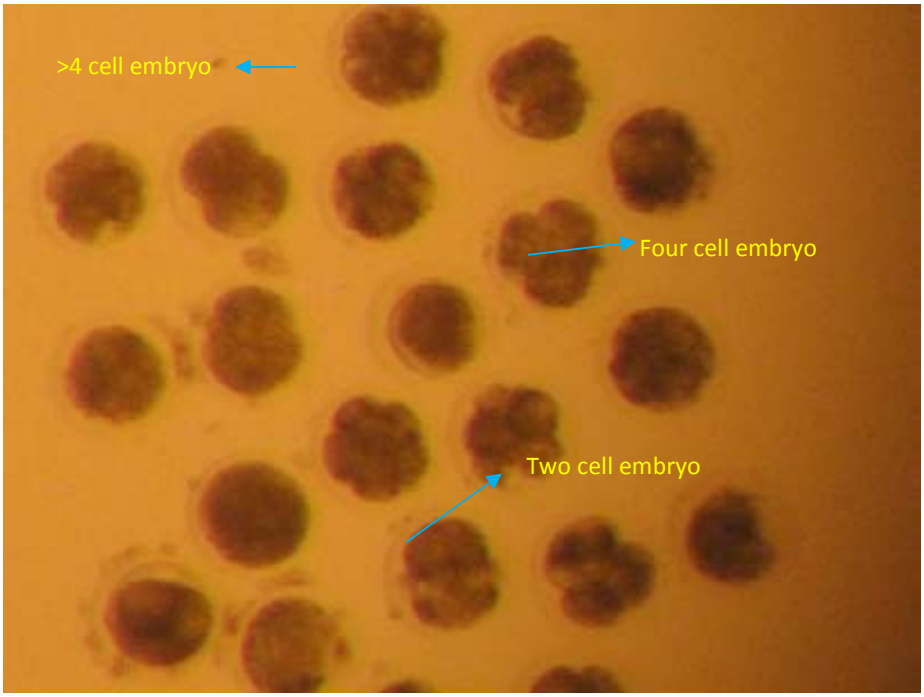


Fig. 10 Stages of embryo development 48h after fertilization produced *in vitro* in cow

2.1.4. Embryo Cryopreservation

The development of reliable techniques for long-time preservation of domestic animals embryos would enormously facilitate application of embryo transplantation in animal breeding programmes. Also, to maintain a high quality of embryos during the long term storage process of *in vitro* or *in vivo* produced embryos for commercial use, it is necessary to use freezing equipment [25, 78]. Embryo freezing has applied in different domestic animal species such as sheep [70, 79-81], cattle [25, 82-83], goat [84-85], equine [86-87], porcine [88] and human [89]. Cryopreservation would extensively enhance the usage of IVEP technologies, cryo-survival of embryos produced via IVP or micro-manipulated embryos. Also, vitrification technique provides direct transfer of embryos with reduction of the time, equipment and knowledge required to handle embryos [90-91]. Cryopreservation can be carried out via conventional slow freezing, programmable freezing and vitrification. Vitrification technique widely uses by scientists including open pulled straw (OPS) [92], superfine OPS [93], cryo-loop [94] and cryo-top [95]. Greater advances have been provided by vitrification technique, a technique that bypasses ice crystal formation and places the embryo in glass-like state with maximum

protection from cryo-damage [96]. The main factors affecting the cryopreservation of embryos are the animal species, concentration of cryoprotectant (ethylene glycol, propylene glycol, glycerol and dimethyl sulphoxide), freezing/thawing methods and the stage of embryos that are cryopreserved. On the other hand, toxicity of cryo-protective agents, composition of the cryopreservation solution, the time of exposure and the protein supplemented media (BSA and FCS) affects viability of embryos [71]. The basic media also called holding media are TCM199 and phosphate buffer solution (PBS) commonly supplemented with BSA and FCS [25]. The recently used OPS vitrification technique has successfully been used for vitrifying of embryos, IVP embryos and oocytes; however, the most commonly used method for vitrification with good rates of viability and survival after thawing are the use of cryoprotectants such as ethylene glycol (EG, is known as a most permeable cryoprotectant) [97] and Dimethyl sulphoxide (DMSO, is a better glass forming molecule) [98], and sucrose as a non permeating saccharide is another key ingredient of embryo cryopreservation protocols [96]. It has shown that sheep embryos in early stages have lower survival rate (33%) compared to 52% in embryos in advanced stages [22]. This difference may be explained by a less efficient evaluation of embryo quality for early stages of development leading to the cryopreservation of embryos of lower quality or by a higher sensitivity of these embryos to the different cryopreservation methods. On the other hand, a link between developmental kinetics and viability of the embryos was also reported for in vitro produced embryos and this may explain the lower cryoresistance of less advanced *in vivo* produced embryos [22]. Szell et al. [99] have shown that the best results were achieved when the embryos were cryopreserved after 30 or 60 second exposure to a vitrification medium containing a mixture of 3.5 M glycerol + 3.5 M propylene glycol. Under these conditions 52% (22/42) of rapidly cryopreserved sheep embryos developed into lambs. Dobrinsky et al. [100] have shown that pregnancy rate after non surgical embryo transfer were 44.5% for vitrified embryos and 45.1% for conventionally frozen controls.

In this study we have used OPS technique for cryopreservation of sheep blastocysts.

2.1.5. Serum

Serum is one of the most widely used supplements for in vitro culture cell experiments [101-106], which contains unknown substances that make it difficult to accurately describe the contents of the culture media. Moreover, it has been shown that embryos cultured in supplemented media with serum have large numbers of lipid droplets [107]. Also embryos have abnormally accumulated lipids in their cytoplasm that make the embryos sensitize to cryo tolerance [108]. It has been demonstrated that the quality of oocytes might be influence the developmental potential to the blastocyst stage; new findings show that the IVF environment is an important key in blastocyst quality. It has been suggested that serum offers energy sources, amino acids, vitamins, growth factors, and heavy-metal chelators; also, the concentrations of serum and different batches can be differed. Furthermore, supplemented media can cause morphological and physiological functions; these include increased number and size of lipid droplets and differences in embryo quality [109]. Equally, the post fertilization culture media of the developing embryo may affect the incidence and severity of mixoploidy [110]. To stop the mentioned consequences of serum in our gains we tried to work in entirely serum free media. Various serums have used for supplementing the in vitro media. Serum normally is heated at 56^{o}C for 30 minutes, supposedly to inactivate unfavorable factors like complements. Besides being of nutritive value, serum nurtures the cells surrounding the oocyte of the zona hardening when the oocyte is liberated from the follicular environment. Usually ESS, FCS, human serum is used to supplement culture media for in vitro researches (reviewed by [111]). It has been reported that the human serum to be more effective when using as supplementation in one cell sheep embryo culture compared to ESS itself [112]. The percentage of blastocyst/IVM-oocyte found in our lab using serum in each step of the in vitro prepubertal sheep embryo production protocol showed 13.6% and 13.1% for IVF and ICSI, respectively [17]. Also, in another study on sheep it has been observed 15.4% blastocyst rate [76]. The total cell number of blastocysts on prepubertal sheep was (56.8±4.8 in IVF system and 62.7±7.8 in ICSI) [17].

2.1.6. Prepubertal female *in vitro* embryo technology

Nowadays, *in vitro* maturation systems have allowed oocytes maturation in prepubertal animals to undergo fertilization and make qualified embryos to grow up to upper steps of embryo development [62], of course still have deficiencies compared to oocytes from adult females.

It has been illustrated that age of oocyte donor is an important factor affecting oocyte capability and function of IVEP; the time for collecting the ovary sample to take from slaughtered prepubertal sheep to the lab to be used on it has been considered to be around 3 months old, that it is the commercial lamb age in Spain [62]. The juvenile *in vitro* embryo technology (JIVET) has already been considered to be used as a oocyte source for production of embryos due to its advantages in shortening the generation intervals via prepubertal animals beside its accelerations on genetic achievement via transgenesis technique [113]. On the other hand, IVEP techniques have an important role in conservation of genes whether in ART of genetically valuable females in different applications such as breeding or endangered species (reviewed by [62]). It has been reported that prepubertal sheep have lower birth compared to adult (33 vs. 73 %, respectively) [114]. On the other hand, ovaries from prepubertal animals have higher numbers of oocytes than those from adult females [115]. However, *in vitro* embryo production from prepubertal animals is not as successful as adult females (reviewed by [62]). Thus, it has been shown that *in vitro* produced blastocysts is lower in prepubertal than in adult females (1.2 vs. 2.2 blastocyst per ovary) in cows [116]. Also the percentage of blastocysts obtained from IVM oocytes is lower in prepubertal ewes (20% vs. 40%) [117] and in goats (24% vs. 34%) [118].

2.2. Fatty Acids (FA)

Fats have traditionally been implied as a calorie-dense nutrient and as a source for essential fatty acids (FAs), which are biological compounds regulators [119]. The structure of FAs determines its performances that are formed regarding to the length of the acyl chain, the number of double bonds in the chain, and types of isomers formed by

each double bond. Different groups of FAs have formed regarding to their double bounds in acyl chain including saturated and unsaturated fatty acids [120].

Fatty acids have 2 or more *cis* double bonds that are separated from each other by a single methylene bridge (-CH2-unit). The essential fatty acids are all omega-3 and -6 methylene-interrupted fatty acids. Fatty acids have shown to be important for the body but only the essential ones are necessary for good health and cannot be completely synthesised in the body. Only two fatty acids are known to be essential, linoleic acid (LA) and alpha-linolenic acid (ALA) as a short chain. Some other fatty acids are sometimes classified as "conditionally essential," meaning that they can become essential under some developmental or disease conditions including eicosapentaenoic acid (EPA), docosahexaenoic acids (DHA) and arachidonic acid (AA) that form through a number of steps involving desaturation and elongation from short chain fatty acids (Table 1 [36]).

Table 1 n-3 and n-6 fatty acid families and their metabolic transformation (modified from [34])

Diet		***Diet***
n-6		**n-3**
Linoleic acid (LA)		**α-Linolenic acid (ALA)**
18:2		**18:3**
↓	← ***Δ-6-Desaturase*** →	↓
Gamma (γ)-Linolenic acid 18:3		**Stearidonic acid (SDA) 18:4**
↓	← ***Elongase*** →	↓
Di-Homo-γ-Linolenic acid (DGLA)		**Eicosatetraenoic acid (ETA) 20:4**
20:3		
↓	← ***Δ-5-Desaturase*** →	↓
Arachidonic acid		**Eicosapentaenoic acid (EPA)**
20:4		**20:5**
1–series PGs (PGE1 and PGF1α) and 2-series PGs (PGE2 and PGF2α)		***3-series PGs (PGE3 and PGF3α)***

2.2.1. Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) are generally synthesized by the modification of saturated fatty acid precursors that are products of FAs synthase. The desaturase enzymes insert double bonds at specific carbon atoms in the fatty acid chain and the fatty acid elongation system elongates the precursors in two-carbon increments (reviewed by [121]). PUFAs such as EPA and AA, are the precursors for eicosanoids including PGs, prostacyclins (PGI), thromboxanes (TX) and leukotrienes (LT) [122]. The removal of two double bonds from AA by prostaglandin H synthase (PGHS) leaves two double bounds and leads to the formation of series-2 eicosanoids, while the removal of two double bounds from EPA leads to formation of series-3 eicosanoids (Fig. 11) [36, 122]. The series-1 and series-2 are less inflammatory, whereas series-2 is more inflammatory [36, 123].

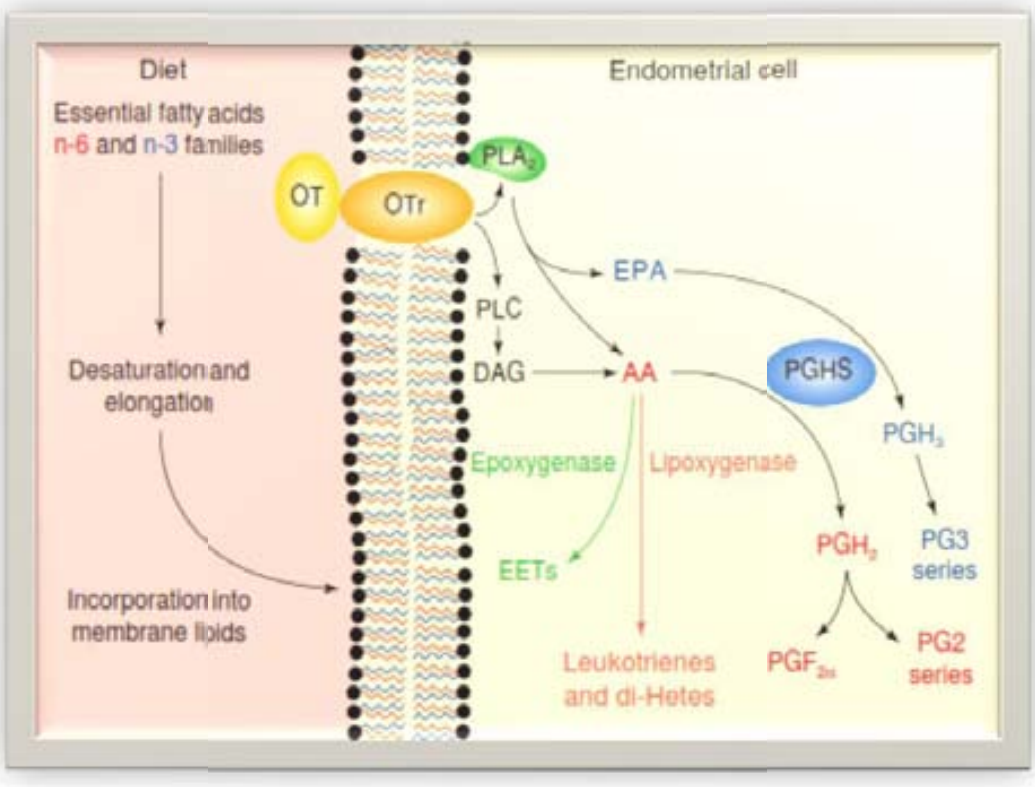


Fig. 11 Schematic representation of the metabolism of n-6 and n-3 PUFAs, and potential mechanisms for regulation of PGF secretion, Dietary PUFAs and their desaturation and elongation products are incorporated into phospholipids of the plasma membrane. The amount of each fatty acid incorporated depends on the

amount of precursor present in the diet. Eicosapentaenoic acid (EPA; C20:5, n-3) is processed by PGHS to generate prostaglandins of the 3 series. Arachidonic acid (AA; C20:4 n-6) can be processed by PGHS, epoxygenase and lipoxygenase to generate prostaglandins of the 2 series, epoxyeicosatrienoic acids (EETs), leukotrienes and hydroxyleicosatetraenoic acids (di-HETEs), respectively.

2.2.2. Importance of PUFAs on reproduction

PUFAs are considered in many physiological performances and activities of the animal reproductive system [32]. PUFAs have important effects on the structure and physical properties of localized membrane domains and also are important in producing of eicosanoids [124]. It has been shown that PUFAs have important affects on reproductive hormones, follicle development, oocyte maturation and quality, embryo survival, pregnancy rate, onset of oestrous and ovulation rate, gestation rate and parturition. Also, it has been shown that the PUFAs might affect maturation, embryo development and cryopreservation [37]. ALA is produced by the ovarian follicles and it is present in the follicular fluid that is involved in oocyte maturation and presumably in oocyte competence for developing to blastocyst stage (reviewed by [36]). ALA, one of the important PUFAs, is involved in oocyte growth and differentiation, and it has reported to have a role in the regulation of meiotic arrest at the germinal vesicle (GV) stage [37]. Marei et al. [68] have shown that ALA in 50µM in IVM media in cow significantly increased the percentage of oocytes at the MII stage. Also these authors determined that higher concentrations of ALA were detrimental. Marei et al. [68] have demonstrated that ALA supplemented in IVM media in cow had increased cleavage rate compared to control (77% vs. 69%), blastocyst rate (36% vs. 23%) and better quality embryos. PUFA-enriched serum from ewes (ewes were offered omega-3 or omega-6 PUFA-enriched in diet) have shown that enhance the FA content of day 7 sheep blastocysts but the quality of the embryos were poorer morphologically, Hughes et al. [125] have resulted that this poor morphological grades was associated with super oxide dismutase 1 (SOD1) transcript expression, indicative of enhanced superoxide toxicity. Marei et al. [69] using LA in IVM media have shown that in cow treatment of cumulus–oocyte complexes (COCs) with LA significantly inhibited cumulus cell expansion and retarded development of the oocytes to the metaphase II (MII) stage in a dose-dependent manner. This effect was reversible, and the oocytes developed to the MII stage after extended culture in the

absence of LA. Treatment of COCs with LA also resulted in a significantly lower percentage of cleaved embryos and blastocyst yield.

2.2.3. PUFAs and Eicosanoids

Prostaglandin E2 is the dominant PG in the pre-ovulatory follicles [126]. Several studies have demonstrated that PGE_2 plays important roles in cumulus cell expansion and oocyte maturation in rodents [127-130] and cattle [131]. Inhibition of PGE2 using PG-endoperoxide synthase 2 in mice decreases cumulus expansion and oocyte maturation [132]. PGE2 is considered as a luteotrophic factor in the early luteal phase. Marei et al. [133] have shown that PGE2 supplementation in GnRH free media increased cumulus expansion and MII stage in oocytes matured in IVM, also have shown that embryo development using PGE2 in IVM has not altered embryo development. These authors have not specified the effects of PGE2 in GnRH free media on embryo development. Marei et al. [68] have shown that ALA significantly increased the concentration of PGE2. Furthermore, Marei et al. [69] using LA in IVM media determined that COCs treated with LA had significant effects compared with controls increasing prostaglandin E_2 concentration in the medium, while supplementation of oocyte maturation media with LA at all concentrations did not have any significant effect on the production of $PGF_{2\alpha}$ by COCs. Later in the cycle, PGF2α is the main luteolytic agent (reviewed by [37]). Sayre et al. [134] have shown that using flushed caprine embryos at day 6 following estrous synchronization and super ovulation, in 25µl droplets of TCM-199 enriched with BSA (8 mg/ml) for 6 days at 38.5 °C in a 5% CO_2 in air with one of the following treatments: (1) control (0.0002% EtOH), (2) PGE_2 (7 ng/ml), (3) $PGF_{2\alpha}$ (7 ng/ml), (4) low PGE_2:high $PGF_{2\alpha}$(3.5 ng/ml:14 ng/ml), (5) balanced PGE_2:$PGF_{2\alpha}$ (7 ng/ml:7 ng/ml), or (6) high PGE_2:low $PGF_{2\alpha}$(14 ng/ml:3.5 ng/ml). Treatment with PGE_2 alone reduced ($P < 0.05$) the hatching rate (1/15; 7%). The hatching rate of embryos treated with $PGF_{2\alpha}$ alone (9/18; 50%), low PGE_2:high $PGF_{2\alpha}$ (8/16; 50%), and balanced PGE_2:$PGF_{2\alpha}$ (11/16; 69%) were similar to control (6/18; 33%). In contrast, the hatching rate was non-significantly increased (13/18; 72%) with the high PGE_2:low $PGF_{2\alpha}$ treatment. None of the treatments affected development from the morula to blastocyst stage. These authors concluded that PGE2 alone reduced hatching rate and PGF2α alone had no effect on the development of caprine embryos.

High concentrations of PGE2 with PGF2α improved the hatching rates. These authors described that uterine concentrations of PGE2 may need to reach a threshold level to improve embryo hatching, as previously reported, while increased uterine concentrations of PGF2α during early pregnancy would not affect development of the embryo. Ovine embryos metabolize arachidonic acid to PGE_2 by day 4 of pregnancy, which continue beyond day 14, while $PGF_{2\alpha}$ production begins at day 10 [135]. The cleavage rate of bovine embryos has been shown to be lower in groups with reduced concentrations of PGE_2 secreted into the culture media during oocyte maturation [136], and the hatching rate of ovine embryos increased with treatment of PGE_2 [135]. Inhibitors of prostaglandin production reduced the fertilization rate in mice [137]. Endometrial concentrations of $PGF_{2\alpha}$ can be increased by various stressors and when prematurely increased were associated with embryonic deaths in the cow [138]. Likewise, $PGF_{2\alpha}$ reduces blastocyst formation and the hatching rate in bovine [139], rabbit [140] embryos. In contrast, Soto et al. [141] reported $PGF_{2\alpha}$to inhibit embryonic development only if treatment was during oocyte maturation or fertilization, but it did not affect the development if treatment was at stages after fertilization. The ratio of PGE_2 to $PGF_{2\alpha}$ during early pregnancy may be involved in the maintenance of pregnancy. Evidence in humans indicates that follicular ratio of PGE_2 to $PGF_{2\alpha}$ was associated with the pregnancy rate after IVF with increased concentrations of PGE_2 to $PGF_{2\alpha}$ was being associated with a greater rate of pregnancy [35]. During the period of signaling to the mother to maintain the pregnancy, uterine concentrations of PGE_2 were increased and the typical peak in concentration of $PGF_{2\alpha}$ for luteolysis was reduced. Endometrial production of PGE_2 in the ewe was increased with embryonic production of interferon-τ (IFNτ), and the enzyme involved in conversion of PGE_2 to $PGF_{2\alpha}$, 9-keto-PGE reductase, was reduced with IFN tau [142]. Both of which leads to an overall increase in uterine concentration of $PGE_2$and an increased PGE_2:$PGF_{2\alpha}$ ratio. Likewise, estradiol production increased the uterine PGE_2:$PGF_{2\alpha}$ ratio during establishment of pregnancy in the pig [143]. The effects of ALA and LA on prepubertal sheep oocytes maturation and development are not studied yet.

2.2.4. PUFAs and Steroid hormones

Progesterone (P) and estradiol (E2) are required for successful conception, both to prepare the endometrium for blastocyst implantation and pregnancy. During IVF-ET, controlled ovarian hyperstimulation results in excessive follicular development and supraphysiologic serum concentrations of E2 and P4 [144]. P4 is a cholesterol-derived, phylogenetically old steroid hormone. It is synthesized during the steroid hormone metabolizing pathways from androgens, estrogens, and glucocorticoids within several cell types such as the corpus luteum, placenta and adrenal gland. P4 is an intra follicular steroid that plays critical roles in ovulation, implantation and maintenance of pregnancy (reviewed by [145]). Resumption of meiosis in oocytes is triggered by steroid hormones, specifically P4, in certain species [146]. The resumption of meiosis and its progression to MII in several mammalian species such as cows, sheep and pigs is steroid dependent and the inhibition of steroidogenesis in ovine follicles leads to impairment of resumption of meiosis and progression to MII. According to research, levels of P4 in follicular fluid and its ratio to the estrogen levels are strongly associated with oocyte quality and maturity in other word E2 is a critical factor for oocyte quality that subsequent embryo development [147]. However, controversy exists regarding the effect of P4 on *in vitro* oocyte maturation. Investigations have shown that addition of P4 (10, 38, 50, 100μM) in IVM of mouse GV oocytes could not improve maturation rates and developmental competence of COCs and cumulus denuded oocytes (CDOs) at any of the tested concentrations when compared to the control groups. When we increased P4 from 10 to 100μM in the culture medium, the maturation rate decreased in a dose dependent manner and the GV arrested rates increased. Research has shown that the effect of P4 in inhibition of meiotic resumption was more effective in COC than CDO [148]. It seems there were intensive interactions between oocytes and the surrounding cumulus cells. Oocytes could affect cumulus cell functions.

Vanderhyden et al. [149] have shown that mouse oocytes modulate steroid production by the surrounding cumulus cells. These observations have suggested that oocytes secret a factor(s) which control cumulus cell production of E2 and P4. In contrast, Jamnongjit et al. [150] observed that testosterone or P4 and epidermal growth factor induced meiotic resumption in mouse oocytes during their *in vitro* maturation; the

effect of these steroids could have been inhibited by specific receptor antagonists. Fukui et al. [151] demonstrated that P4 supplementation of IVM culture systems decreased the rate of bovine oocyte maturation and that addition of P4 to fertilization culture medium did not improve the number of cleavage stage embryos. Carter et al. [152] have shown that addition of P4 to culture medium did not affect the proportion of *in vitro* matured/*in vitro* fertilized oocytes that developed to the blastocyst stage *in vitro*. There was no effect on conceptus elongation following transfer to synchronized recipient heifers.

The role of steroids has been shown to be involved in the acquirement of meiotic competence and the ability to undergo normal fertilization and development to the blastocyst stage [153]. In humans and rhesus monkeys, high ratios of P4 to E2 in follicular fluid were associated with better embryo development [154].

The effect of P4 in concentrations similar to preovulatory follicular fluid (10 and 38μM) on the developmental competence of mouse GV oocytes and subsequent fertilization potential has evidenced. P4 could not increase the fertilization rate and development of the embryo to the blastocyst stage [148]. The result of this experiment was inconsistent with other studies [155-157]. Silva and Knight [158] have shown that the addition of P4 to bovine oocyte *in vitro* maturation medium reduced the rate of blastocyst formation.

Mattioli et al. [59] reported that presence of P4 in porcine oocyte maturation medium increased subsequent sperm head decondensation and male pronuclei formation. Zhang and Armstrong [159] reported that the addition of P4 to porcine oocyte maturation medium could increase both fertilization and cleavage rates, whereas E2 could not. P4 had the opposite effect in ovine oocytes [156].

To answer the question of whether P4 directly affects embryo development or there is an indirect effect via changes in the endometrium, some researchers have added P4 to embryo culture medium *in vitro* and examined development to the blastocyst stage, with contradictory results [160-162]. These contradictory observations might be attributed to different culture systems which have been used.

In vitro and *in vivo* experiments by Clemente et al. [163] showed that the effects of P4 on conceptus elongation could be due to a direct effect of P4 on the embryo. They demonstrated that a P4 receptor was expressed in all stages of embryo development. These researchers showed the direct effect of P4 on embryo development. Supplementation of simple or co-culture embryo culture systems with P4 did not affect on the embryo development and blastocyst cell number. However, *in vitro*-derived embryo transfer to a recipient treated with P4 resulted in a four-fold increase in conceptus length on day 14. These data confirmed the hypothesis that conceptus elongation in cattle was related to P4- induced changes in the uterine environment [163]. This finding agreed with a study by Geisert et al. [164] who showed that administration of P4 early in the estrous cycle advanced uterine receptivity for the transfer of older asynchronous embryos. Supplementation of embryo culture medium with lipid soluble P4 resulted in an increase in the numbers of embryos that developed to the blastocyst stage [165-168]. However different observations were reported by other studies [158, 166, 169].

Ferguson et al. [161] have demonstrated that addition of physiological concentrations of P4 to embryo culture medium at three days post-insemination benefitted embryo development in several ways. Thus, they have concluded that P4 has a direct positive effect on the developing *in vitro* culture of bovine embryos. P4 supplementation increased the number of *in vitro* culture embryos that developed to the grade 1 blastocyst stages well as the number of hatched blastocysts. It was shown that co-culture of an embryo with endometrial tissue cultured in the presence of P4 and E2 benefitted embryo development [170].

These results have shown that P4 could be a factor for embryonic survival and improve *in vivo* embryo development and implantation, both directly and indirectly. The effect of P4 on oocyte maturation and embryo development may be dependent on its concentration and the mammalian species [145]. It has been shown that ALA and LA from PUFAs may affect a number of factors related to the synthesis and metabolism of main reproductive hormones such as progesterone (P4) and estradiol (E2). Cumulus cells secrete E2 and P4 [171]. E2 is necessary for development of follicles and oocyte maturation *in vivo* [172] and also for fertilization [173]. Follicular E2 production was

higher in cows supplemented with ALA [174], and some (but not all) studies have found depressed P4 concentrations in the early luteal phase in treated animals with PUFAs (reviewed by [37]). Similarly, ALA increases P4 production in theca cells [171].These actions are either mediated directly through effects of PUFAs on steroid synthesis, for example through altered steroidogenic acute regulatory protein (STAR) expression [175], or indirectly through altered PG production. Wonnacott et al. [176] have shown that follicle number and size were altered when they were used omega-3 and omega-6 PUFAs in diet feeding for sheep, whereas, follicular fluid P4 concentration in ewes fed with omega-3 was higher than omega-6. The ratio of E2/P4 in follicular fluid has been used as a surrogate marker of healthy and atretic follicles [177]. It has been illustrated that successful pregnancies were more likely to occur in human IVF using oocytes from follicles containing high levels of E2 and a higher ratio of E2/P4 [178]. Supplementation of oocyte maturation media by P4 inhibitor decreased the number of GVBD in porcine in a reversible manner [167]. However, supplementation of P4 in the maturation medium has a reduction effect on blastocyst production [158]. A drop in P4 level removes its negative feedback inhibition of GnRH from hypothalamus, thus pulses of GnRH increases the E2 secretion. This increase in E2 concentration up-regulates ovarian receptors for FSH and LH [179]. It has been shown that E2 is necessary for development of follicles and maturation *in vivo*.

2.2.5. Steroids related to eicosanoids in vivo and in vitro

In ruminants, oxytocin (OT), P4, and E_2 regulate the uterine secretion of $PGF_{2\alpha}$ that causes luteolysis [180]. OT, from the pituitary and corpus luteum (CL), stimulates the pulsatile release of $PGF_{2\alpha}$ via the OT receptor (OTR) in the luminal epithelium of the endometrium, resulting in regression of the CL (reviewed by [181]). Progesterone can regulate $PGF_{2\alpha}$ secretion in different ways. Prolonged exposure to P4 promotes the endometrial accumulation of arachidonic acid and cyclooxygenase (COX) necessary for the synthesis of $PGF_{2\alpha}$. Progesterone also exerts a suppressive effect on $PGF_{2\alpha}$ secretion, which wanes after prolonged exposure [180]. This suppressive effect is due to an inhibitory action on OTR gene expression during the early and midluteal phase of the estrous cycle (reviewed by [181]). Estradiol is also important for the timing of luteolysis because removal of E_2results in a prolonged cycle [182] and administration of

E_2 in midcycle initiates luteolysis by increasing plasma PGFM (13,14,-dihydro-15-keto-$PGF_{2\alpha}$) concentration [183], presumably by raising endometrial OTR concentration [184]. The effect of E_2 is dependent on P4 because it is only observed after the endometrium has been primed with P4 for a certain period of time (reviewed by [181]). E_2 can also affect the magnitude, timing, and pattern of $PGF_{2\alpha}$ pulses in response to OT [185]. During the early and midluteal phases, estrogen receptor (ER) expression is suppressed in the ovine endometrium except in the deep glands, presumably by an inhibitory action of rising P4 levels [186]. It has been known for a long time that progesterone stimulates prostaglandin synthesis in the endometrium of the ewe [187]. Some *in vivo* studies have been carried out to try to determine the regulation of the enzymes involved in prostaglandin synthesis. cPLA2 is increased at Day 11 of the cycle in sheep [188]. The cPLA2 is probably regulated by steroid hormones because E_2 increases PLA2 activity in the rat uterus [189], but no studies on the regulation of PLA2 in the ruminant endometrium have been reported and no studies have examined the possible role of sPLA2. COX-2 is up-regulated during the estrous cycle in sheep and cattle [190-191]. The expression of COX-1 appears to differ between the two species, in cattle COX-1 is undetectable [191], whereas in sheep, COX-1 is highly expressed but does not change during the cycle. Progesterone up-regulates COX-2, and E_2, either alone or together with P4, has no effect [190]. PGFS is also up-regulated during the estrous cycle in the cow [192], but no studies have been performed *in vivo* to determine if steroid hormones are involved. The 9-keto-PGE reductase is also present in the ovine endometrium and it increases during the late luteal phase [193].

In vitro studies have been used to try to examine the mechanisms involved in the regulation of prostaglandin synthesis. In general, P4 stimulates $PGF_{2\alpha}$secretion from endometrial epithelial cells [194] and endometrial strips [195]. The observed stimulation of prostaglandin synthesis by P4 in endometrial epithelial cells was not due to an increase in either COX-2 or PGFS mRNA expression. Thus, the increase in COX-2 induced by P4 *in vivo* could be due to an action of P4 on other cells of the endometrium. The effect of E_2 in vitro is more variable. Although studies on human endometrium have demonstrated that estrogens enhance PG production in epithelial cells by elevating PG synthase activity, no stimulatory effect of E_2 on the expression of either COX-1 or -2 has been observed in ruminants [196]. This agrees with the *in vivo* results obtained by Charpigny et al. [190]. E_2inhibits $PGF_{2\alpha}$ secretion from endometrial cells but had no

effect on endometrial strips. This inhibitory effect of E_2 on isolated cells could be explained by an inhibition of COX-2, but not PGFS, expression. The situation is further complicated by the observation that, in endometrial tissue taken from long-term ovariectomized cows, estradiol stimulates prostaglandin secretion [197].

2.3. Hyaluronic Acid (HA)

Hyaluronan (Hyaluronic acid, HA) is a carbohydrate; more specifically is a polysaccharide with long polymer chains of sugars and is found in the extracellular matrix of most animal tissues [98] Also, HA is found in the intercellular matrix of mammalian tissues, like bovine vitreous humor, rooster combs and umbilical cords, and also produced by Streptococcus zooepidemicus [97]. Furthermore, HA is one of the most plentiful Glycosaminoglycan (GAG) in the female reproductive tract like uterine, oviduct and follicular fluids in human [198], cattle [199] and pig [200]. HA in the oocyte is secreted by granulosa and expanded cumulus cells [101]. The main functions of Hyaluronan are involved in cell to cell adhesion [201], sperm motility, capacitating and the acrosome reaction [202]. It has been shown that HA may play a role in delaying death of the oocyte, prevention of fragmentation of porcine oocytes [203] and improves *in vitro* bovine embryo development to the blastocyst stage [44]. It has also been demonstrated that increasing cryo-survival of embryos may be due to the addition of HA to freezing media in murine and bovine [204], possibly by decreasing the formation of intracellular ice crystals [102]. Physiological concentrations of Hyaluronan in the female reproductive tract such as follicular fluid, oviduct and uterine of pigs is from 0.04 to 1.83 mg/ml [205]. Also the concentration of HA in COC is between 0.5 to 1 mg/ml [206] and in follicular fluid in human is 55.1 ± 2.4 ng/ml which is used as an indicator to predict oocyte viability for fertilization [207]. Based on this knowledge, the concentration up to 1 mg/ml HA in Synthetic Oviduct Fluid (SOF) was selected for my subsequent studies.

Hyaluronan is a high molecular weight polymer that can be cleaved to low molecular weight fragments of oligosaccharides. Hyaluronic acid is a linear and unbranched polymer that is produced from simple repeated disaccharides. The disaccharides are formed of N-acetyl-D-glucosamine (GlcNAc) and D-glucuronic acid (GlcA) connected through a β1-4 glycosidic bond, to make an HA chain (Fig. 2).

Therefore, Hyaluronan is a homogeneous polymer with a molecular size which spans the range (10^5 - 10^7 Da) [208].

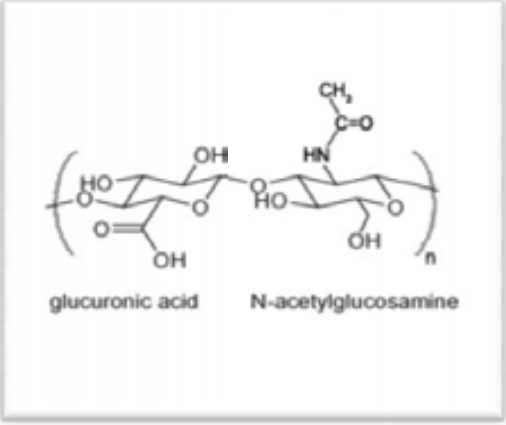


Fig. 2 Structure of Hyaluronan, composed of two monosaccharides β (1, 4) – *N* acetyl D glucosamine and β (1, 3) – D - glucuronic acid http://www.glycosan.com/ha_science/what_hyaluronan.html

The synthesis of HA occurs at the inner face of the plasma membrane and exists as a free linear polymer, in contrast to other GAGs, produced by Golgi apparatus enzymes and covalently attached to protein cores. HA synthesis is due to a group of integral plasma membrane Glycosyltransferases, known as HA synthases (HAS) of which three are identified, and are suggested to both polymerize and translocate HA from the inner face to the extracellular matrix [209]. These synthases have the ability to synthesize HA and are dual headed transferases which use the two UDP-sugar substrates, UDP-glucuronic acid and UDP-N-acetylglucosamine [210]. There are three independently expressed HA synthase genes (HAS1, HAS2 and HAS3) that have been identified in the mammalian genome encoded by three separate but related genes [209-210] HAS2 in human genome is present on chromosome (Chr) 8q24.1 and mouse Chr 15, while HAS1 and HAS3 are in loci 19q13.4 and 16q22.1 and in mouse Chr 17 and Chr 8, respectively [211]. HAS isoforms synthesize HA of different sizes *in vitro* in the range of 2×10^5 to 2×10^6 Da by HAS1, 2×10^6 Da by HAS2, 1×10^5 to 1×10^6 Da by HAS3 [212].

2.3.1. Sizes of Hyaluronic Acid

The large molecular polymers of HA are extracellular, space-filling and have a role in regulatory and structural characterizations. The small fragments are angiogenic,

inflammatory and immune stimulatory. The high concentration of HA in the fetal circulation and amniotic fluid can act as an immunosuppressant during fetal growth. The large molecular weight HA is found during ovulation, fertilization and embryogenesis [213]. Tetrasaccharides produce expression of heat shock proteins and are anti apoptotic and suppress cell death [214]. The physical and biological functions of HA is affected by concentration and chain length. It is suggested, high molecular weight (HMW) HA at high concentrations suppresses endothelial cell growth, while low molecular weight (LMW) HA stimulates endothelial cell growth leading to induction of angiogenesis. Moreover, both HMW and LMW are expressed in the ovine cervix and are shown to increase at estrous [215]. It has been shown intracervical application of LMW HA, 52 hr after sponge removal increased cervical penetration up to 3.4 cm to allow transcervical intrauterine artificial insemination in sheep [216]. Hyaluronic acid is degraded through three pathways, the hyaluronidase (Hyal) enzyme is important in Hyaluronan catabolism [213]. The Hyaluronidases can be classified by their reaction products: 1. Bacterial Hyaluronidases (EC 4.2.99.1), 2. Endo-β-glucuronidase (EC 3.2.1.36) [209], 3. Mammalian types of Hyaluronidases (EC 3.2.1.35) which are part of the glycoside hydrolase group 56, a group of enzymes that cleave Hyaluronic acid at the β1-4 linkage between N-acetyl-β-D-glucosamine and D-glucuronic acid to generate smaller oligosaccharides [217]. Six genes are supposed to encode Hyals, theses genes include: HYAL1, HYAL2, and HYAL3 on human Chr 3p21.3 and in mouse on Chr 9F-F2 and HYAL4, SPAM1, and HYALP1 on human Chr 7q31.3 and in mouse on Chr 6 A2. Among the described Hyaluronidases Hyal2 is more important, Hyal2 has a glycophosphotidylinositol (GPI) anchored form that is localized to the outer membrane of cells [218]. Also Hyal2 cleaves HMW HA to a limit product about 20 kDa or almost 50 disaccharides units [209]. Through specific cell surface receptors, Hyaluronic acid is known to contribute to cell signaling [219].

2.3.2. HA Receptor

CD44 is the key, primary cell surface receptor for Hyaluronan. It is a member of a big group of HA-binding proteins (HABP) called Hyal-adherins. These have important functions in different cellular events that include cell-cell and cell-extracellular matrix reactions like proliferation, adhesion and migration. Moreover, this receptor has been shown to be involved in the binding and presence of certain growth factors [220]. Another receptor for HA is HA mediated motility (RHAMM) that has been shown to

promote cell growth and movement, sperm motility, angiogenesis and embryonic development [221]. The third receptor for HA is HA receptor for endocytosis, termed HARE. This receptor is able to internalize Hyaluronan by endocytosis through the clathrin coated mark pathway, shown in liver sinusoids; which attenuates the clearance of systemic circulating HA by HARE [222].

2.3.3. HA Functions

HA is shown to have positive effects in improving the number of reached embryos to blastocyst stage from *in vitro* matured (IVM) bovine oocytes [44]. Furthermore, the addition of HA to the culture media on the third and fifth day strengthened cryo tolerance of blastocysts and also improved rates of lambing in sheep [101]. HA has stimulated mouse embryo development in culture media [223]. Researchers have shown that recombinant albumin and HA increased the ability of embryos to re-expand and hatch following slow freezing [204]. Addition of Hyaluronic acid to media after thawing has improved the motility parameters of sperm and increased the fertility rate. Furthermore, it has been reported that addition of 1250 µg/ml HA has negative effects but 750 µg/ml of HA has the greatest effect on the motility, vitality and fertilization rate after cryopreservation [224]. Additionally, added HA in high concentrations has increased the viscosity of SOF media (6mg/ml) in comparison to SOF media containing Bovine serum albumin (BSA), and on day 8 the rate of blastocysts was increased in SOF supplemented with HA compared with BSA [102].

CHAPTER 3:

EFFECT OF ALFA-LINOLENIC ACID (ALA) ON OOCYTE MATURATION AND EMBRYO DEVELOPMENT OF PREPUBERTAL SHEEP OOCYTES

3. Effect of Alfa-linolenic acid (ALA) on oocyte maturation and embryo development of prepubertal sheep oocytes

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Abstract

The purpose of this study was to evaluate the effect of omega-3 α-Linolenic acid (ALA) added to the IVM medium on embryo development of prepubertal sheep oocytes. Experiment 1 investigated the effect of ALA at different concentrations (0 [control], 50, 100 and 200 µM) and DMSO (100 µM) in IVM media on cumulus cell expansion and oocyte nuclear maturation and on synthesis of prostaglandins (PGE2 and PGF2α). Experiment 2 investigated the effects of ALA at different concentrations in the IVM medium on oocytes fertilization, cleavage and developmental potential to blastocyst stage and changes in estradiol and progesterone concentrations in the spent IVM media. IVM oocytes were fertilized with frozen-thawed spermatozoa capacitated in a serum-free sperm medium. Presumptive zygotes were cultured for 8 days in synthetic oviductal fluid (SOF) medium without serum. Blastocyst quality was assessed by counting total cell number and the number of apoptotic cells using Hoechst and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Nuclear maturation of oocytes and the number of fully expanded cumulus cells were reduced after treatment with 200 µM of ALA compared to other groups ($P \leq 0.05$). Supplementation with ALA increased both PGE2 and PGF2α concentrations in the spent medium ($P \leq 0.05$). No differences were observed in blastocyst development among control (12.2%) and 50, 100 and 200 µM of ALA groups (6.9, 11.5 and 14.0%, respectively). However, total cell number (46.50±5.85, 67.94±6.71, 45.20±6.37, and 59.80±5.51, respectively, $P \leq 0.05$) and apoptotic cell numbers (6.45±0.89, 2.48±0.81, 4.02±1.15, and 3.67±1.15, respectively, $P \leq 0.05$) were significantly improved. After IVM, estradiol concentration was lower and progesterone concentration was higher in ALA groups compared to control group ($P \leq 0.05$). In conclusion, these results revealed that ALA affects prepubertal sheep embryo quality associated with alteration of releasing reproductive hormones.

Keywords: α-linolenic acid, prepubertal sheep, oocytes, embryos, IVM.

3.1. Introduction

Reproduction in ruminants is associated with the accessibility of energy. Dietary fats support reproductive functions by supplying energy and by actions on reproductive performances. For example, increasing the amount of fatty acids (FAs) in diets increases steroid and eicosanoids secretion which can alter ovarian and uterine functions and affect pregnancy rates (reviewed by [120]). The FA component of the diet also affects the oviductal and uterine environments (gene expression) to promote embryo development [225]. The animals fed with FAs have altered proportions of FAs in the follicular fluid [226] and show improved reproductive functions in bovine [227-228] and sheep [125, 229]. Fatty acids are important sources of energy for oocytes and embryos [227]. Fouladi-Nashta *et al* [230] reported that an increased level of rumen-inert FA in diet improved the developmental potential of bovine oocytes to the blastocyst stage and also embryo quality. However, no changes in the morphology of the oocytes (grades 1–4) or cleavage rate were found. But the blastocysts from the high-fat group contained considerably more total inner cell mass and trophectoderm cells compared to the low-fat group.

Polyunsaturated fatty acids (PUFAs) including omega-6 and omega-3 essential FAs are important for health but cannot be synthesized in the body and must be provided by diet (reviewed by [124]). They affect reproduction in both males and females. It has been shown that the PUFA content of oocytes might affect maturation, embryo development and cryopreservation [37]. Alfa-linolenic acid (ALA) belongs to the omega-3 group (C18:3, n-3) and is involved in oocyte growth and differentiation and was reported to have a role in the regulation of meiotic arrest at the germinal vesicle (GV) stage (reviewed by [37]). In the body, ALA is transformed to eicosapentaenoic acid (EPA, C20:5).

The long-chain polyunsaturated FAs, EPA and ALA, are the precursors of eicosanoids including prostaglandins (PGs) which play important roles in reproduction functions such as ovulation, estrus, embryo survival, and parturition [36]. A-Linolenic acid is produced by the ovarian follicles and is present in the follicular fluid. It is involved in oocyte maturation and presumably in oocyte competence in development to the

blastocyst stage (reviewed by [36]). Fatty acids may affect the maturation of oocytes by altering the structure of lipids in oocytes [230] or through changes in the type and concentrations of PGs or other metabolites present in the follicular fluid surrounding the oocyte [231]. Prostaglandin E2 is the dominant PG in the preovulatory follicles [126]. Several studies have reported that PGE2 plays important roles in cumulus cell expansion and oocyte maturation in rodents [127-130] and cattle [131]. Inhibition of PGE2 using PG-endoperoxide synthase 2 in mice decreases cumulus expansion and oocyte maturation [132]. PGE2 is considered as a luteotrophic factor in the early luteal phase. Later in the cycle, PGF2α is the main luteolytic agent. It has been reported that ALA and EPA can inhibit PGF2α release in bovine endometrial cells (reviewed by [37]). The effects of ALA and its metabolite PGE on prepubertal sheep oocytes maturation and development are not clear.

Cumulus cells secrete the steroid hormones estradiol (E2) and progesterone (P4) [171]. PUFAs can influence ovarian steroid synthesis. For example, follicular E2 production was higher in cows supplemented with ALA [174], and some (but not all) studies have found low P4 concentrations in the early luteal phase in PUFA-treated animals (reviewed by [37]). Similarly, ALA increases P4 production in theca cells [171].These actions are either mediated directly through the effects of PUFAs on steroid synthesis, e.g., through altered steroidogenic acute regulatory protein expression [175] or indirectly through altered PG production. The ratio of E2-to-P4 in follicular fluid has been used as a surrogate marker of healthy and atretic follicles [177].

The use of Juvenile *in vitro* Embryo Technology (JIVET) in breeding programs is beneficial as it decreases the generation intervals and enhances the genetic gain. On the other hand, ovaries from prepubertal animals have higher numbers of oocytes than those from adult females [115]. However, *in vitro* embryo production from prepubertal animals is not as successful as adult females (reviewed by [62]). Thus, it has been shown that *in vitro* produced blastocysts is lower in prepubertal than in adult females (1.2 vs. 2.2 blastocyst per ovary) in cows [116]. Also the percentage of blastocysts obtained from IVM oocytes is lower in prepubertal ewes (20% vs. 40%) [117] and in goats (24% vs. 34%) [118]. Considering the beneficial effects of ALA supplementation in improving oocyte maturation and developmental potential, we have conducted

experiments to assess whether supplementation of different ALA concentrations to prepubertal sheep oocyte *IVM* media can improve their fertilization rate and development to the blastocyst stage. We have also analyzed the concentrations of PGs and steroids in the spent media.

3.2. Materials and Methods

3.2.1. Materials

Except otherwise mentioned, all chemicals were obtained from Sigma Chemical Co (St. Louis, MO, USA). Embryo culture media were incubated at 38.5^{o}C under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 for at least 2 hours before culture. Washing and Maturation media were incubated at 38.5^{o}C under a humidified atmosphere of 5% CO_2 in air for at least 2 hours before use for slicing and culture.

3.2.2. Oocyte collection

Ovaries from 3-month old prepubertal sheep were obtained from a local abattoir and brought to the laboratory within 1 hour in PBS at 30^{o}C to 35^{o}C. Cumulus oocyte complexes (COCs) were retrieved by slicing the ovaries in 55-mm Petri-dish containing washing media basically Tissue culture medium 199 supplemented with 20 mM of Hepes, and 4 mg/ml FA-free BSA [68]. Afterwards, the COCs were transferred into a 35-mm Petri dish and washed twice before moving to the maturation medium. The COCs with 2 layers or more of compact cumulus cells were selected for maturation.

3.2.3. In Vitro Maturation

A group of COCs were cultured (20 COCs for each treatment for nuclear maturation experiments and 45 - 50 for embryo development experiments) in 500 µl of maturation medium according to the experimental design. Maturation medium was prepared as previously reported by Shirazi *et al.* [70] with minor modifications. Tissue culture medium 199 was used as a basic media supplemented with 5 µg/ml of FSH

[Follitropin; Bioniche Animal Health, Belleville, ON, Canada], 5 µg/ml of LH [Leutropin; Bioniche Animal Health], 1 µg/ml estradiol 17 β (E2), 0.2 mM Sodium Pyruvate, 2 mM L-glutamine, 50 µg/ml gentamicin and 6 mg/ml FA-free BSA. The COCs were incubated in 4-well dishes (NUNC, VWR international, Milan, Italy) for 24 hours at 38.5^{o}C in a humidified atmosphere of 5% CO_2 in air.

3.2.4. Oocyte Maturation Assessment: Cumulus Expansion and Nuclear Stage

Cumulus expansion was determined at 24 hours by subjected descriptions as not expanded, partially expanded or fully expanded under a light microscope (Olympus SZH, DF plan 1X; Olympus). Oocyte nuclear stage in meiosis was determined after aceto-orcein staining [68]. Briefly, the oocytes were denuded by gentle pipetting and placed on a clean glass slide and overlaid with a square cover slip that was held up by four droplets of Vaseline-paraffin mixture (40:1). Afterwards, they were fixed for at least 24 hours in glacial acetic acid (GAA) in methanol fixative solution (1:3). Thereafter, the oocytes were stained for 2 minutes with 1% orcein in 45% GAA mixture before washing with a mixture of distilled water, glycerol and GAA (3:1:1). Finally, the nuclear maturation was recorded under a phase contrast microscope (Olympus, B201; Olympus).

3.2.5. Assessment of PGs by RIA

Concentrations of PGF2α and PGE2 in the spent maturation media were measured by RIA technique according to Cheng *et al.* [68, 232]. Briefly, the standards (range 0.05–10 ng/ml for PGE2 and 0.025-5 ng/ml for PGF2) or samples were mixed with anti-PGE2 and PGF2α serum (from Dr. N.L. Poyser; University of Edinburgh, Edinburgh, UK), and tritiated tracer ([5, 6, 8, 11, 12, 14, 15 (n)-^{3}H]-PGE2 and PGF2α; Amersham International plc, Amersham [cat no, TRK431 and TRK464, respectively]) in duplicates. After 24 hours incubation at 4^{o}C, dextran-coated charcoal suspension including 0.4% dextran (T-70; Amersham Pharmacia Biotech) and 2% neutralized charcoal were added to all tubes except the total count. They were incubated at 4^{o}C for 10 minutes and centrifuged at 2000× g for 10 minutes. The supernatant was removed into 6 ml scintillation vials containing 4 ml scintillant (Ultima gold; Packard Bioscience

BV, Pangbourne, Berks, UK) and counted for 2 minutes. The minimum detection was 2pg per tube for PGE2 and 1 pg/tube for PGF2α. The intra-assay coefficients of variation were 3.5% and 4.1%, while the inter-assay coefficients were 6.3%and 9.6%, respectively.

3.2.6. In Vitro Fertilization

One hour before the end of the 24 hours oocyte maturation period, the swim up method was commenced for preparation of semen for fertilization. For separation of motile sperms, Tervit *et al.* [73] protocol was used with modifications that briefly included 107.70 mM NaCl, 7.16 mM KCl, 1.19 mM KH_2PO_4, 0.49 mM $MgCl_2$, 1.71 mM $CaCl_2$, 3.3 mM Na-Lactate, 4 mM $NaHCO_3$, 0.33 mM Na-Pyruvate, 0.103 mM L-Glutamine, 2.4 mM D-Glucose, 21 mM Hepes, 20 µl/ml Penicillin-Streptomycin and 3 mg/ml BSA fraction V. Swim up was performed according to the Shirazi *et al.* [70] method with some minor modifications in which straws were thawed in a 37^{o}C water bath for 30 sec and the sperm contents were divided into four doses of 85 µl which were added under 3 ml of sperm washing media in a 15 ml falcon tube. The tubes were placed at 45^{o} angle in racks, which are placed in a 38.5^{o}C humidified incubator with 5% CO_2 in air for 45 minutes. Then the supernatants were removed and transferred to a new 15 ml falcon tube and centrifuged at 200× g for 7 minutes at room temperature. After centrifugation, the sperm pellet was re-suspended in fertilization media containing 3.42 mM of $CaCl_2$, 9.9 mM of Na-lactate, 0.99 mM of Na-pyruvate, 1 mM of L-glutamine, 10 µl/ml of penicillin/streptomycin and 2 mg/ml of BSA fraction V. Sperm concentration was determined by using the Neubauer counting chamber. *In vitro* fertilization was carried out by coincubating the matured COCs with 2 to 2.5 × 10^6 sperm per milliliter for 24 hours at 38.5^{o}C in a humidified atmosphere of 5% CO_2 in air.

3.2.7. Assessment of nuclear stage of zygotes

Twenty hours after fertilization, the presumptive zygotes were stained with 125 ng/ml of 4,6-diamidino-2-phenylindole (Vysis Inc., Downer's Grove, IL, USA) to assess fertilization rate by observing the presence of male and female pronuclei. Briefly, the presumptive zygotes were gently denuded by pipetting and fixed in 4%

Paraformaldehyde for 30 minutes without washing they were mounted on 4,6-diamidino-2-phenylindole droplet and overlaid with cover slip. The pro-nucleus formation was recorded using a fluorescent microscope (Olympus B201, Olympus). The presence of male and female pronuclei was counted as a two-pronucleus (2PN) stage or normal fertilization. Polyspermy was oocytes with two or more sperm heads or more than two-pronuclei, and asynchronous fertilization oocytes with the female pronucleus and a condensed sperm head.

3.2.8. In vitro embryo culture

After 20 hours fertilization, presumptive zygotes were denuded from remaining cumulus cells by gentle pipetting and then washed in SOF media [73] containing 9.9 mM of Na-lactate, 0.99 mM of Na-pyruvate, 1 mM of L-glutamine, 2.24% of basal medium eagle (BME) essential amino acids, 0.5% of minimal essential medium (MEM) non essential amino acids, 0.34 mM of trisodium citrate, 4.6 mM of myo-inositol and 4 mg/ml of BSA fraction V, three times in 100 µl drops. Thereafter, presumptive zygotes were transferred to 500 µl SOF media and cultured until day 8 after fertilization (fertilization day=0) at 38.5^{o}C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. The culture medium was refreshed every 48 hours (as nutrients in the medium could be depleted and also there is a possibility of accumulation of toxic products that can affect negatively further embryo development). At day 8 (fertilization day=0) produced blastocysts were used to assess embryo quality.

3.2.9. Assessment of embryo quality

In vitro produced prepubertal sheep blastocysts were stained using Hoechst combined with TUNEL according to the Fouladi-Nashta et al. [233] protocol with some modifications to count total cells and the number of apoptotic cells. Briefly, blastocysts were fixed in 4% Para-formaldehyde containing 30 µg/ml of Hoechst for 10 minutes, washed three times in SOF-BSA media, and then permeabilized in 0.1% Triton 100X in SOF-BSA for 5 minutes. TUNEL staining using a fluorescein isothiocyanate (FITC)-conjugated in situ cell death detection kit (Roche, Penzberg, Germany) was performed according to the manufacturer's instructions without washing. The embryos were

transferred into 10 µl droplets of a 1:10 dilution of the enzyme solution (Terminal deoxynucleotidyl transferase from calf thymus) in dilution solution (Nucleotide mixture) and incubated in a humid chamber for 1 hour at 38.5^{o}C. After incubation, the blastocysts were washed three times and mounted on 3 µl drops of glycerol based mounting media (Vectashield; Vector laboratories, Burlingame, CA, USA) and examined under an Olympus B201 fluorescent microscope. The number of blue cells determined the total cells and the green cells as apoptotic cells.

3.2.10. Measurement of E2 and P4 by ELISA

The steroidogenic activity of cumulus cells was assessed in the maturation medium according to the procedure previously described by Maya-Soriano *et al.* [234]. Briefly, E2 and P4 concentrations were determined after 24 hours of IVM using commercial enzyme immunoassay Kits (Estradiol ELISA Kit 402210 and Ultra Progesterone ELISA kit 402410, respectively; Neogen Corporation, Lexington, USA) following the manufacturer's instructions. The assay was validated by determination of assay specificity (dilutional parallelism), accuracy from spike recovery (101.1% and 89.6% for E2 and P4 assays, respectively), precision from intra-assay variability (3.8% and 3.2% for E2 and P4 assays, respectively), and sensitivity (0.03 and 0.2 ng/ml for E2 and P4 assays, respectively).

3.2.11. Experimental design

Experiment 1 was done in three replicates with different concentrations of ALA (0 [control], 50, 100, 200 µM and 100 µM of DMSO (it was used for dissolving ALA) in IVM medium. After 24 hours of maturation, 303 COCs were used for evaluation of cumulus cell expansion and 286 COCs were denuded and used for nuclear division evaluation. The maturation medium was collected and frozen for measuring the PGE2 and PGF2α.

Experiment 2 was performed in four replicates to assess the effects of ALA concentrations (0 [control], 50, 100 and 200 µM) on *IVF*, oocyte cleavage and blastocyst development and quality in the IVM medium. A total of 574 prepubertal sheep oocytes were fertilized and cultured *in vitro*. The DMSO group was not used in this experiment

because according to experiment 1 we did not observe any effect of DMSO. The maturation medium was collected and frozen for measuring the E2 and P4.

3.2.12. Statistical analysis

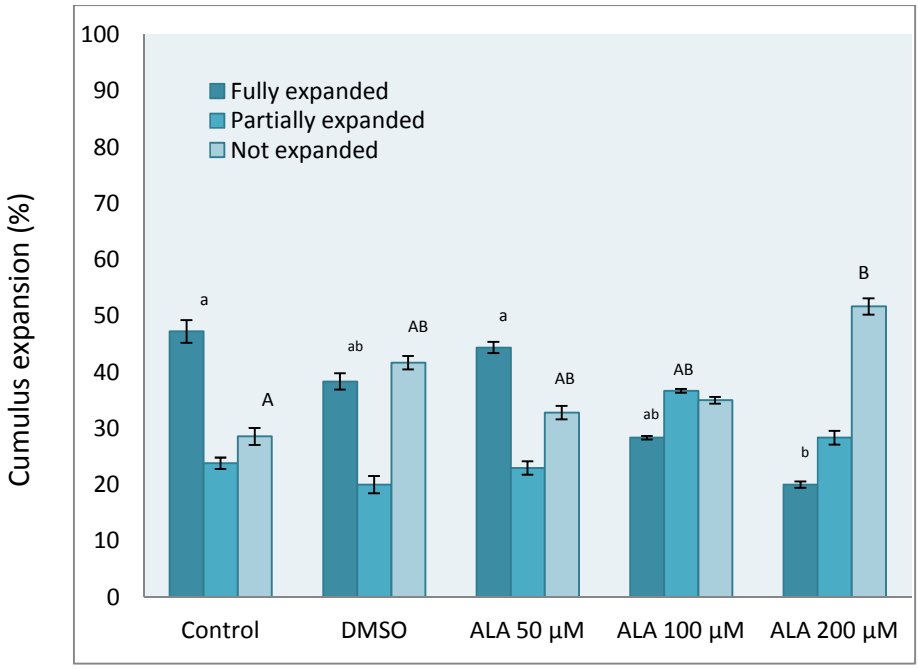
Statistical analysis was carried out using IBM SPSS Statistic for windows, version 20.0 (IBM Corp. Armonk, NY, USA). Linear mixed model was used for analyzing the number of total cells and apoptotic cells. The data for cumulus expansion, nuclear division (metaphase of the second meiotic division [MII]) is presented as the Mean±SEM, and two-pronuclei (2PN), polyspermy status, cleavage and blastocysts rate were analyzed by general linear model ANOVA and reported as a Percentage±SEM. The data for PGF2α and PGE2 were analyzed using ANOVA to compare the treatments of ALA with different concentrations (0 [control], 50, 100, 200 µM). The data for PGF2α, PGE2 and ratio of PGE2-to-PGF2α were transformed to $\log_{10}$ and E2, P4 and E2-to-P4 ratio to $\log_X$ before analyze.

3.3. Results

3.3.1. Experiment 1: effect of different ALA concentrations on oocyte maturation and PGs secretions

The highest concentration (200 µM) of ALA significantly decreased the rate of fully expanded cumulus cells 24 hours after IVM compared with the control and 50 µM ALA ($P \leq 0.05$, fig. 1).

Fig.1 effect of ALA concentrations (50, 100 and 200 µM) added at the IVM media on cumulus cell expansion of prepubertal sheep oocyte 24h after *in vitro* maturation



Data are prepared as a Mean±SEM. a, b letters indicate significant differences (P≤0.05) among experimental groups for Fully Expanded Cumulus Oocytes. A, B letters indicate significant differences (P≤0.05) among experimental groups for Not Expanded Cumulus Oocytes, Partially Expanded Cumulus Cell Oocytes were not significant different among experimental groups. DMSO, *Dimethyl Sulphoxide*; ALA, Alpha Linolenic Acid

The results from oocyte maturation did not show differences on oocyte degeneration, GV, GV Breakdown, MI (Metaphase I), AI (Anaphase I), and TI (Telophase I) rates at 24 hours of IVM among experimental groups (Table 1). On the contrary, significantly lower rate of MII oocytes were found in the 200 µM ALA group compared with the control group (82.9% and 93.1%, respectively; $P \leq 0.05$, Table 1).

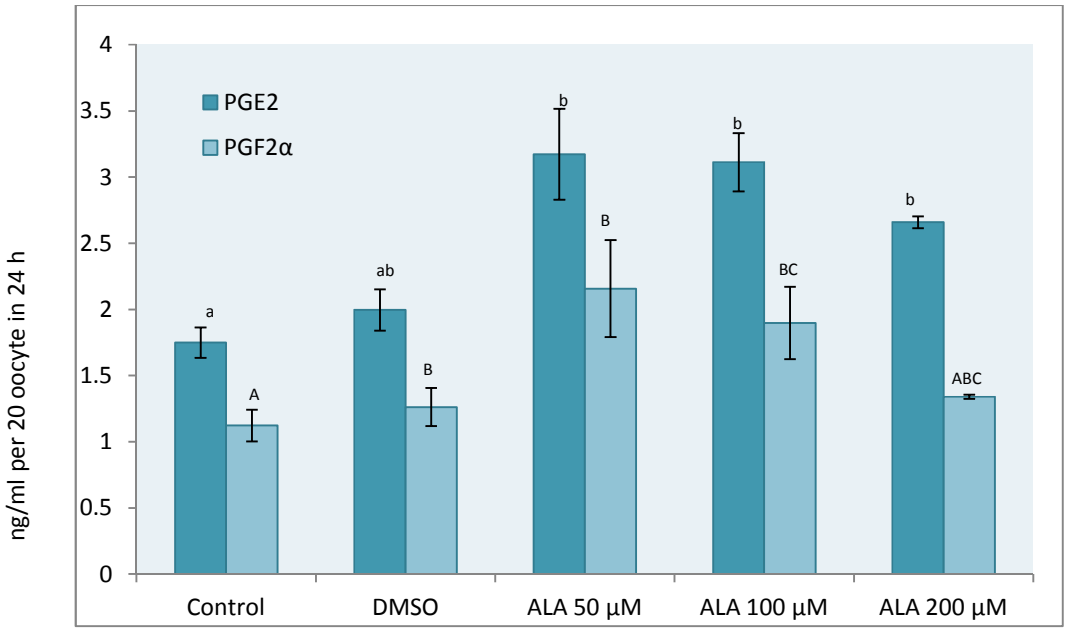
Table 1 effect of ALA concentrations (50, 100 and 200 µM) added to IVM media on nuclear stages of prepubertal sheep oocytes 24h after *in vitro* maturation

Treatments	Total oocytes	GV n(%)	GVBD n(%)	MI n(%)	AI n(%)	TI n(%)	MII n(%)	Degenerated oocytes n(%)
Control	58	3 (5.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	54 (93.1)[a]	1 (1.7)
DMSO	58	2 (3.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	54 (93.1)[a]	2 (3.4)
ALA 50µM	58	2 (3.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	56 (96.6)[a]	0 (0.0)
ALA 100µM	58	2 (3.4)	1 (1.7)	0 (0.0)	0 (0.0)	2 (3.4)	53 (91.4)[ab]	0 (0.0)
ALA 200µM	52	2 (3.8)	0 (0.0)	0 (0.0)	0 (0.0)	4 (7.7)	43 (82.7)[b]	3 (5.8)

GV, Germinal Vesicle; GVBD, Germinal vesicle breakdown; M-I, Meta-phase of the first meiotic division; A-I, Ana-phase of the first meiotic division; T-I, Telo-phase of the first meiotic division; M-II, Meta-phase of the second meiotic division; DMSO, *Dimethyl Sulphoxide*; ALA, Alpha Linolenic Acid. Data are prepared as a Percentage and Different superscript letters indicate significantly different among experimental groups (P≤0.05)

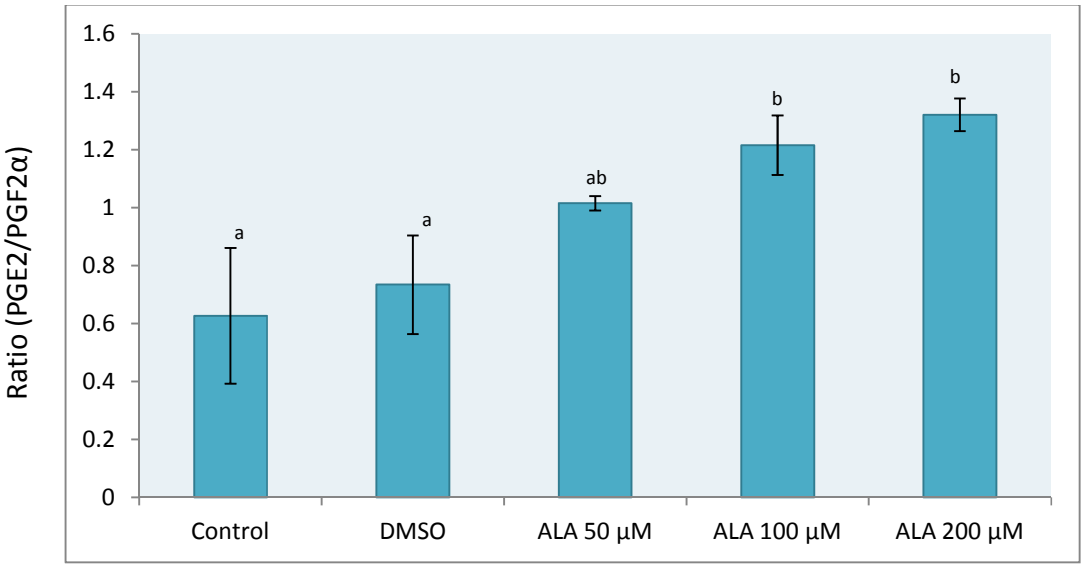
Figure 2 shows the concentration of PGE2 in the maturation media. After 24 hours of IVM, ALA treatment groups had significantly higher concentration of PGE2 than the control group (P≤0.05). No differences were observed between control and DMSO groups. Figure 2 shows PGF2α concentration after IVM. The 50 and 100 µM of ALA groups significantly increased PGF2α concentration compared with the control and DMSO groups (P≤0.05). No changes were observed in the PGF2α between 200 µM ALA and control groups. Significant differences were observed in the ratio of PGE2-to-PGF2α between ALA groups (100 and 200 µM) and the control group (P≤0.05, fig. 3).

Fig.2 Effect of ALA concentrations (50, 100 and 200 µM) added to IVM media on Prostaglandin E2 (PGE2) and prostaglandin F2α (PGF2α) synthesis.



Results are presented as a Mean±SEM with $\log_{10}$. a, b letters indicate significant differences for PGE2 concentrations among experimental groups (P≤0.05). A, B, C letters indicate significant differences for PGF2α concentration among experimental groups (P≤0.05). DMSO, *Dimethyl Sulphoxide*; ALA, Alpha Linolenic Acid

Fig.3 Effect of ALA concentrations added (50, 100 and 200 µM) to IVM media on the ratio of PGE2/PGF2α



Results are presented as a Mean±SEM with log_{10}. Different superscripts indicate significantly different among experimental groups (P≤0.05)

3.3.2. Experiment 2. effects of different ALA concentrations on embryo development and synthesis of E2 and P4

In experiment 2, samples of IVF oocytes were stained to test the nuclear stage of the presumptive zygotes at 20 hours after fertilization. No differences were found in the total number of fertilized oocytes among the control and ALA groups. The percentage of normal zygotes (2PN) was statistically higher at 200 µM of ALA group than the control group (60.0 vs. 47.8%, respectively; P≤0.05). However, this difference was not observed between 50 and 100 µM ALA groups. The percentage of polyspermic oocytes was significantly (P≤0.05) higher in ALA groups (16%, 12%, and 5%, respectively, Table 2) than the control group (26%). There was no difference in total unfertilized oocytes including degenerated and parthenogenetic oocytes among groups.

Table 2 Effect of ALA (50, 100 and 200 µM) added to IVM media on *in vitro* fertilization at 20h after insemination

	Total oocytes	Unfertilized oocytes			Fertilized oocytes			
		Unfertilized n(%)	Parthenogenetic n(%)	Degenerated n(%)	Fertilized oocytes n(%)	Twopronuclei n(%)	Polyspermic n(%)	Asynchronous n(%)
Control	46	4 (8.69)	2 (4.34)	2 (4.34)	38 (82.60)	22 (47.82)^{a}	6 (13.04)^{a}	10 (26.3)^{a}
ALA 50 µM	41	2 (4.87)	0 (0.0)	2 (4.87)	37 (90.24)	19 (46.34)^{a}	12 (29.26)^{b}	6 (16.2)^{b}
ALA 100 µM	45	4 (8.88)	1 (2.22)	1 (2.22)	39 (86.66)	23 (51.11)^{a}	11 (24.44)^{b}	5 (12.8)^{bc}
ALA 200 µM	45	5 (11.11)	1 (2.22)	1 (2.22)	38 (84.44)	27 (60.00)^{b}	9 (20.00)^{b}	2 (05.2)^{c}

2PN: oocytes with male and female pronucleus or normal fertilized zygote; PS: Polyspermic fertilized oocytes; AS: Asynchronous fertilized oocyte. Different letters mean significant differences among experimental groups (P≤0.05).

Table 3 shows the percentage of cleavage and embryo development at days 6, 7 and 8 after fertilization (fertilization day=0). No differences were found among the ALA

and control groups in cleavage and blastocyst development at day 6, 7 and 8 after fertilization.

Table 3 Effect of ALA (50, 100 and 200 µM) concentrations added to IVM medium on cleavage and blastocyst development at 6, 7 and 8 days after insemination

Treatment	Total Oocytes	Total Cleaved (%)	Two cell (%)	Four cell (%)	Eight cell (%)	Blastocyst/ Cleaved n(%) day 6	Blastocyst/ Cleaved n(%) day 7	Blastocyst/ Cleaved n(%) day 8
Control	139	108 (77.69)	5 (3.59)	28 (20.14)	75 (53.96)	3 (2.77)	9 (8.33)	17 (15.74)
ALA 50 µM	143	101 (70.62)	11 (7.69)	23 (16.08)	77 (53.84)	1 (0.99)	4 (3.96)	07 (6.93)
ALA 100 µM	149	122 (81.87)	9 (6.04)	24 (16.10)	89 (59.73)	2 (1.63)	7 (5.73)	14 (11.47)
ALA 200 µM	143	107 (74.82)	12 (8.39)	16 (11.18)	79 (55.24)	2 (1.86)	8 (7.47)	15 (14.01)

The quality of blastocysts 8 days after fertilization assessed by the number of total cells and the number of apoptotic cells is shown in Table 4. The total cell number in the control group was lower than the 50 and 200 µM ALA groups (46.5 vs. 67 and 59, respectively; P≤0.05). The number of apoptotic cells was significantly lower in 50 and 200 µM ALA groups compared with the control group (67.9, 58.8, and 46.5, respectively, P≤0.05, Table 4).

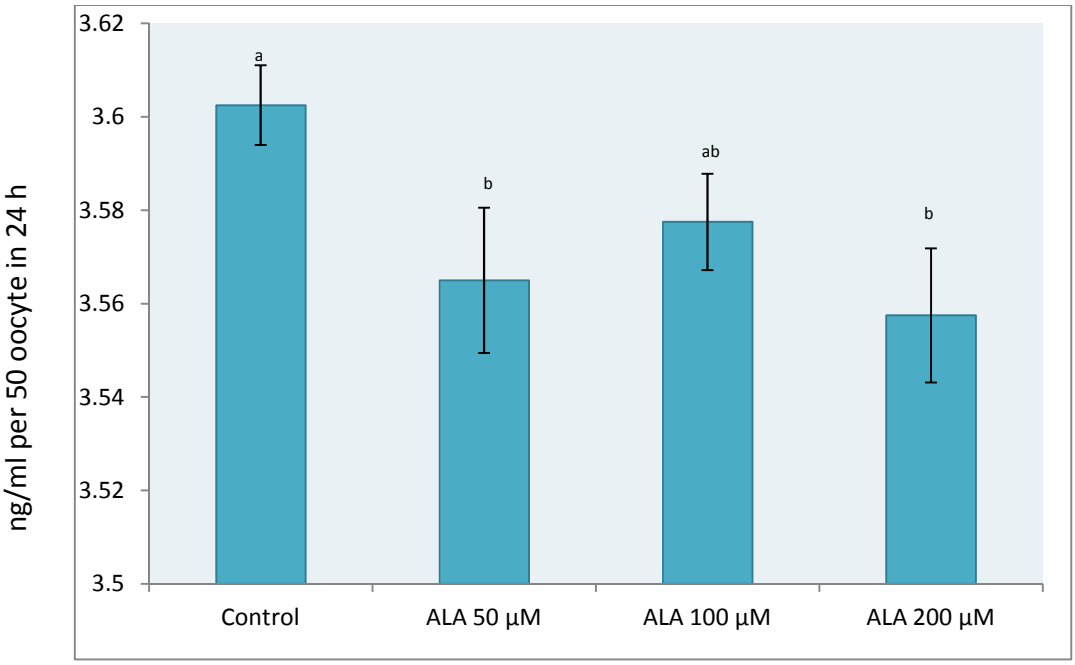
Table 4 Effect of ALA (50, 100 and 200 µM) concentrations added to IVM medium on blastocyst quality assessed by total cell number and apoptotic cells

Treatments	Total blastocyst	Total cell	Apoptotic cell
Control	9	46.50 ± 5.85^{A}	6.45 ± 0.89^{a}
ALA 50 µM	5	67.94 ± 6.71^{B}	2.48 ± 0.81^{b}
ALA 100 µM	6	45.20 ± 6.37^{A}	4.02 ± 1.15^{a}
ALA 200 µM	10	59.80 ± 5.51^{B}	3.67 ± 1.15^{b}

Results are expressed as a Mean±SEM for treatments. A, B letters mean significant differences in total cells among experimental groups. a, b letters mean significant differences in apoptotic cells among experimental groups (P≤0.05).

The concentration of E2 measured in the spent media was significantly lower in 50 and 200 µM ALA groups compared with the control group (P≤0.05, Fig. 4). However, 100 µM ALA group was not different compared with the control group.

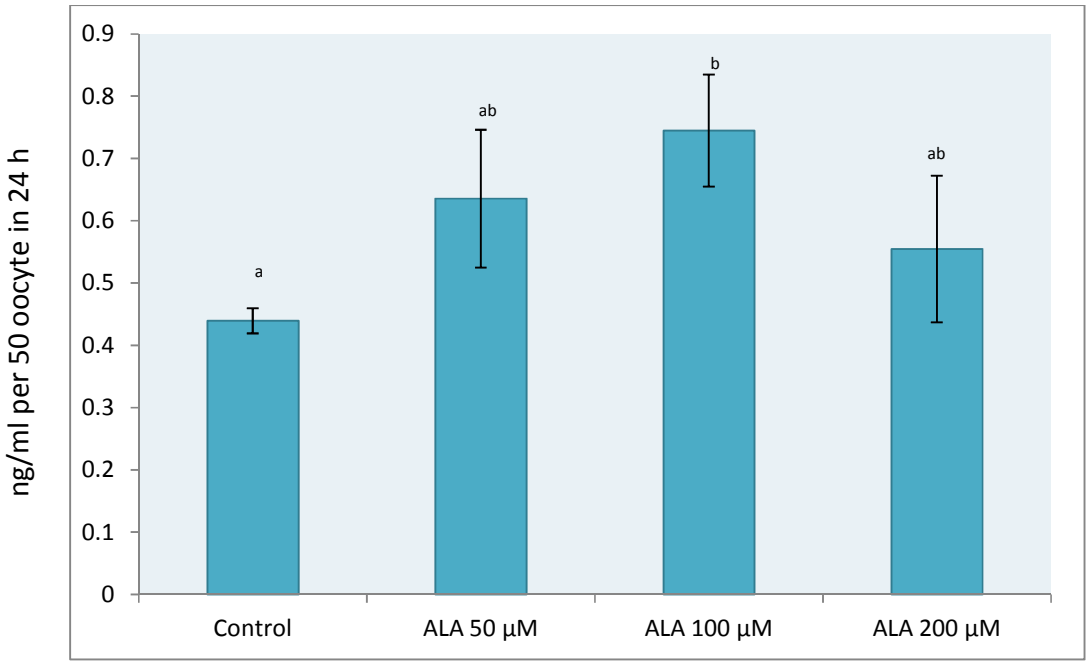
Fig.4 Effect of ALA (50, 100 and 200 µM) concentrations added to IVM medium on Estradiol (E2) concentration in the spend maturation media.



Results are presented as a Mean ± SEM with $\log_X$. Different superscripts indicate significantly different among experimental groups (P≤0.05)

The concentration of P4 significantly increased after treatment with 100 µM of ALA compared with the control group (P≤0.05, Fig. 5). This difference with the control group was not observed at 50 and 200 µM (Fig. 5).

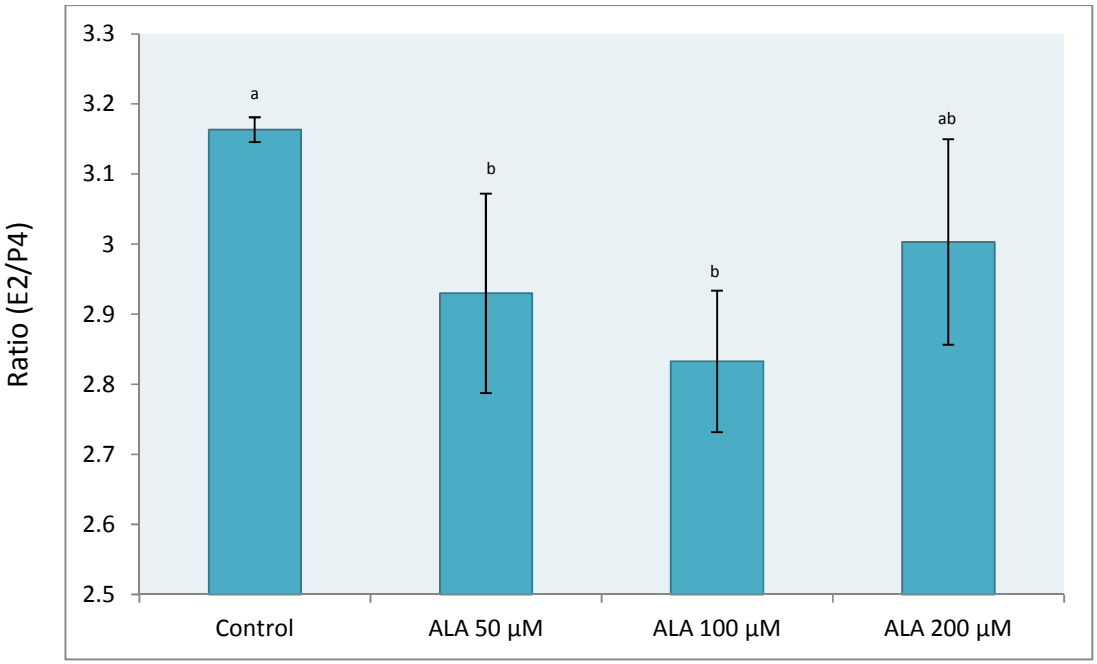
Fig.5 Effect of ALA (50, 100 and 200 µM) concentrations added to IVM medium on progesterone (P4) concentration in the spend maturation media.



Results are presented as a Mean±SEM with $\log_X$. Different superscripts indicate significantly different among experimental groups (P≤0.05).

Furthermore, the E2-to-P4 ratio was statistically lower at 50 and 100 µM ALA groups compared with the control group (P≤0.05, Fig. 6), but 200 µM group did not show a significant change.

Fig.6 Effect of ALA (50, 100 and 200 µM) concentrations added to IVM medium on the ratio of E2/P4 concentration in the spend maturation media.



Results are presented as a Mean ± SEM with $\log_X$. Different superscripts indicate significantly different among experimental groups (P≤0.05).

3.4. Discussion

Nuclear and cytoplasmic maturation are critical factors in determining developmental potential of oocytes after fertilization. Oocytes from prepubertal animals are often recovered from small follicles and are smaller in diameter [235]. These oocytes are deficient in the vital factors in the cytoplasm [62] and are not exposed to the hormonal microenvironment of ovulatory follicles. Provision of a suitable IVM media could overcome some of these disadvantages and improve their developmental potential. Romaguera *et al.* [16] observed a similar percentage of blastocyst development between oocytes coming from follicles larger than 3 mm of prepubertal females and oocytes from adult goats. Likewise, Bender *et al.* [231] via analyzing follicular fluid composition found significantly higher ALA concentration in cow compared with heifer (29 vs. 14 µg/ml, respectively). Thus, addition of ALA to IVM media of prepubertal sheep oocytes could improve their embryo development. In cattle, the addition of 50 µM of ALA to IVM has improved oocyte nuclear maturation and embryo

development [68]. This effect is mediated both directly through Mitogen-activated protein kinase pathway and indirectly through PGE2 synthesis [68] and changing mitochondrial distribution and activity [69]. In the present study, we did not find differences in blastocyst production among the ALA-treated and control groups. However, the blastocyst quality, assessed by the number of total cells number and apoptotic cells, indicated an improvement in embryo matured with ALA supplementation.

Our results revealed that ALA at high concentration (200µM) reduced the number of COCs with fully expanded cumulus cells. This was in agreement with the published results on maturation of bovine oocytes using this high ALA concentration [68]. There was also a reduction in the rate of oocytes reaching MII stage. Thus, it seems that 200 µM is an excessive concentration compared with physiological levels measured in sheep and bovine [229, 236-237]. In cattle ALA levels of 0.04 mg/ml (143.66 µM) in plasma, 0.02 mg/ml (71.8 µM) in uterine endometrial tissue and 0.02 mg/ml (71.8 µM) in follicular fluid were reported [236]. In sheep serum, n-3 High-density lipoprotein (HDL) concentration was 6.6 µM [229]. In our laboratory, we have observed ALA concentration of 40.8 and 22.7 µM in large (>3 mm) and small follicles, respectively in prepubertal goats.

The results from the present study revealed that the addition of ALA to IVM media of prepubertal sheep oocytes increased PGE2 synthesis at a highly significant level in all ALA groups. A similar effect was reported in 50 µM ALA group in a bovine study [68]. Although, the main source of PGE2 production is the granulosa cells, also sheep and cattle COCs are able to synthesis it *in vitro* [133, 238]. Similar findings were observed for prepubertal sheep in the studies presented here. In another study, researchers found that ALA at 100 µM significantly induced PGE2 synthesis in ovine amnion cells cultured *in vitro* [239]. Kirkup *et al.* [239] found that supplementation of 100 µM of PUFA (ALA, stearidonic acid, arachidonic acid, dihomo-γ-linoleic acid and γ-linoleic acid) affected the type and quantity of PGs synthesized, thus arachidonic acid produced more two-series PGs and dihomo-γ-linoleic acid produced one-series PGs. The PGF2α is also important in several aspects of reproduction such as ovulation, estrus, embryo survival and parturition [122]. In our study, ALA concentrations of 50 and 100 µM significantly increased PGF2α secretion but the 200 µM was not statistically

different compared with the control group. This reduction in PGF2α synthesis in this group could be associated with the low cumulus expansion and MII stage of oocytes matured with 200 µM of ALA. In conclusion, addition of ALA to the IVM medium of prepubertal sheep oocytes has increased the PGs synthesis, both PGE2 and PGF2α, but this increase has not affected oocyte nuclear maturation. The lack of positive effects of 50 and 100 µM ALA on nuclear maturation, as it was observed in cattle [68] could be because of the specific characteristics of oocytes coming from ovaries of prepubertal lambs. The highest ALA concentration, 200 µM, has negatively affected nuclear maturation and cumulus expansion as it was observed in cattle.

In experiment 2, we examined the effect of ALA addition to IVM media on IVF and embryo development of prepubertal sheep oocytes. A-Linolenic acid concentrations had no effect on the total fertilized oocytes however, 200 µM of ALA increased the number of normal zygotes (2PN) compared with the control group. The rate of polyspermic zygotes was significantly higher in all ALA concentrations compared with the control group. Interestingly, the percentage of asynchronous zygotes (one female pronucleus and one condensed sperm head) was significantly lower in ALA treatments compared with the control group. This could be considered as an improvement of male pronucleus formation under ALA treatments, whereas there was no difference in total fertilization rate between ALA treatments and the control group. Similarly, Childs *et al.* [240] have shown no effect of palmitic acid and *n*-3 PUFA used in diet on embryo yield and quality in heifer beef cows [240]. In contrast, Marei *et al.* [68] observed positive effects using ALA in IVM of bovine oocytes on embryo development. Likewise, Hughes *et al.* [125] using n-3 PUFA-enriched serum (5%, v/v) in the SOF culture medium observed an increase in blastocyst production, increased transcript expression for the antioxidant enzyme superoxide dismutase 1, but there was also an increase in morphologically poor embryos. Our results in prepubertal sheep oocytes revealed that there are no significant differences in cleavage and blastocyst rates among the control and ALA experimental groups (Table 3). This is in agreement with a report from Wonnacott *et al.* [229] who did not find any improvement in cleavage and blastocyst rates when adding omega 3 or 6 HDL to *IVM* media of sheep oocytes. Marei *et al.* [68] supplementing 50 µM of ALA to the IVM medium of bovine oocytes have shown an increase in cleavage, blastocyst and hatched blastocyst rates. Similar to this report, we

have observed better quality blastocysts produced from oocytes matured in the presence of 50 µM of ALA and assessed by the number of cells and apoptotic cells (Table 4). In conclusion, ALA has improved male pronuclear formation in zygotes and blastocyst quality, which could reflect changes in the cytoplasmic maturation of prepubertal sheep oocytes and needs further investigation.

After 24 hours of IVM, the concentration of E2 was lower and the concentration of P4 was higher in the maturation of ALA groups compared with the control group. Hughes *et al.* [125] found that using *n-3* PUFA increases the synthesis of P4 by ovine theca cells. Also, Bao *et al.* [241] reported that HDL enhanced P4 production. Likewise, Wonnacott *et al.* [229] observed a high P4 and low E2 synthesis from granulosa cells cultured *in vitro* for 96h coming from small and medium size follicles of sheep ovaries fed with *n-3* and *n-6* HDL. It has been described that bovine healthy follicles have higher E2 level compared to atretic follicles, and atretic follicles have substantially elevated P4 level or theca products such as testosterone or androstenedione for the same size of follicle (reviewed by [242]). In rhesus monkey Zhen *et al.* [157] have shown that the absence of E2 and P4 in IVM caused a failure of oocytes to develop to blastocyst stage. In our study, we continued the maturation period to assess embryo developmental competence subsequent to ALA supplementation and the results revealed that embryo development was not affected by the level of steroid hormones during IVM (Figs. 4 and 5). Silva *and Knight* [158] concluded that the addition of P4 to IVM of bovine oocytes reduced the proportion of embryos forming blastocysts. The increased P4 concentration under ALA supplementation of maturation media might be because of increased mRNA and protein expression of steroid acute regulator in the cumulus cells [37] or differentiation-luteinisation of cumulus cells after 24 hours of culture (reviewed by [243]). The increase of P4 in the maturation medium had a negative effect on oocyte developmental potential leading to the production of poor quality embryos [37]. As the oocytes used in these experiments were derived from follicles smaller than 3 mm, COCs collected from small follicles in prepubertal ewes have machinery to produce both steroids and respond to gonadotrophic hormones FSH and LH present in the maturation medium to produce more P4 compared with E2. The higher level of P4 in the maturation medium affected maturation of oocytes and may have contributed to the low blastocyst rate and quality. However, it has recently been shown that oocyte competence lead to blastocyst stage is not related to E2 and P4 concentrations in the follicular fluid [244].

It has been shown that lower E2 levels and higher P4-to-E2 ratios were associated with fast-cleaving embryos [154], which in other words higher 2PN in ALA treatments could be related to the lower E2-to-P4 ratio in this study. However, in our study, the high concentration of P4 in ALA groups did not have a negative effect on blastocyst yield or quality.

The percentage of blastocyst-oocyte found in our study in the control group was 12.23%. In another study, from our group, Catala *et al.* [17] using serum in each step of the *in vitro* embryo production protocol found the percentage of blastocysts obtained from prepubertal ewes was 13.6% and 13.1% in IVF and intra-cytoplasmic sperm injection system, respectively. Also, O'Brien *et al.* [76] observed a 15.4% blastocyst rate. The total cell number of blastocysts in the present study was 46.50±5.8 while Catala *et al.* [17] observed blastocysts with higher number of cells (56.8±4.8 in the IVF system and 62.7±7.8 in the intra-cytoplasmic sperm injection system). Therefore, the lower number of cells found in the present study in blastocysts at 8 days after insemination could be because of serum omission in our protocols that were entirely serum-free for all experiments. Rizos *et al.* [245] have shown that using serum in IVM and IVC media bovine embryo development affects the speed of embryo development and quality of the forming blastocysts. Also, Lonergan *et al.* [110] have found that using fetal calf serum in after insemination culture media for embryo development affects the incidence and severity of mixoploidy in the achieved blastocyst.

3.4.1. Conclusion

Evidence from the present study revealed that the addition of 200 µM of ALA to the IVM medium of prepubertal sheep oocytes had a negative effect on nuclear maturation and cumulus cell expansion. This negative effect was not observed at 50 and 100 µM ALA concentrations. Oocytes matured with ALA improved male pronucleus formation and blastocyst quality; however no effects were found on cleavage and blastocyst rates. The concentration of PGE2 and PGF2α in the IVM media was higher in ALA groups compared with the control group. E2 concentration was reduced and P4 concentration increased in ALA groups compared with the control group. In our entirely

free-serum medium, the results on blastocyst production were similar to other research groups but the lack of serum has affected the number of total cells of the blastocysts.

3.4.2. Acknowledgement

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CHAPTER 4:

EFFECT OF LINOLEIC ACID (LA) ON OOCYTE MATURATION AND EMBRYO DEVELOPMENT OF PREPUBERTAL SHEEP OOCYTES

4. Effect of Linoleic acid (LA) on oocyte maturation and embryo development of prepubertal sheep oocytes

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Effect of Linoleic acid (LA) on oocyte maturation

Abstract

Linoleic acid (LA) is the polyunsaturated fatty acid (PUFA) most abundant in the follicular fluid (FF). Moreover, LA affects oocyte maturation, embryo development and steroidogenesis. The aim of this study was to test the effect of different LA concentrations added to the IVM media (0 [control], 50, 100 and 200µM) in experiment 1 on cumulus cell expansion, nuclear maturation and prostaglandins (PGE2 and PGF2α) secretions; and in experiment 2 on fertilization, cleavage and emberyo development rate and steroids (estradiol and progesterone) secretions on prepubertal sheep oocytes. Oocytes matured using LA treatments for a period of 24 hours for experiment 1; and for the experiment 2 matured oocytes were inseminated with frozen-thawed sheep sperm in a serum-free, BSA supplemented media subsequently presumptive zygotes were cultured for 8 days in SOF serum-free media. The number of blastocyst at day 8 was recorded and its quality was assessed by the counting of total cell and apoptotic cell. No changes were observed in the number of oocytes reaching to MII stage (91.8%, 91.6%, 87.9%, 93.1% and 93.1%, respectively). Production of PGE2 and PGF2α were increased in all LA treatments compared with the control group ($P \leq 0.05$). The ratio of PGE2-to-PGF2α was not altered. LA at 50µM significantly was improved the rate of two-pronuclei (2PN) compared with the control group (57.89% vs. 45.45%, respectively, $P \leq 0.05$). There were no differences in the rate of cleaved embryos and blastocyst at day 8 of fertilization. However, embryo quality was improved using lowest concentration of LA (50µM) with increasing the number of total cell compared with the control group (63.88±4.54 vs. 53.35±3.64, $P \leq 0.05$, respectively). There was no difference in apoptotic cell number in all treatments. Also, production of E2 was decreased significantly while there were not differences in P4 production and the rate of E2-to-P4 ratio. In conclusion, LA supplementation to the IVM media on the prepubertal sheep oocyte negatively altered the fully expanded cumulus cell expansion significantly without inhibition of MII nuclear stage of prepubertal sheep matured oocyte. The results from the present study provided evidences in increasing the number of zygotes with normal 2PN; also showed beneficial effects of low level LA on embryo quality of blastocyst at 8 day of fertilization in serum-free, BSA suplemented media.

Keywords: Linoleic acid, prepubertal sheep, oocyte, embryo, IVM.

4.1. Introduction

In vitro embryo production (IVEP) allows producing of large number of embryos from ovaries collected at slaughterhouse. Embryos produced from oocytes of prepubertal females can play an important role in livestock breeding programs increasing the genetic gain by shortening the generational interval [246]. However, the efficiency of IVEP of oocytes from prepubertal females is still lower than those obtained from adult females, including ovine [157, 246-248]. Polyunsaturated fatty acids (PUFAs) are noticed to have positive influences on human health [249] and animal reproduction [250]. Several studies reported improvements on reproductive functions by PUFAs *in vivo* [228, 230] and *in vitro* [68-69]. It has been suggested that these improvements could be due to the effects on pituitary, ovaries and uterus axis [251]. Recently, Mckeegan et al [252] have reviewed that endogenous and exogenous fatty acids (FA) play diverse roles in mammalian oocytes and embryos. Endogenous FAs are important source of energy for oocytes and embryos [227]. FAs may also affect oocyte maturation directly by altering the lipid structure in the oocyte [231] (typically FAs are in the cell cytoplasm in the form of triacylglycerol) or by indirectly affecting prostaglandins (PGs) concentrations and other metabolites in the FF surrounding the oocytes [230]. Linoleic acid (LA) is the most abundant FA in the FF. The LA concentration in bovine FF has been determined around 0.20 mg/ml (713.15µM) [236]. Analyzing FF collected from different sizes of follicles in bovine for determining the LA concentration has shown a significant decrease in the concentration of LA in large follicles compared with small follicles [226]. It has been shown that the physiological retardation of LA in FF might be important for oocyte maturation, likely, LA may have a role in controlling oocyte maturation [69]. In cattle has been shown that supplementation of maturation medium with 100µM of LA was reduced the fully expanded cumulus cell expansion and oocyte maturation, and moreover, a significantly lower percentage of cleaved embryos (58±2.8% vs. 74±3.9%) and lower blastocyst (8±4.0% vs. 33±4.0%) were obtained compared with the control group [69]. Different authors using PUFAs in diet have found contrary results. Bilby et al [225] demonstrated that LA has no effect on oocyte morphology, number of cleaved embryos and embryo development when was

compared with the monounsaturated FAs used in diet. Also, Carro et al [253] have shown that 100µM did not change oocyte nuclear maturation rate (especially MII stage) in bovine oocytes. In contradictory, Thangavelu et al [254] could improve blastocyst quality when they were compared with the palmitic and stearic acids. Adamiak et al [237] have suggested that the contradictory results on oocyte development in different studies relates to the different factors such as metabolic status of the animal and season [255] or others that make difficulties in determining effects of specified FAs on oocyte development.

LA, an essential PUFA, with eighteen carbons (C:18) [256], which cannot be produced in the body and must be fed through diet [257], is metabolized to long chain PUFAs such as arachidonic acid (AA, 20:4n-3) and dihomo γ linolenic acid (DGLA, 20:3n-6) through desaturation and elongation processes [258]. These long chain PUFAs are the precursors for eicosanoids like PGs, prostacyclins, thromboxanes and leukotrienes [122].

PGs play key roles in reproductive activities such as ovulation, estrous and parturition. PGs are not stored in the tissues of the body, they are produced in the reaction to the physiological stimulations by prostaglandin H synthase (PGHS) [258]. Prostaglandin F2α (PGF2α) and Prostaglandin E2 (PGE2) are important mediators of the progression of ovulation that their concentration arise rapidly before ovulation in FF. It has been observed that fat supplements in diet may modulate PGs secretion and increase the pregnancy rate at the early stages of conception *in vivo* (reviewed by [251]). PGE2 is an important mediator for oocyte maturation [68, 232]; in mice, inhibition of PGE2 by PG-endoperoxide synthase 2 has been shown to reduce cumulus cell expansion (CE) and oocyte maturation [132]. Recently, 3 isoforms of PGE synthase, just after follicle aspiration, have been detected in bovine cumulus oocyte complexes (COCs), but these isoforms have not similarly detected for PGF synthase. Furthermore, bovine COCs using PGE2 in the absence of gonadotrophins have successfully matured to MII stage [133]. It has also been described that PGE2 is an autocrine-paracrine mediator which regulates CE and nuclear maturation [132].

Steroid hormones such estradiol (E2) and progesterone (P4) have a key role in regulating the estrous cycle *in vivo*. E2 is necessary for developing of follicles and oocyte maturation [259] and fertilization *in vivo* [173]. Enhancement of E2 concentration up-regulates ovarian receptors for follicle stimulating hormone (FSH) and luteinizing hormone (LH) [260]. Supplementation of oocyte maturation medium *in vitro* using P4 inhibitor decreased germinal vesicle breakdown (GVBD) in porcine in a reversible manner [167]. However, using P4 in IVM has a reduction effect on blastocyst production [158]. Decreasing P4 concentration removes its negative feedback on gonadotrophin releasing hormone (GnRH) from hypothalamus, thus pulses of GnRH increases the E2 secretion. It has been illustrated that successful pregnancies were more likely to occur in human IVF using oocytes from follicles containing high levels of E2 and a higher ratio of E2-to-P4 [261]. We have designed two experiments to assess different LA concentrations (0 [control], 50, 100 and 200µM) using in IVM of prepubertal sheep oocyte for checking oocyte maturation (experiment 1), fertilization and embryo development to blastocyst stage (experiment 2), and whether LA can improve the quality of produced embryos *in vitro* in a serum-free, BSA supplemented media. We have also analyzed the concentrations of PGs and steroids in the spent maturation media in both experiments to find out the relationship to oocyte maturation and embryo development, respectively.

4.2. Materials and Methods

4.2.1. Materials

All chemicals, *except* ones *mentioned,* were obtained from Sigma Chemical Co (St. Louis, MO, USA). Culture media was incubated at 38.5 ^{o}C under a humidified atmosphere of CO_2 5%, O_2 5% and N_2 90% for at least 2 hours before culture. Also, Washing and maturation, swim up and fertilization media before using were incubated for at least 2 hours at 38.5 ^{o}C under a humidified atmosphere of CO_2 5% in air.

4.2.2. Oocyte collection

Prepubertal sheep ovaries were obtained from the local abattoir and were transferred in a thermal flask containing 30 ^{o}C to 35 ^{o}C in PBS within 1 hour. COCs were retrieved via slicing follicles smaller than 3 mm in diameter in a 55 mm petri-dish containing washing media. Briefly, tissue culture medium 199 (TCM199) was used as a basic media supplemented with 20 mM Hepes buffered 1M and 4 mg/ml FA-free bovine serum albumin (FAF-BSA) [262]. Afterwards, the COCs containing 2 or more layers of compact cumulus cell were chosen to wash and use for IVM.

4.2.3. *In Vitro* Maturation

A group of COCs was matured (20 COCs for each treatment for nuclear maturation experiments and 45 to 50 for embryo development experiments) into 500µl of maturation medium that was prepared as established by Shirazi et al [71]. Briefly, TCM199 was used with 5µg/ml of FSH (Follitropin; Bioniche Animal Health, Belleville, ON, Canada) and 5µg/ml of LH (Leutropin; Bioniche Animal Health), 1µg/ml of estradiol 17 β, 0.2 mM of sodium pyruvate, 2 mM of L-glutamine, 50µg/ml of gentamicin and 6mg/ml of FA-free BSA. The COCs were incubated in four well dishes (NUNC, VWR international, Milan, Italy) for 24 hours at 38.5 ^{o}C in a humidified atmosphere of 5% CO_2 in air.

4.2.4. Oocyte maturation assessment: cumulus expansion and nuclear stage

Cumulus cell expansion was monitored in 24 hours of IVM described subjectively as a “not expanded”, “partially expanded” and “fully expanded” under a light microscope (Olympus SZH, DF plan 1X; Olympus) [68-69, 262]. The MII stage was examined using aceto-orcein staining as described by [262]. Briefly, oocytes were denuded via gentle pipetting, then placed on a glass slide and used a cover slip that was supported through four droplets of Vaseline-paraffin mixture (40:1). In a period of 24 hours suspending in glacial acetic acid (GAA)-methanol fixative (1:3 (v/v)), oocytes stained for 2 minutes using 1% orcein in a 45% GAA mixture, and then a mixture of distilled water in glycerol

and GAA (3:1:1) used for washing the stained oocytes. The MII stage recorded under a phase contrast microscope (Olympus B201; Olympus).

4.2.5. Assessment of PGs by RIA

The spent IVM media was analyzed by RIA for PGF2α and PGE2 concentrations as previously described by Cheng et al [232] Briefly, the standards (range 0.05–10 ng/ml for PGE2 and 0.025-5 ng/ml for PGF2α) or samples were mixed with anti-PGE2 and PGF2α serum (from Dr N L Poyser; University of Edinburgh, UK), and tritiated tracer ([5, 6, 8, 11, 12, 14, 15 (n)-^{3}H]-PGE2 and PGF2α; Amersham International plc, Amersham (cat no: TRK431 and TRK464, respectively)) in duplicates. In a period of 24 hours incubation at 4 ^{o}C, dextran coated charcoal suspension including 0.4% dextran (T-70; Amersham Pharmacia Biotech) and 2% neutralized charcoal added to all tubes except the total count. The tubes were incubated at 4 ^{o}C for 10 minutes before being centrifuged at 2000× g for 10 minutes. Then the supernatants were transferred into 6 ml scintillation pony vials containing 4 ml scintillant (Ultima gold; Packard Bioscience BV, Pangbourne, Berks, UK) and counted in a beta counter for 2 minutes. The detection limit was 2pg for PGE2 and 1pg for PGF2α. The intra-assay coefficients of variation were 3.5% and 4.1%, while the inter-assay coefficients were 6.3% and 9.6% respectively.

4.2.6. *In Vitro* Fertilization

One hour before the end of 24 hours oocyte maturation, sperm swim up was carried out. The sperm washing media was prepared using Tervit et al [73] protocol contained 107.70 mM of NaCl, 7.16 mM of KCl, 1.19 mM of KH_2PO_4, 0.49 of mM $MgCl_2$, 1.71 of mM $CaCl_2$, 3.3 mM of Na-Lactate, 4 mM of $NaHCO_3$, 0.33 mM of Na-Pyruvate, 0.103 mM of L-Glutamine, 2.4 mM of D-Glucose, 21 mM of Hepes, 20µl/ml of Penicillin-Streptomycin and 3 mg/ml of BSA fraction V. Swim up procedure was carried out according to the method established by Shirazi et al [71]. Briefly, sperm straws were thawed in a 37 ^{o}C water for 30 second then poured in 1 ml eppendorf tube and 85µl of the thawed semen kept under 3 ml of sperm washing media containing 3 mg/ml BSA fraction V in 15 ml conical tubes at 38.5 ^{o}C in a humidified atmosphere of 5% CO_2 in air for up to 45 minutes. The supernatants were pulled by 1 ml pipette to an another 15 ml

tube and centrifuged in 200× g for 7 minutes at room temperature (20 ^{o}C to 25 ^{o}C). The pellet was re-suspended in fertilization medium [73] containing 3.42 mM of $CaCl_2$, 9.9 mM of Na-Lactate, 0.99 mM of Na-Pyruvate, 1 mM of L-Glutamine, 10µl/ml of Penicillin-Streptomycin and 2 mg/ml of BSA fraction V. The COCs were fertilized with 2 × 10^6 sperm per ml for 20 hours at 38.5 ^{o}C in a humidified atmosphere of 5% CO_2 in air.

4.2.7. Assessment of zygotes nuclear stage

Presumptive zygotes were denuded from cumulus cells and were fixed in 4% Para-formaldehyde for 30 minutes and then were mounted in 3µl of 125 ng/mL of 4,6-diamidino-2-phenylindole (Vysis Inc., Downer's Grove, IL, USA) droplets for nuclear staining. The droplets were overlaid with cover-slip and presence of pronuclei were confirmed under a fluorescent microscope (Olympus, B201, Olympus) [263]. The zygotes with male and female pronuclei were counted as two-pronuclei (2PN), whereas oocytes with two or more sperm tails or more than two-pronuclei were counted as a polyspermy (PS). Oocytes with only one-pronuclei and a non decondensed sperm head were considered as asynchronous (AS).

4.2.8. *In Vitro* Culture

Within 20 hours of fertilization, zygotes were denuded from the remained cumulus cells in fertilization medium via gentle pipetting and washed in synthetic oviductal fluid (SOF) [73] medium and cultured for 8 days in 500µl SOF medium containing 4 mg/ml of BSA fraction V at 38.5 ^{o}C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The culture medium was refreshed every 48 hours. At day 8 produced blastocysts used for differential staining combined with Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to assess blastocyst quality [233].

4.2.9. Assessment of embryo quality

Blastocysts were fixed in 4% PFA containing 30µg/ml Hoechst for 10 minutes that was prepared in PBS. Afterwards, blastocysts washed 3 times then permeabilized in 0.1% Triton 100X in SOF-BSA media for 5 minutes. TUNEL labeling using an fluorescein isothiocyanate (FITC)-conjugated *in situ* cell death detection kit (Roche, Penzberg, Germany) was performed according to the manufacturer's instructions without washing for assessing the apoptotic cells. TUNEL labeling was carried out with incubating embryos in 10µl of 1:10 dilution of the enzyme solution Terminal deoxynucleotidyl transferase from calf thymus) in dilution solution (Nucleotide mixture) in a humid chamber for 1 hour at 38.5 ^{o}C. Finally, blastocysts were washed three times and mounted in 3µl droplets of glycerol based mounting media (Vectashield, Vector laboratories, Burlingame, CA, USA) for examination under a fluorescent microscope (Olympus, B201, Olympus). All works were carried out at room temperature and on a hot stage at 38.5 ^{o}C. Blastocyst quality was assessed counting bluish cell as a total cell and green cell as apoptotic cell.

4.2.10. Measurement of E2 and P4 by ELISA

The steroidogenic activity of cumulus cells was evaluated in the spent IVM as previously described by Maya-Soriano et al [264] Briefly, E2 and P4 levels were measured in the IVM media that were used for the period of 24 hours using commercial enzyme immunoassay Kits (Estradiol ELISA Kit 402210 and Ultra Progesterone ELISA kit 402410, respectively; Neogen Corporation, Lexington, USA) according to the manufacturer's instructions. The assay was validated by determination of assay specificity (dilutional parallelism), accuracy from spike recovery (101.1% and 89.6% for E2 and P4 assays, respectively), precision from intra-assay variability (3.8% and 3.2% for estradiol and progesterone assays, respectively), and sensitivity (0.03 ng/ml and 0.2 ng/ml for estradiol and progesterone assays, respectively).

4.2.11. **Experimental design**

Experiment 1 was performed in three replicates with increasing LA concentrations (0 [control], 50, 100, 200µM). We have also included another control using dimethyl sulphoxide (DMSO; 100µM, maximum concentration used for dissolving LA). Totally 295 COCs were matured for a period of 24 hours to evaluate cumulus cell expansion and nuclear maturation of the oocyte in metaphase II stage. IVM media of treatments were collected at the end of maturation and were kept in -20 ^{o}C for RIA assessments.

Experiment 2 was carried out in three replicates to show the influences of increasing concentrations of LA (0 [control], 50, 100, 200µM) 24 hours of maturation on cleavage rate and embryo development to blastocyst stage at day 8 of *in vitro* fertilization.

In experiment 2 a total of 458 prepubertal sheep oocytes were fertilized and cultured *in vitro*. In these experiments we removed the DMSO from our treatments because according to the experiment 1 we did not observe any differences on cumulus cell expansion and oocyte maturation. Furthermore, we have not observed any differences in our previous experiments with alpha-linolenic acid (ALA) on prepubertal sheep COCs [262] and also in previous works from our lab DMSO was not affected the embryo development in bovine [68]. The maturation medium was collected and frozen for measuring the E2 and P4. Embryo quality was assessed by counting total cell and apoptotic cell in blastocyst.

4.2.12. Statistical Analysis

Statistical analysis was carried out using IBM SPSS statistic for windows, version 20.0 (IBM Corp. Armonk, NY, USA). The Linear mixed model was run for total cell and apoptotic cell number. General linear model ANOVA was used to analyze changes for cumulus cell expansion, nuclear maturation stages, two-pronuclei, asynchrony, polyspermy, cleaved embryo and blastocyst rate. The data of PGF2α and PGE2 were

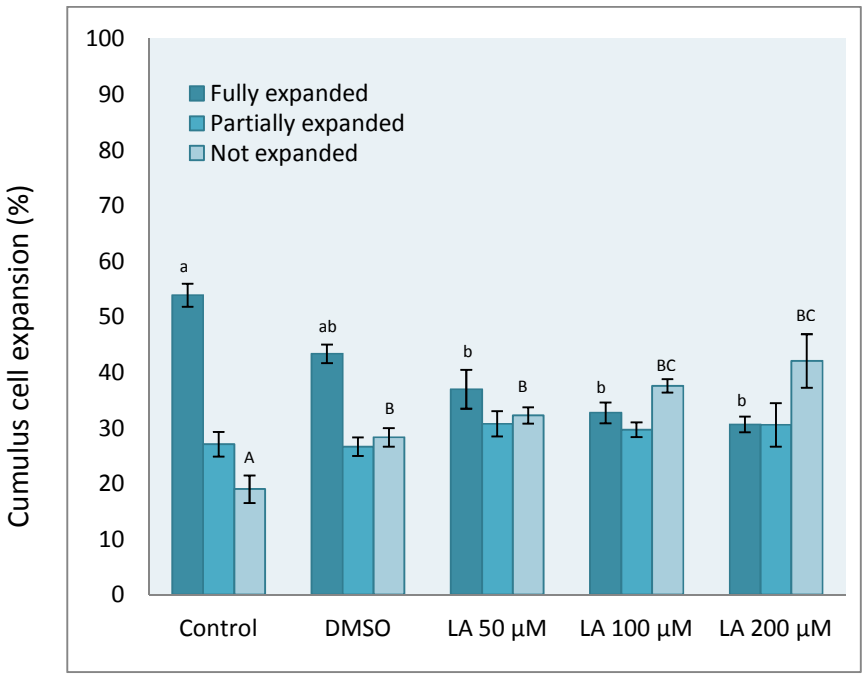
analyzed using two-way ANOVA. The data for PGF2α, PGE2 and ratio of PGE2-to-PGF2α was transformed to log $_{10}$ and E2, P4 and E2-to-P4 ratio to log $_{X}$ prior to analyze.

4.3. Results

4.3.1. Experiment 1: effect of LA concentrations on oocyte maturation and PGs secretions

In the experiment 1 a total of 295 oocytes were assessed within 24 hours of IVM to test CE and percentage of oocytes reaching MII stage. Supplementation maturation media containing prepubertal sheep oocyte with LA significantly decreased the fully expanded COCs compared with the control group (P ≤ 0.05, Fig. 1), however, there was no difference compared with the DMSO group.

Fig. 1. Effect of LA concentrations (0 [control], 50, 100 and 200µM) added to the IVM medium on cumulus cell expansion of prepubertal sheep oocytes in a period of 24 hours of IVM.



Data are presented as percentage. Letters a, b indicate significant differences among experimental groups for fully expanded cumulus cell oocytes (P ≤ 0.05). Letters A, B indicate

significant differences among experimental groups for not expanded cumulus cell oocytes ($P \leq 0.05$). Partially expanded cumulus cell oocytes were not significantly different among experimental groups. DMSO, *dimethyl sulphoxide*; LA, Linoleic acid.

No differences were observed for MII stage of nuclear maturation of the oocytes treated with LA concentrations compared with the control and DMSO groups (Table 1).

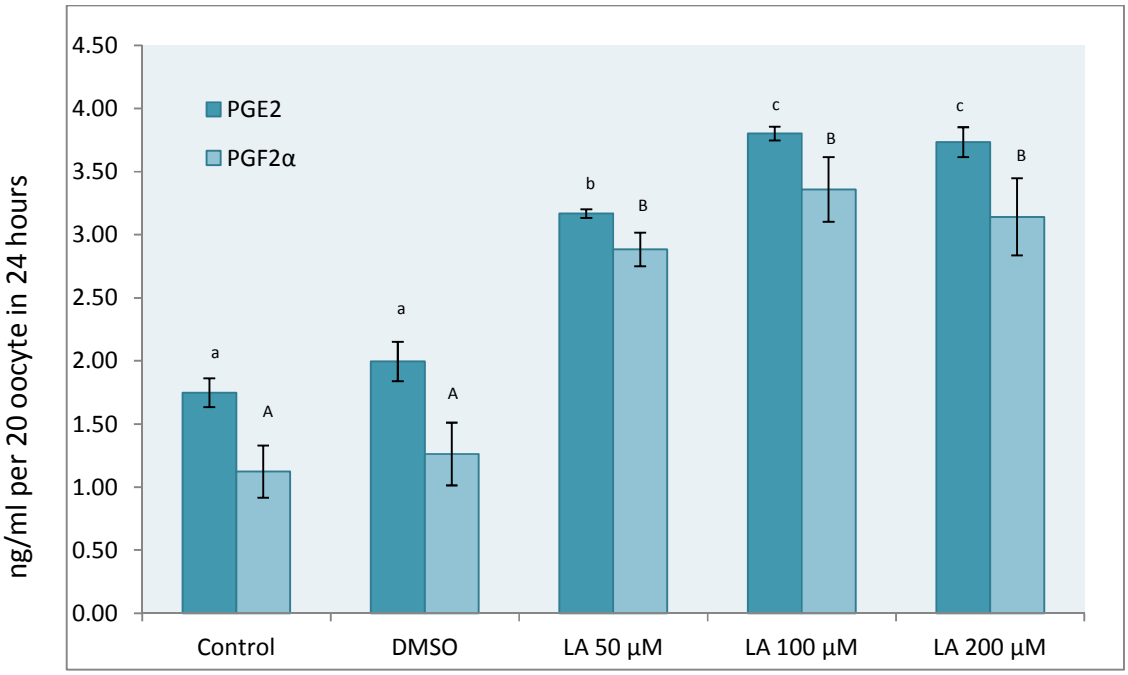
Table 1. Effect of LA concentrations (0 [control], 50, 100 and 200µM) added to the IVM medium on nuclear stages of prepubertal sheep oocytes in a period of 24 hours of IVM.

Treatments	Total oocytes	GV, n (%)	GVBD, n (%)	MI, n (%)	AI, n (%)	TI, n (%)	MII, n (%)	Degenerated oocytes, n (%)
Control	58	3 (5.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	54 (93.1)	1 (1.7)
DMSO	58	2 (3.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	54 (93.1)	2 (3.4)
LA 50µM	61	2 (3.27)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.66)	56 (91.80)	2 (3.27)
LA 100µM	60	1 (1.66)	0 (0.0)	0 (0.0)	0 (0.0)	3 (5.00)	55 (91.66)	1 (1.66)
LA 200µM	58	2 (3.44)	0 (0.0)	0 (0.0)	0 (0.0)	3 (5.17)	51 (87.93)	2 (3.44)

Data are presented as percentage. Abbreviations: A-I, anaphase of the first meiotic division; DMSO, *dimethyl sulphoxide*; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase of the first meiotic division; MII, metaphase of the second meiotic division; TI, telophase of the first meiotic division; LA, Linoleic acid.

LA in all concentrations significantly increased the production of PGE2 in a period of 24 hours culture in a serum-free, BSA supplemented maturation media (1.9 ng/ml per 20 matured oocytes, Fig. 2).

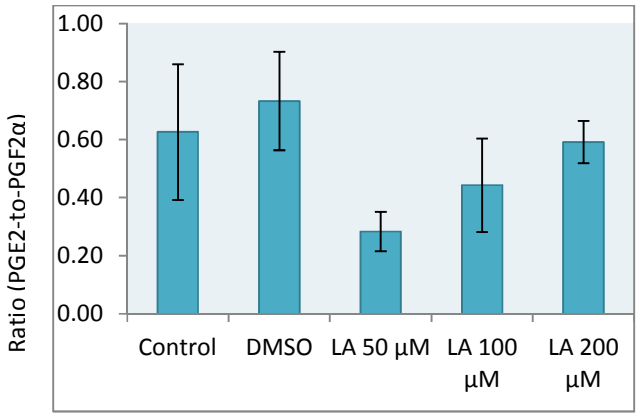
Fig. 2. Effect of LA concentrations (0 [control], 50, 100 and 200µM) added to the IVM medium on PGE2 and PGF2α synthesis.



Results are presented as the Mean ± SEM with log $_{10}$. Letters a, b, c indicate significant differences for PGE2 concentrations among experimental groups ($P \leq 0.05$). Letters A, B indicate significant differences for PGF2α concentrations among experimental groups ($P \leq 0.05$). LA, Linoleic Acid; DMSO, *dimethyl sulphoxide*; SEM, standard error of the mean.

Similarly, PGF2α production increased significantly by LA supplementation (1.1 ng/ml per 20 matured oocytes, Fig. 2). However, there was no significant difference in the ratio of PGE2-to-PGF2α between LA treatments compared with the control and DMSO groups (Fig. 3).

Fig. 3. Effect of LA concentrations (0 [control], 50, 100 and 200µM) added to the IVM medium on the ratio of PGE2-to-PGF2α.



Results are presented as the Mean ± SEM with log $_{10}$. LA, Linoleic acid; SEM, standard error of the mean.

4.3.2. Experiment 2. effects of LA concentrations on embryo development and synthesis of E2 and P4

Table 2 shows the results of 145 presumptive zygotes following a period of 20 hours exposing matured oocytes in a media containing motile sheep sperms. In experiment 2 the lowest concentration of LA (50µM) produced more 2PN compared with the control group (57.8% vs. 45.4%, respectively, P ≤ 0.05); but there was no difference in the percentage of polyspermy. The rate of asynchronous was lower in second concentration of LA (100µM) compared with the control group. There was no difference in the total number of fertilized zygotes between controls and the LA treatments (Table 2).

Table 2. Effect of LA concentrations (0 [control], 50, 100 and 200µM) added to the IVM medium on *in vitro* fertilization in a period of 20 hours of insemination.

Experiments	Total oocytes	Unfertilized oocytes			Fertilized oocytes			
		Unfertilized, n (%)	Parthenogenetic, n (%)	Degenerated, n (%)	Fertilized oocytes, n (%)	2PN, n (%)	PS, n (%)	AS, n (%)
Control	33	3 (9.09)	3 (9.09)	2 (6.06)	25 (75.75)	15 (45.45)^{A}	7 (21.21)	3 (09.09)^{ab}
LA 50µM	38	2 (5.26)	2 (5.26)	3 (7.89)	31 (81.57)	22 (57.89)^{B}	4 (10.52)	5 (13.15)^{ab}
LA 100µM	40	4 (10.00)	2 (5.00)	1 (2.50)	33 (82.50)	19 (47.50)^{A}	12 (30.00)	2 (05.00)^{b}
LA 200µM	34	2 (5.88)	1 (2.94)	2 (5.88)	29 (85.29)	17 (50.50)^{A}	6 (17.64)	6 (17.64)^{a}

Data are presented as percentage. Letters A, B indicate significant differences among experimental groups at the same column for two-pronuclei embryos (P ≤ 0.05). Letters a, b indicate significant differences among experimental groups at the same column for Asynchronous embryos (P ≤ 0.05). Abbreviations: AS, asynchronous zygote, an oocyte with one female pronucleous and a condensed sperm head; Fertilized oocyte, an oocyte with atleast one sperm penetrated; PS, polyspermy zygote, an oocyte with several sperm head or atleast three pronucleous; 2PN, normal zygote, oocyte with two pronuclei, male and female pronuclei and one sperm tail or; Degenerated oocyte, an oocyte morphologically abnomal without signs of sperm; Parthenogenetic zygote, a zygote that is created solely from a female oocyte without any genetic contribution from a male; unfertilized oocyte, oocytes without sperm penetration.

Likewise, no difference was observed between LA treatments and the control group on total cleaved embryos and blastocyst production at day 8 of fertilization (Table 3).

Table 3. Effect of LA concentrations (0 [control], 50, 100 and 200µM) added to the IVM medium on cleavage and blastocyst development at 8 days of insemination.

Experiments	**Total oocytes**	**Total cleaved (%)**	**Two cell (%)**	**Four cell (%)**	**Eight cell (%)**	**Blastocyst/Total Cleaved n (%) Day 8**
Control	113	96 (84.95)	9 (7.96)*	21 (18.58)^{A}	66 (58.40)^{a}	15 (15.62)
LA 50µM	111	93 (83.78)	10 (9.00)*	24 (21.62)^{A}	59 (53.15)^{a}	13 (13.97)
LA 100µM	111	91 (81.98)	6 (5.40)**	27 (24.32)^{A}	56 (50.45)^{b}	12 (13.18)
LA 200µM	123	108 (87.80)	12 (9.75)*	37 (30.08)^{B}	59 (47.96)^{b}	12 (11.11)

Asterisks *, ** indicate significant differences among experimental groups at the same column for tow cell embryos (P ≤ 0.05). Letters A, B indicate significant differences among experimental groups at the same column for four cell embryos (P ≤ 0.05). Letters a, b indicate significant differences among experimental groups at the same column for eight cell embryos (P ≤ 0.05).

LA at the lowest concentration significantly improved embryo quality compared with the control group by increasing total cell number at day 8 blastocyst (63.8% vs. 53.3%, respectively, P ≤ 0.05, Table 4) compared with the control group. However, the number of apoptotic cell in LA group did not differ compared with the control group.

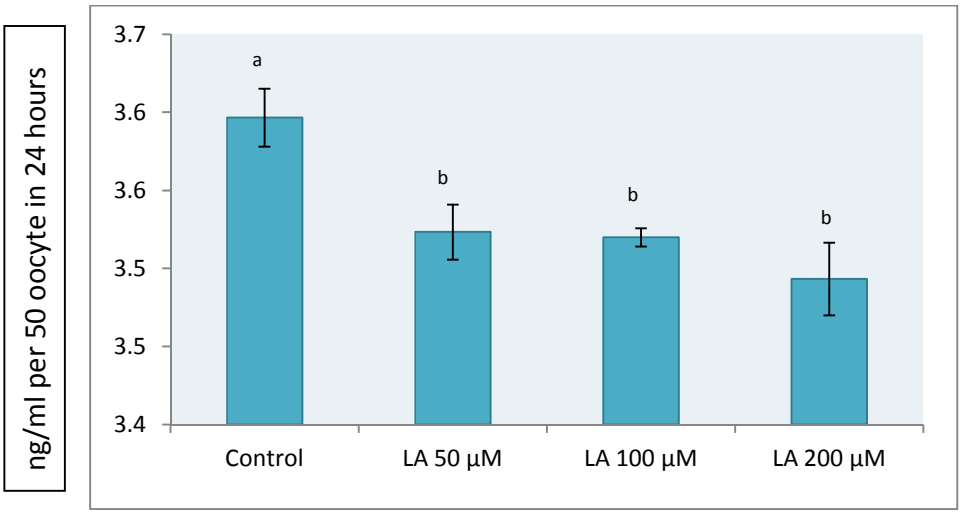
Table 4. Effect of LA concentrations (0 [control], 50, 100 and 200µM) added to the IVM medium on blastocyst quality assessed by the number of total cells and apoptotic cells.

Treatments	Total Blastocyst	Total cell	Apoptotic cell
Control	14	53.35±3.64 [a]	4.57±1.51
LA 50µM	9	63.88±4.54 [b]	3.99±1.60
LA 100µM	9	54.22±4.54 [a]	3.94±1.56
LA 200µM	12	57.38±3.78 [a]	5.13±1.60

Results are expressed as the Mean ± SEM for treatments. Different superscripts letters indicate significant differences among experimental groups for total cells (P ≤ 0.05). Abreviations: LA, Linoleic acid; SEM, standard error of the mean.

Supplementation of oocyte maturation media with concentrations of LA in experiment 2 significantly reduced E2 production in IVM spent media of LA treatments compared with the control group (Fig. 4).

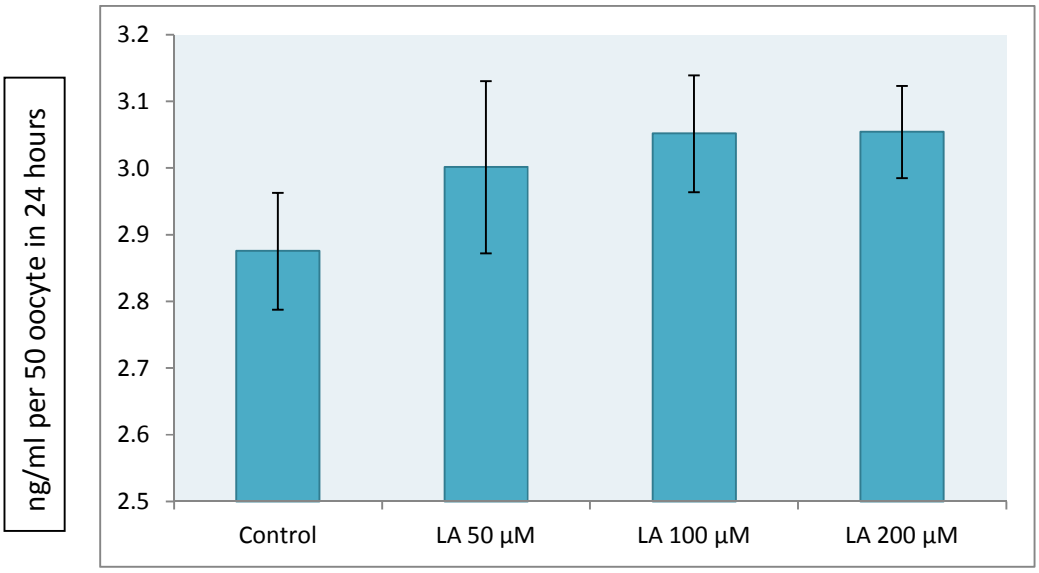
Fig. 4. Effect of LA concentrations (0 [control], 50, 100 and 200µM) added to the IVM medium on Estradiol concentration in the spend maturation media.



Results are presented as the Mean ± SEM with log $_X$. Different superscripts letters indicate significant differences among experimental groups (P ≤ 0.05). LA, Linoleic acid; SEM, standard error of the mean.

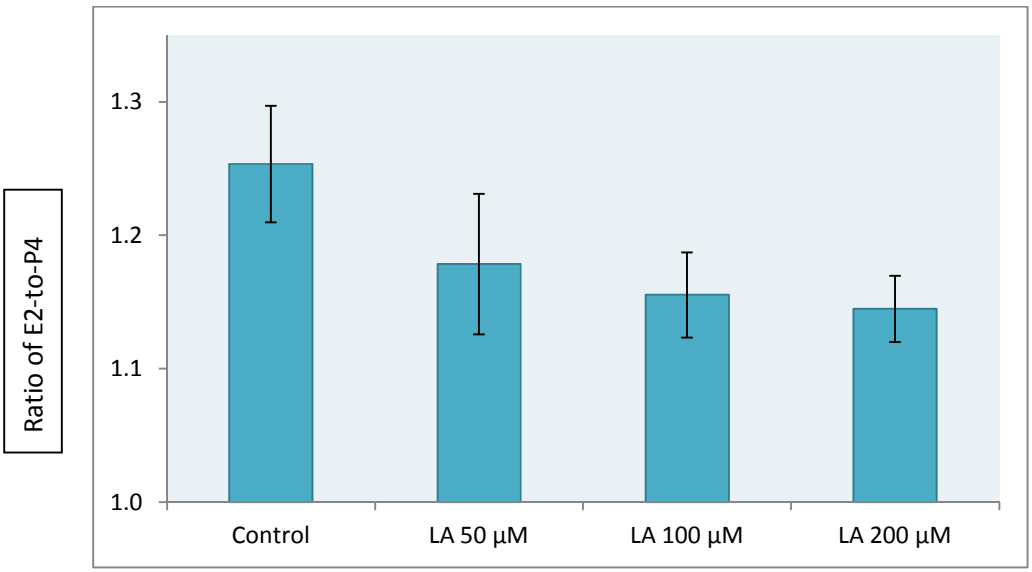
Concentration of P4 was not altered (Fig. 5) and also the E2-to-P4 ratio was not statistically changed in LA group compared with the control group (Fig. 6).

Fig. 5. Effect of LA concentrations (0 [control], 50, 100 and 200µM) added to the IVM medium on progesterone concentration in the spend maturation media.



Results are presented as a Mean ± SEM with log $_X$. Abbreviations: LA, Linoleic acid; SEM, standard error of the mean.

Fig. 6. Effect of LA concentrations (0 [control], 50, 100 and 200µM) added to the IVM medium on the ratio of E2-to-P4 concentration in the spend maturation media.



Results are presented as a Mean ± SEM with log $_x$. Abbreviations: LA, linoleic acid; SEM, standard error of the mean.

4.4. Discussion

Oocyte nuclear and cytoplasmic maturation are critical factors determining its embryo developmental potential after fertilization. Oocytes from prepubertal animals are often recovered from small follicles and are smaller in diameter [235], deficient in the vital factors in the cytoplasm which regulate oocyte nuclear maturation, are not exposed to the hormonal microenvironment of ovulatory follicles. Therefore, it is necessary to find suitable compound which can overcome some of these disadvantages and improve their developmental potential *in vitro* culture environments.

It is known that oocytes with small diameter have lower developmental competence due to an insufficient cytoplasmic maturation, which explains the lack of success in getting blastocyst because of deficiency in accumulation of RNA and proteins required to maintain embryonic development beside embryonic genome activation [265]. Thus, it seems that most of the oocytes from prepubertal female ovaries have not yet

completed their growth. Therefore, we have tried an energy source to find its effects on oocyte competence, embryo development and quality.

The present study investigated the effects of LA on cumulus cell expansion and nuclear maturation of prepubertal sheep oocyte cultured *in vitro* and their subsequent developmental competence to the blastocyst stage. The results showed that LA decreased the CE in all LA treatments (50, 100 and 200µM) compared with the control group ($P \leq 0.05$). Accordingly, it has been reported that the rate of CE has a relationship in a dose-dependent decrease with 50, 100 and 200µM of LA in bovine oocytes [69]. In the present study, we did not find significant difference in prepubertal sheep oocyte reaching to the Metaphase II stage in LA treatments compared with the control group. LA in bovine have reduced the number of oocyte reaching to MII stage using 100µM [69]. Carro et al [253] did not find negative effects on MII oocytes using low concentrations (9 and 16µM) of LA but at 100µM was negatively affected. In another study, addition of antioxidants to IVM media supplemented with LA did not affect MII and cumulus cell expansion [266]. In the present study, prepubertal sheep oocytes were not affected development of oocytes to MII stage by LA treatments compared with the control group (91±1.7, 91±4.8, 87±2.2 vs. 93±3.3, respectively) that could be because of cultured oocytes in the media containing antioxidants (0.2 mM of sodium pyruvate, 2 mM of l-glutamine) [267] or maybe prepubertal sheep oocytes are not affected by LA concentration like in adult bovine oocytes. In a recent study with ALA we have shown that 200µM significantly has decreased CE and MII in prepubertal sheep oocyte [262]. In bovine, it has been shown that changes leading to cumulus cell expansion are independent from oocyte nuclear maturation [268]. This is in agreement with the results obtained from present study on prepubertal sheep COCs.

Concentrations of LA were previously reported in bovine including 0.20 mg/ml (713.15µM) in FF, 0.56 mg/ml (1996.82µM) in plasma, and 0.27 mg/ml (926.75µM) in uterine endometrial tissue [236] and 0.188 mg/ml (670.36µM) in plasma by [237]. In our laboratory, in prepubertal goats, the LA concentrations were 304.03±15.18 µM and 274.94±27.62 µM in small and large follicles (larger than 3mm), respectively. There is no report about physiological levels of LA in lamb reproductive system. Homa and Brown [226] demonstrated that proportions of LA were significantly lower in FF of large follicles

(31.1±1.2% of total FA) than small follicles (34.8±0.7% of total FA) ($P \leq 0.05$) and there was a significant inverse correlation between follicle diameter and percentage of LA in FF. Though the similarity of oocytes nuclear development between the LA treated and the control group may imply lack of adverse effect of LA on lamb oocyte within the range of concentration used in the present study which can be considered lower than the physiological concentrations.

The results from present study indicated that LA supplementation to IVM media has stimulated PGE2 secretion to a highly significant level compared with the control group. PGF2α has important function as a signaling molecule involved in a number of activities in the body such as inflammation [258]. It is also a key factor in several aspects of reproduction involving ovulation, estrous and birth [122]. It has been reported that 100μM LA significantly stimulated release of PGE2 from COCs as compared with the control group, however no difference was observed in PGF2α production [69]. It has recently been shown that PGE2 in the absence of gonadotrophins resulted in a concentration-dependent enhancement of oocytes reaching MII stage and little but significant increase in cumulus cell expansion for 24 hours of maturation in bovine [133]. There was no difference in PGE2-to-PGF2α ratio between LA treatments compared with the control group. The results may indicate that PGE2 may affect CE more than the oocyte nuclear maturation. In addition, the data indicated that prepubertal sheep oocytes are able to uptake the LA supplemented to the maturation media and utilize it as a precursor for PG production.

We have also examined impact of LA on fertilization of prepubertal sheep oocytes by staining of presumptive zygotes in a period of 20 hours of fertilization. LA supplementation did not affect the total number of fertilized oocyte however, at 50μM concentration improved the rate of normal fertilized zygote (2PN) compared with the control group. This is in agreement with our recent results about ALA which has also increased the 2PN compared with the control group. Our results showed that there is no difference in cleavage and blastocyst rates at day 8 of fertilization (Table 3). Marei et al [69] showed that the cleavage (58±2.8% vs. 74±3.9%) and blastocyst rates (8±4.0% vs. 33±4.0%) decreased significantly in bovine oocyte matured in media supplemented with 100μM of LA compared with the control group. Wonnacott et al [176] have also shown

that the diet rich in n-6 high density lipoprotein (HDL) does not affect cleavage and blastocyst rates in sheep. The results presented here show that the quality of prepubertal sheep blastocyst at day 8 was better with 50µM LA compared with the control group (Table 4). Analyzing the E2 and P4 from the spent maturation media showed that LA decreased the E2 concentration without changing the concentration of P4 production; also the E2-to-P4 ratio was not affected. It has been illustrated that the oocyte denuded from cumulus cell, has produced 29% less E2, but 40 fold more P4, suggesting that oocyte secretes a factor(s) that stimulates E2 production and inhibits P4 synthesis by cumulus cell [269]. We hypothesis that reduction of cumulus cell and decreasing E2 concentration beside increasing PGE2 production in maturation media in the presence of LA might affect embryo quality of prepubertal sheep blastocyst at day 8 via improving the number of total cell at the 8 day blastocyst.

In the present study, we have used serum-free, BSA supplemented media for IVM, IVF and IVC of prepubertal sheep oocyte and have achieved 15.62% blastocyst rate per cleaved in the control group. The results for embryo development and quality are comparable with previous studies on prepubertal sheep which oocytes have matured in serum-contained media. In a study by Catala et al [17] using serum in all stages of embryo production about prepubertal sheep in IVF and ICSI system could achieve 13.6% and 13.1% blastocyst rate per oocyte, respectively. Also, O'Brien et al [246] have also produced blastocyst 15.4% using serum. The number of total cell in our study was 53.35±3.64 which is comparable with a previous report by [17] who produced blastocyst with 62.7±7.8 cell by ICSI and 56.8±4.8 cell by IVF techniques. These indicate that PUFA can be a suitable replacement for serum during oocyte maturation. Omission of serum would benefit the quality of embryo and outcomes of embryo transfer by avoiding syndromes like large offspring [270].

4.4.1. Conclusion

Results from the present study showed that the addition of 50, 100 and 200µM of LA to the IVM media has not affected oocyte nuclear maturation and blastocyst development of prepubertal sheep oocyte. Furthermore, we have observed significantly higher rate for 2PN and higher quality blastocyst from oocyte matured with 50µM of LA

compared with the control group. The synthesis of PGE2 and PGF2α were significantly increased in oocytes matured in LA treatments compared with the control group. Oocytes treated with LA reduced significantly E2 concentration in the IVM media. But, P4 concentration was unchanged among LA and control group. In our free-serum, BSA supplemented media (IVM, IVF and IVC media); the results for blastocyst production and quality were similar to the routine IVEP methods in adult and prepubertal sheep using serum in the different stages of embryo production.

CHAPTER 5:

EFFECT OF LARGE MOLECULAR SIZE HYALURONAN ON SHEEP BLASTOCYST DEVELOPMENT AND SURVIVAL AFTER CRYOPRESERVATION

5. Effect of large molecular size hyaluronan on sheep blastocyst development and survival after cryopreservation

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Abstract

Hyaluronan (HA), a component of extracellular matrix in mammalian tissues including reproductive system, has been shown to support embryo development. HA is produced in various sizes with distinct physiological functions. For embryo development *in vitro* matured 489 oocytes were fertilized for a period of 25 hours and then 272 embryos at stages 4, 8 and 16 cells cleaved sheep embryos were cultured in serum-free synthetic oviductal fluid (SOF) with concentrations (0 [control], 0.125, 0.25 and 0.5 mg/ml) of large size HA (5×10^6 Da). Development to blastocyst stage was recorded at days 7, 8 and 9 after fertilization. A group of embryos were fixed and stained by differential staining combined with terminal deoxynucleotidyl transferase dUTP nick end labelling to analyze embryo quality and the remained blastocysts from each group were vitrified in open pulled straws (OPS). Thereafter, thawed embryos cultured for an extra period of 48 hours to record survival rate and quality of blastocysts after cryopreservation. HA numerically increased blastocyst percentage at day 7 (33±5.7, 32±6.0, 35±5.5 vs. 25±5.2; $P\geq0.05$) and survival rates 48 hours after culture in serum-free media (63±17.1, 83±15.2, 58±14.2 vs. 38±17; $P\geq0.05$) compared with the control group. HA increased the total cell (TC) number (100.7±3.8, 97.2±3.7, 105.0±3.9 vs. 83.6±4.6; $P<0.05$) and trophectoderm cells (TE) (74.2±3.2, 75.6±3.3, 80.1±3.4 vs. 58.4±3.8; $P<0.05$) at day 7 embryos. Survived embryos had higher TC (130.8±3.6, 113.9±5.2, 149.8±5.4 vs. 63.2±3.7; $P<0.05$), TE (96.7±3.1, 85.2±4.5, 111.9±4.7 vs. 42.9±3.0; $P<0.05$) and ICM (32.9±1.8, 27.7±2.6, 36.5±2.7 vs. 20.3±2.2; $P<0.05$). The results indicate that large molecular size HA improves the embryo development numerically and viability. Quality of fresh and survived blastocysts in highly significant level improved which might have implication for improving embryo transfer.

Key words: Hyaluronan, ovine, blastocyst, embryo quality, cryopreservation

5.1. Introduction

Hyaluronan (Hyaluronic acid, HA) is a carbohydrate with long polymer chains of sugars that is found in the extracellular matrix of most animal tissues [271]. It is the most abundant glycosaminoglycan (GAG) in the female reproductive tract such as uterine, oviduct and follicular fluids in human [198], cattle [272] and pig [273]. In mammalians HA is synthesized by three different mammalian hyaluronic acid synthase (HAS) enzymes [274-277]; the HAS enzymes synthesize HA of different sizes *in vitro* in the range of 2×10^5 to 2×10^6 Da by HAS1, 2×10^6 Da by HAS2, 1×10^5 to 1×10^6 Da by HAS3 [212, 278], located at the inner cytoplasmic face of the plasma membrane [279]. Recently, Marei et al [67] have demonstrated existence of HAS2 and HAS3 in all stages of embryo development whereas HAS1 mostly was expressed in 2 and 4 cells embryos. The expression of Hayl2 is detected at days 6 and 7 of bovine *in vitro* cultured morula and blastocysts. It has already reported that the biological effect of HA depends on its molecular size [213, 280-281]. The large size polymers (>1000-5000 disaccharides) are structural component of tissues and function as hydrating molecules that have a role in maintenance of cell membrane integrity [282]. The presence of hyaluronan in tissues is responsible for maintaining spaces between individual cells and at the same time allows the diffusion of small size materials [283]. The large size HA can be retained by CD44 receptor on the outside of the cell membrane, where it reduces the membrane potential [284] or regulates the cell volume [285]. The large molecular size HA is found during ovulation, fertilization and embryogenesis [213]. It has been shown that HA may play a role in delaying death of the oocyte with prevention of fragmentation of porcine oocytes [286] and improves *in vitro* bovine [44] and murine [223] embryo development to the blastocyst stage. Supplementation of HA to the culture media on the third and fifth day of culture media improved lambing rate in sheep [287]. HA polymers are also space-filling molecules that hydrate tissues, and are antiangiogenic. Furthermore, HA polymers are also anti-inflammatory and immunosuppressive as well. The fetal circulation and amniotic fluid contain high concentrations of HA. This may account for some of the immune-suppression in the developing fetus.

Hyal2 (Hyaluronidase) is an enzyme that cleaves HA to small fragments with decreasing sizes by enzymatic hydrolysis, which include hyal1, hyal2, hyal3, hyal4,

hyalp1 and PH20 isoforms [40]. The 20-kDa fragments derive by hyal2 is highly angiogenic, and stimulates synthesis of inflammatory cytokines [288]. Interaction between hyal2 and CD44 (HA receptor) promoting endocytosis of HA to further degradation by lysosomal hyal1 into smaller HA fragments enabling cellular migration, proliferation and mitosis [289]. Tetrasaccharides from smaller size HA induce expression of heat shock proteins and are anti-apoptotic and suppressing cell death [214]. In addition, HA has improved the motility of frozen sperm after thawing and increased its fertilization rate. This effect was concentration dependent and 0.75 mg/ml of HA had the greatest effect on sperm motility, vitality and fertilization ability [290]. Furthermore, high concentrations of HA (6 mg/ml) increased the viscosity of embryo culture media and resulted in higher day 8 blastocysts rate as compared to media supplemented with bovine serum albumin (BSA, 1 mg/ml) [291]. Physiological concentrations of HA in the female reproductive tract such as follicular fluid, oviduct and uterus of pig is from 0.04 to 1.83 mg/ml [205]. As well as the concentration of HA in cumulus oocyte complexes (COC) is between 0.5 to 1 mg/ml [206] and in follicular fluid in human is 55.1 ± 2.4 ng/ml which is used as an indicator to predict oocyte viability for fertilization [207]. Based on this knowledge, the concentration up to 0.5 mg/ml from large size HA in SOF serum-free was used for this study.

The studies presented here investigated the effect of large size HA on sheep embryo developmental competence from *in vitro* fertilized and assessed survival rate of the blastocysts after cryopreservation. Quality of the blastocysts was also assessed on fresh and frozen/thawed blastocysts.

5.2. Materials and Methods

5.2.1. Materials

All chemicals, *except* ones *mentioned,* were obtained from Sigma Chemical Co (St. Louis, MO, USA). SOF medium was incubated at 38.5 ^{o}C under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for at least 2 hours before using. Oocyte washing media, maturation, sperm washing and fertilization media were incubated at

38.5 ^{o}C under a humidified atmosphere of 5% CO_2 in air for at least 2 hours before using.

5.2.2. Oocyte collection

Sheep ovaries were obtained from a local abattoir and brought to the laboratory within 1 hour in 1% PBS at 30 ^{o}C to 35 ^{o}C. Cumulus oocyte complexes (COCs) were collected by aspirating follicles of 2 mm to 6 mm diameter with 10 ml syringe containing 2 ml washing media, composed of tissue culture medium (TCM) 199 as a basic media with 20 mM Hepes and 4 mg/ml BSA fraction V [292] supplemented with 0.225 IU/ml heparin sodium salt. The aspirated COCs were then allowed to settle for a maximum of 10 minutes before transfer to a 90 mm petri dish for recovery under a dissecting microscope.

5.2.3. *In Vitro* Maturation

The collected COCs were washed two times in washing media and then once in serum-free maturation media established by Shirazi et al [71] containing TCM199 with 2 mM L-glutamine, 0.02 mg/ml cysteamine, 0.2 mM sodium pyruvate, 5 µg/ml FSH [Follitrpin, Bioniche animal health, Belleville, ON, Canada], 5 µg/ml LH [Leutropin, Bioniche animal health, Belleville, ON, Canada], 10 ng/ml epidermal growth factor, 1 µg/ml E2, 50 µg/ml gentamicin and 6 mg/ml BSA fraction V. Groups of 40-50 COCs were transferred to 500 µl of maturation media in 4-well dishes (NUNC, VWR international, Milan, Italy) and incubated for a period of 25 hours at 38.5 ^{o}C in a humidified atmosphere containing 5% CO_2.

5.2.4. Sperm Preparation and *In Vitro* Fertilization

One hour before the end of the oocyte *in vitro* maturation (IVM) period, the swim up method was commenced for separation of motile sperms (SC009 OV TEXEL PJP 09 071, UK) for fertilization. Sperms were washed in SOF medium developed by Tervit et al [73] with some modifications that contained 3.3 mM of Na-lactate, 4 mM of $NaHCO_3$,

0.33 mM of Na-pyruvate, 0.103 mM of L-glutamine, 2.4 mM of D-glucose, 21 mM of Hepes, 20 µl/ml of penicillin-streptomycin and 3 mg/ml of BSA fraction V. Swim up was performed according to the Shirazi et al method [71, 293] with some minor modifications in which 60 µl of frozen thawed sperm was kept under 3 ml of sperm washing media in conical Falcon tube at 38.5 ^{o}C in a humidified atmosphere of 5% CO_2 in air for up to 45 minutes and then supernatant moved to another tube and centrifuged in 200 × g for 7 minutes in 25 ^{o}C. After centrifugation, 100 µl remained pellet re-suspended with 900 µl of fertilization medium. Fertilization medium was prepared according to the Tervit et al [73] protocol with some modifications containing 3.42 mM of $CaCl_2$, 9.9 mM of Na-lactate, 0.99 mM of Na-pyruvate, 1 mM of L-glutamine, 10 µl/ml of penicillin-streptomycin and 2 mg/ml of BSA fraction V. Insemination of matured oocytes were carried out by culturing matured COCs with 1 × 10^6 sperm per ml enriched with 15% heat inactivated estrus sheep serum (ESS) for 18 to 20 hours at 38.5 ^{o}C in a humidified atmosphere containing 5% CO_2.

5.2.5. *In Vitro* Culture

Following fertilization, presumptive zygotes were denuded from remaining cumulus cells by gentle pipetting and then washed three times in 100 µl drops of SOF medium [73] containing 9.9 mM of na-lactate, 0.99 mM of na-pyruvate, 1 mM of l-glutamine, 2.24% of basal medium eagle (BME) essential amino acids, 0.5% of minimal essential medium (MEM) non-essential amino acids, 0.34 mM of trisodium citrate, 4.6 mM of myo-inositol and 4 mg/ml of BSA fraction V. Thereafter, zygotes were transferred to 500 µl SOF medium and cultured for 7 days (fertilization day=0) at 38.5 ^{o}C in 5% CO_2, 5% O_2 and 90% N_2 and maximum humidity. The culture medium was refreshed every 48 hours.

At day 7 *in vitro* produced embryos were graded according to the International embryo transfer society (IETS) embryo grading method [294]. Embryos at different stages (expanded blastocyst, hatching and/or hatched blastocyst) were used for differential staining combined with by differential staining combined with terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) [293] and vitrification.

5.2.6. Vitrification and warming

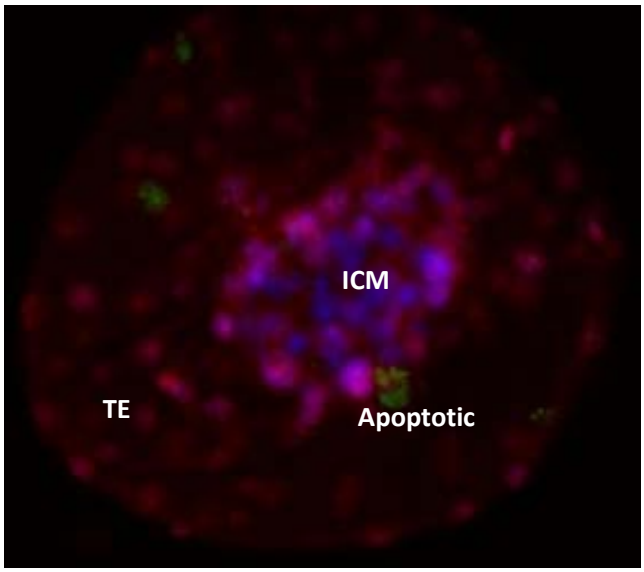
Vitrification by open pulled straw (OPS) technique was used for *in vitro* produced sheep embryos, according to the Vajta et al [25] technique with minor modifications. Briefly, TCM199 was used as a basic medium and supplemented with 1 M of Hepes buffered saline and 20% fetal bovine serum (FBS) and used as a holding media (HM). The embryos were exposed for 7 minutes in HM in 150 µl drops and then transferred to the dilution media (DM) in 100 µl drops that was prepared with 7.5% ethylene glycol (EG) (v/v) and 7.5 % dimethyl sulphoxide (DMSO) (v/v) in HM for 3 minutes and thereafter followed by 25 seconds exposure to the vitrification medium (VM) in 30 µl drops that were prepared with 16.5% EG and 16.5% DMSO in HM. Two embryos were loaded into the OPS via capillary, by placing the narrowest end of the straw into the vitrification media. The straws were immersed immediately into LN_2. The straws were thawed by placing the end of the straws directly in 1.5 ml HM that was supplemented with 0.25 M sucrose for 1 minute and then transferred to HM in 150 µl drops containing 0.15 M sucrose for 5 minutes. Thereafter, embryos were continuously washed two times in HM in 150 µl drops for 5 minutes. The embryos in different stages were cultured for 48 hours and vitality of embryos checked every 2, 6, 12, 24 and 48 hours and the result recorded.

5.2.7. Determination of embryo quality

Differential staining combined with TUNEL labelling was used to assess blastocyst quality by counting the total cells, inner cell mass (ICM), trophectoderm (TE) and apoptotic cells. *In vitro* produced sheep blastocysts were stained at day 7 of culture according to the Fouladi-Nashta et al [293] with some modifications. Briefly, to permeabilize and stain TE cells, blastocysts were incubated for 20 seconds in SOF medium containing 0.2% of triton 100X and 30 µg/ml of propidium iodide (PI). The embryos were then washed three times in SOF medium before fixing for 20 minutes in 2% (w/v) of paraformaldehyde in PBS supplemented with 30 µg/ml of bisbenzimide (Hoechst, 33422). Afterwards, the blastocysts were washed 3 times, and re-permeabilized in 0.1 % (v/v) of triton 100X in SOF medium for 5 minutes before transfer to 10 µl droplets of 1:10 dilution of TUNEL staining solution (Roche, Penzberg,

Germany) in a humid chamber for 1 hour at 38.5 ^{o}C. The blastocysts were then washed three times and mounted in 3 µl drops of glycerol based mounting media (Vectashield, Vector laboratories, Burlingame, CA) for examination under a fluorescent microscope (Leica, Wetzlar, Germany). Images of stained blastocysts were taken for counting the number of TE (red), ICM (blue) and apoptotic (green) cells (Fig. 1).

Fig. 1 Differential stained and TUNEL labeled of sheep embryo.



The picture shows DST staining of a 7 day blastocyst triple-stained merged image with probidium iodide (PI, red) for trophectoderm (TE), Hoechst (blue) for Inner cell mass, and TUNEL (green) for apoptotic nuclei.

5.2.8. Experimental Design

After IVM, the oocytes were fertilized and the cleaved embryos produced after fertilization (≥4 cell) were cultured in a serum-free medium (0 [control], 0.125, 0.25 and 0.5 mg/ml of large size HA). Blastocyst rate was recorded at days 7, 8 and 9 after fertilization. A group of day 7 blastocysts (n=25) were used for assessment of embryo quality. The remained day 7 blastocysts (n=23) were used for cryopreservation by vitrification technique. The survival rate recorded at 2, 6, 12, 24 and 48 hours after vitrifying and warming. The number of total cells, TE, ICM and apoptotic cells were

counted in fresh blastocyst and survived blastocyst after 48 hours of vitrifying-warming. This experiment was carried out in three replicates.

5.2.9. Statistical analysis

Statistical analysis was carried out using IBM SPSS statistic for windows, version 20.0 (IBM Corp. Armonk, NY, USA). The Linear mixed model was run for total cell trophectoderm cell, inner cell mass, ratio of ICM/TE and apoptotic cell number. General linear model ANOVA was used to analyze changes for cleaved embryo, blastocyst and survival rate.

5.3. Results

A total of 489 oocytes were fertilized *in vitro* and after fertilization 272 embryos were cultured. The percentage of blastocyst was significantly higher in the highest concentration of HA compared with the other HA concentrations and control group at day 9 (Table 1, $P \leq 0.05$). There was not different between HA groups in blastocysts rate at days 7 and 8 of embryo culture.

Table 1 The effect of different concentrations of large size HA (0 [control], 0.125, 0.25 and 0.5 mg/ml of large size HA) added to the embryos at ≥4 stage of fertilized embryos in serum-free culture medium for recording sheep blastocyst development.

Day\Treatment	Control	Healon 0.125 mg/ml	Healon 0.25 mg/ml	Healon 0.5 mg/ml
Day 7	17/69 (25±5.2)	23/69 (33±5.7)	19/60 (32±6.0)	26/74 (35±5.5)
Day 8	18/69 (26±5.3)	25/69 (36±5.8)	21/60 (35±6.2)	30/74 (41±5.7)
Day 9	19/69(28±5.4)[a]	25/69 (36±5.8)[a]	21/60 (35±6.2)[a]	32/74 (43±5.8)[b]

Values in the same row with different superscripts (a, b) differ significantly ($P \leq 0.05$)

Table 2 shows significant difference in vitality between HA concentrations and control groups ($P \leq 0.05$). Also the provided data showed that 6 hours after warming survival rate is in the highest range. There was not significant difference between HA groups at different survival recording times compared with the control group.

Table 2 The effect of different concentrations of large size HA (0 [control], 0.125, 0.25 and 0.5 mg/ml of large size HA) added to to the embryos at ≥4 stage of fertilized embryos in the serum-free culture medium on survival rate at different hours post warming of sheep vitrified day 7 blastocysts.

Hour-treatment	Control	Healon 0.12 mg/ml	Healon 0.25 mg/ml	Healon 0.5 mg/ml
2	2/8 (25±15.3)[a]	1/8 (13±11.7)[a]	5/6 (83±15.2)[b]	6/12 (50±14.4)[a]
6	4/8 (50±17.7)[a]	4/8 (50±17.7)[a]	6/6 (100±0.0)[b]	7/12 (58±14.2)[a]
12	3/8 (38±17.1)[a]	5/8 (63±17.1)[a]	5/6 (83±15.2)[b]	7/12 (58±14.2)[a]
24	3/8 (38±17.1)[a]	5/8 (63±17.1)[a]	5/6 (83±15.2)[b]	7/12 (58±14.2)[a]
48	3/8 (38±17.1)[a]	5/8 (62.5±17.1)[a]	5/6 (83±15.2)[b]	7/12 (58±14.2)[a]

Values in the same row with different superscripts (a, b) differ significantly (P ≤ 0.05)

At day 7 the total cell of blastocysts were significantly higher in all HA groups compared with the control group because of increases in the number of trophectoderm cells. HA did not change the ICM and apoptotic cell rates compared with the control group but the ICM/TE ratio was affected significantly (P ≤ 0.05) (table 3).

Table 3 The effect of different concentrations of large size HA (0 [control], 0.125, 0.25 and 0.5 mg/ml of large size HA) added to to the embryos at ≥4 stage of fertilized in the serum-free culture medium on total cells, TE, ICM, apoptotic cells and ICM/TE ratio of day 7 sheep blastocysts.

Parameters-treatments	Control	Healon 0.125mg/ml	Healon 0.25 mg/ml	Healon 0.5 mg/ml
Total Cells	83.67±4.60[a]	100.75±3.81[b]	97.25±3.75[b]	105.07±3.90[b]
Trophectoderm (TE)	58.47±3.83[a]	74.29±3.26[b]	75.66±3.31[b]	80.16±3.41[b]
Inner Cell Mass (ICM)	25.20±2.54	26.38±1.95	21.52±1.76	24.82±1.89
Apoptotic cells	3.62±0.97	2.61±0.61	3.65±0.72	3.79±0.73
ICM/TE Ratio	0.45±0.04[a]	0.36±0.03[a]	0.33±0.03[b]	0.30±0.03[b]

Values in the same row with different superscripts (a, b) differ significantly (P ≤ 0.05)

These results were also observed in survived embryos after 48 hours of vitrifying-warming except that in these embryos ICM was higher in HA groups compared with the control group (Table 4).

Table 4 The effect of different concentrations of large size HA (0 [control], 0.125, 0.25 and 0.5 mg/ml of large size HA) added to the embryos at ≥4 stage of fertilized serum-free culture medium on total cells, TE, ICM, apoptotic cells and ICM/TE ratio of survived sheep embryos after freezing-warming.

Parameters-treatments	Control	Healon 0.125mg/ml	Healon 0.25 mg/ml	Healon 0.5 mg/ml
Total Cells	63.21±3.74[a]	130.80±3.63[b]	113.94±5.28[b]	149.84±5.47[b]
Trophectoderm(TE)	42.98±3.04[a]	96.73±3.12[b]	85.24±4.54[b]	111.94±4.70[b]
Inner Cell Mass (ICM)	20.36±2.24[a]	32.99±1.82[b]	27.74±2.62[b]	36.53±2.74[b]
Apoptotic cells	6.02±1.24	5.92±0.77	6.09±1.24	5.08±1.02
ICM/TE Ratio	0.43±0.08	0.36±0.07	0.37±0.08	0.40±0.07

Values in the same row with different superscripts (a, b) differ significantly (P ≤ 0.05)

5.4. Discussion

In the present study, we have shown, that the addition of large size HA to serum-free culture medium significantly increased sheep blastocyst development, total cell and survival rate of vitrified-warmed blastocysts.

Large size HA ($5x10^6$ Da molecular weight) at 0.5 mg/ml concentration increased significantly the blastocyst development compared with the control group (43.2% vs. 27.5%, respectively, P≤0.05). Previous reports indicated that HA improves blastocyst percentages in combination with BSA in bovine (25.5% vs. 17.9%) [295]. Also the addition of 6 mg/ml HA (1.69×10^6 molecular weight) to SOF culture medium increased blastocyst rate compared with the BSA group (38.2% vs. 29.3%) [291]. HA has also improved blastocyst quality leading to improvement of pregnancy rate in sheep (57.4% vs. 53.6%) [287]. The results from the studies revealed here are in agreement with previous literatures and show that supplementation of HA is beneficial for blastocyst

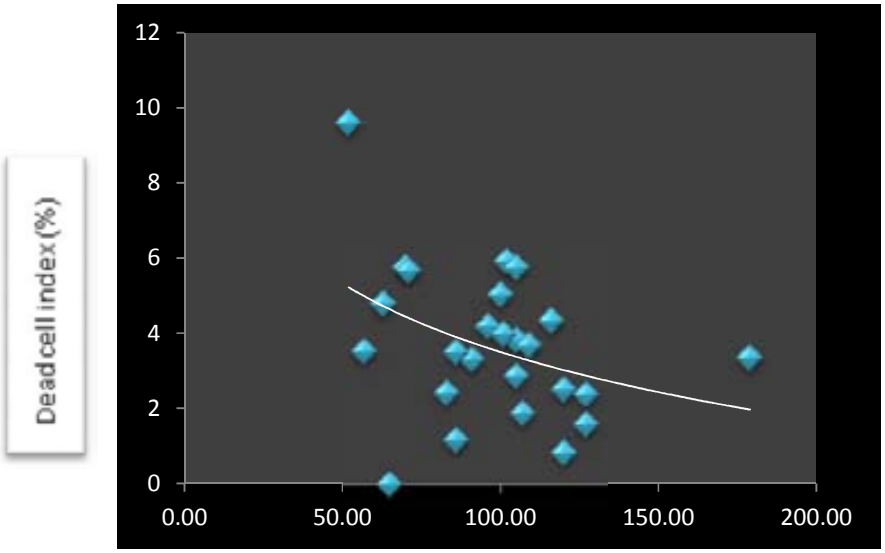
development indicating the importance of differences in concentrations of HA on supporting embryo development. Toyokawa et al [296] speculated that exogenous HA has improved porcine parthenogenetic embryo development *in vitro*, irrespective of its molecular size. Nasr-Esfahani et al [297] have published a study (performed on 50 couples) observing a higher fertilization rate when injecting oocytes with HA treated spermatozoa. Recently, Marei et al [67] have published a study and reported that addition of hyal2 in culture medium 48 hours after insemination in the concentration of 300 IU/ml has significantly increased blastocyst rate. It has been shown that embryos are producing HA during embryo development peculiarly in developing steps [298], even it has been shown that HA actively is secretion during mitosis [299].

Until now, *in vitro* produced embryos have low survival after freezing-thawing compared with the embryos derived from *in vivo* [300]. These differences appear to be exacerbated when embryos achieve from IVM, IVF and IVC containing serum with darker ooplasm, lower density, swollen blastomeres, slower growth rate and sensitivity to increased temperature [300-303], making them more sensitive to freezing, resulting in a lower pregnancy rate compared with the embryos derived from *in vivo* [304]. Stojkovic et al [291] have shown that after freezing-thawing, the total percentage of 8 day embryos that re-expanded and hatched during culture with HA was higher compared with the BSA group (86.8% vs. 47.3%, respectively). These results are similar with the present study that survival rate of sheep vitrified blastocysts after warming were better compared with the control group (Table 2). Also Stojkovic et al [291] have shown that embryos treated with HA have less ultra-structural deviation (formation of the blastocoels, number of organelles in the cytoplasm and irregular forms of embryonic cells) and de-differentiation (the differentiation in trophoblast and ICM cell was no longer visible) after freezing-thawing compared to the embryos cultured along by BSA [291]. Also the beneficial of HA was confirmed on oocytes [305] and embryos in bovine [44, 291, 306] and porcine [306] and in murine with increasing the implantation and foetal development rate after blastocyst transfer [223]. The results from the present study are in agreement with previous reports suggesting that HA could be an alternative agent for replacing serum in ovine embryo culture.

Quality of embryos were evaluated before and after freezing-warming via analysing the data from total cell, TE, ICM, apoptotic cell and the ratio of TE/ICM. Large size HA improved day 7 blastocyst quality as evidenced by higher total cell number and higher percentage of survival rate after freezing-warming (table 3 and 4, $P \leq 0.05$). Similar results were reported by Stojkovic et al [291] who showed significant increase in the number of TE and total cell of blastocysts cultured with HA than using BSA alone. Lane et al [307] reported more ICM cell in expanded blastocysts cultured in the presence of HA compared with other embryos achieved *in vitro*, but the differences were not significant. In contrast, Michelle et al [307] found no beneficial effect of HA supplementation to the medium containing BSA or without BSA in total cell, TE or ICM cells in bovine, although the size of HA was not specified experimentally. Recently, Marei et al [67] have shown that supplementing culture media 48 hours after post insemination with hyal2 with 300 IU/ ml the number of total cell, TE and ICM have significantly increased, whereas, they have gained higher number of blastocyst number as well. These could support the idea that the higher number of cells in embryos are beneficial in getting significantly developed embryos to blastocyst step.

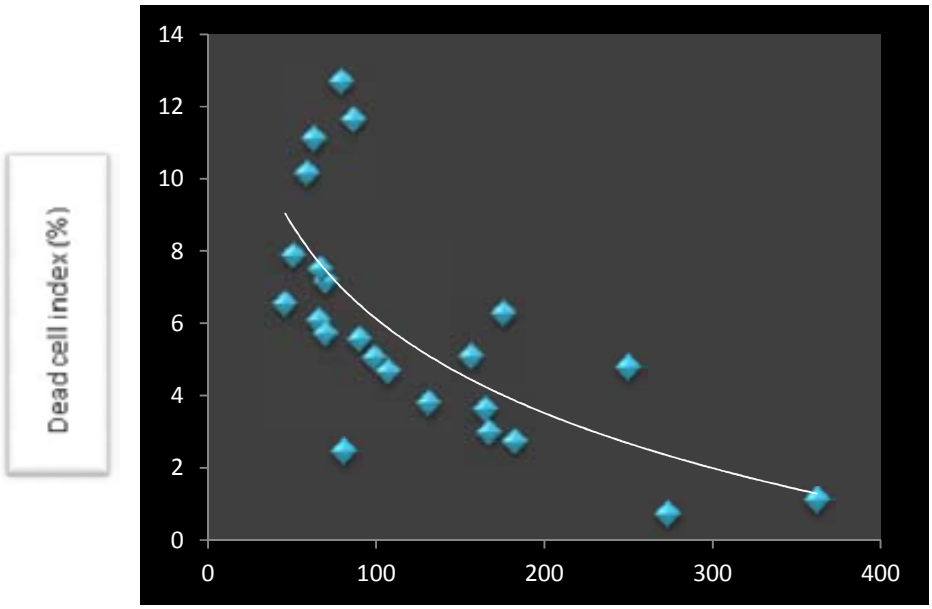
Correlation analyzes between total cell and apoptotic cell index (fig. 1) for fresh embryo at day 7 after fertilization showed an association between these two parameters showing that increasing the number of total cell decrease the apoptotic cell numbers.

Fig. 1. The relationship between total cell number and apoptotic cell index for fresh 7 day embryos.



Also, the increases of total cell number in vitrified-warmed blastocysts and decreasing the number of apoptotic cell evidenced (fig. 2). This effect is possibly mediated through CD44 and activation of mitogen activated protein kinesis as was indicated in granulosa cells [308] showing reduced apoptosis marked with fragmented condensed nuclei. Likewise, the fact of better vitality in survived embryos (48 hours after vitrifying-warming) is related to low number apoptotic cell can be justified by the relationship between the cell proliferations and regeneration or healing phenomenon.

Fig. 2. The relationship between total cell number and apoptotic cell index for survived embryos 48 hours after warming



Palasz et al [309] demonstrated that the using HA in the cryopreservation medium improves survival of bovine blastocysts, also they have postulated that this can be due to a reduced toxicity of cryoprotectant by HA. HA has strong water-binding capacity, the ability to interact with water to form a gel-like structure to reduce water mobility. It means HA decreases the lethal consequences of crystallization, and at the same time, improves the resistance of membranes and organelles to the shock of cryopreservation [291]. The vacuolization of the embryonic cells, the degradation of cell organelles and the formation of intercellular spaces after cryopreservation have shown markedly reduced by using HA *in vitro* culture. Also it has shown that the mitochondria of embryos cultured in the presence of HA have few crystal or even ballooned crystal [291]. This hypothesis coming up that the increases in number of cells cultured *in vitro* is in association with embryo development and embryo survival that making embryos capable to develop and even survive after warming. The advantage of higher survival with higher

number of trophectoderm cells will be in related to conception rate and subsequently pregnancy rate as trophectoderm cells produce interferon tau that is responsible for maternal recognition of pregnancy in early period of conception.

5.4.1. Conclusion

This study shows that the addition of large size HA to the serum-free culture medium improved ovine blastocyst development and quality assessed by number of total cells and embryo survival after vitrification. As well as it can be considered to be used instead of serums in *vitro* embryo production fields.

CHAPTER 6:

GENERAL DISCUSSION

6. General Discussion

The present study has been focused on the finding of the effects of the substances such as Alpha Linolenic Acid (ALA), Linoleic Acid (LA) and Hyaluronic Acid (HA) on oocyte maturation and subsequently embryo development and quality by evaluation of embryo cell number and survival rate after vitrification. As it has stated in the introduction, the present study had three main aims and was conceived to answer three main questions. Accordingly, our first aim was determining the effect of ALA added to the in vitro maturation medium on: a) the nuclear maturation and b) concentration of reproductive hormones such as prostaglandins (PGE2 and PGF2α), and steroids (E2 and P4) and c) subsequently embryo development of prepubertal sheep oocytes and embryo quality by counting blastocyst cells. Our second aim was using LA in maturation media same ALA on prepubertal sheep oocytes. finally, the third aim was using HA in vitro embryo culture media for evaluation of embryo development and embryo quality and later on freezing and thawing blastocysts achieved at day 7 for assessment of survival rate via counting blastocyst cells.

It is well known that many laboratories have been trying to improve the embryo quality and development by supplementing the media in different steps of in vitro embryo production using different substances such as ALA [68], LA [69, 310] and HA [67, 311] in different animal species. However, the results obtained so far have been inconsistent. Indeed, some studies have found that addition of fatty acids have improved the embryo development in terms of blastocyst formation and embryo quality of IVP embryos in terms of total cell number in bovine [68-69, 228, 230].

According to this background, we conducted studies evaluating ALA and LA fatty acids in prepubertal sheep oocytes to see the impact of them in prepubertal animal. Fouladi-Nashta et al [228] using three dietary sources of FAs (Palmitic and oleic, LA and ALA) on developmental potential of oocytes in lactating dairy cows have shown that there was no difference between dietary treatments in the average number of oocytes collected per cow, nor in the distribution of oocytes in each quality grade. Even, diet containing the FAs did not affect the proportions of blastocysts that developed from the number of inseminated oocytes or the number of cleaved oocytes. Furthermore, there

was no effect of dietary treatment on proportions of trophectoderm cells related the total inner cell mass in blastocysts [228]. These authors have justified that their maturation medium was contained FCS to maximize developmental potential of oocytes. However, FCS contains lipids, which might have moderated the effects of dietary FAs on oocyte developmental potential. Marei et al [68] using ALA on bovine oocyte maturation have shown that 50µM of ALA have improved the MII stage compared to control group. Higher doses of ALA reported to be detrimental. These authors also have shown that ALA has increased the percentages of cleavage, blastocyst and embryo quality. Marei et al [68] have concluded that these effects have mediated both directly through MAPK pathway and indirectly through PGE2 synthesis.

Our results using 50, 100 and 200µM of ALA showed that ALA in higher concentrations are harmful for cumulus cell expansion and MII stages of prepubertal sheep oocytes cultured in serum free maturation media. After 24h of maturation the spent maturation media was used to analyze the concentration of prostaglandins. These results showed a significant increase for PGE2 in all ALA concentrations but for PGF2α this increases happened only for first and second concentrations (50 and 100µM). While, the ratio of PGE2/PGF2α significantly increased. It has shown that PGE is a critical mediator of oocyte maturation and cumulus expansion. PGE2 acts via an autocrine/paracrine regulatory pathway mediated by its G-protein coupled receptors (PTGERs) on cumulus cells [312] which stimulates intracellular increase in cAMP as a second messenger [313]. Inhibition of PGE2 production using prostaglandin synthase 2 inhibitors was found to attenuate cumulus expansion and GVBD of mouse oocytes [132]. It has reported that addition of Epidermal Growth Factor in 10ng/ml in maturation medium has produced higher levels of prostaglandin synthase 2 and PGE2 in vitro and that it has been accompanied with higher rates of cumulus expansion and MII stages after 24h of maturation [314]. Also, it has shown that PGE2 in the absence of gonadotrophins resulted in a concentration dependent increase in the percentage of oocytes reaching MII after 24h in maturation media. But supplementation of PGE2 in the presence of gonadotrophins had no effect in the percentage of oocyte maturation in bovine [133]. In this study we had gonadotrophins in our maturation media that regarding the literatures PGE2 in the presence of gonadotrophins does not have any beneficial effects on oocyte maturation. Significant decrease in cumulus expansion and MII stage at the highest concentration of ALA might be related to other factors such as

reproductive hormones. In this study the concentration of E2 has significantly decreased in ALA groups compared to control group while the concentration of P4 has increased significantly in ALA groups. The results achieved for cleavage and blastocyst rate showed there is no difference compared to control group in other word ALA does not have beneficial or detrimental effects on embryo development of prepubertal sheep oocytes after in vitro fertilization. It has shown that successful pregnancies have achieved in human IVF using oocytes from follicles that contained higher concentrations of E2 and a higher E2/P4 ratio [178]. Meanwhile, it has shown that adding E2 and P4 in IVM media has improved developmental competence of primates however, if there is not E2 there was not achieved blastocyst [157]. Embryo quality has improved in ALA concentrations and that was related to low apoptotic cell numbers.

LA is the most abundant fatty acid in bovine follicular fluid, and it was previously reported that LA concentration significantly decreases when follicle size increases. This suggests that LA may have a role in regulation of oocytes maturation [69]. Also, it has shown that increasing dietary fat influences concentrations of prostaglandins, steroid hormones and growth factors. In second paper we have used 50, 100 and 200µM of LA as supplement to the in vitro maturation medium to assess nuclear maturation, prostaglandins (PGE2 and PGF2α) and steroids (E2 and P4) concentrations and blastocyst development. Marei et al [69] using LA in same concentrations (50, 100 and 200µM) in maturation medium on bovine COCs have shown that LA significantly inhibited cumulus cell expansion and retarded development of the oocytes to the MII stage in a dose dependent manner. Carro et al [310] using LA at different concentrations have concluded that LA at 9 and 43µM concentrations did not affected oocyte maturation but LA at 100µM arrested oocyte maturation at Germinal Vesicle Breakdown. Our results showed that cumulus cell expansion was inhibited by LA in all concentrations. That is in agreement with previous reports that LA in higher concentrations is detrimental and harmful for cumulus cell expansion but there was not negative effects on the nuclear maturation and they were reached to MII in all concentrations in the same range of control. The spent maturation media was used to be measured for PGE2 and PGF2α. The assessments showed that both PGs have increased significantly that is in agreement with the published results from Marei et al [69]. Accordingly, increases in the level of PGs showed that they do not affect the nuclear maturation in prepubertal sheep oocytes but cumulus expansion might be related to the concentrations of PGs. As it has

shown that PGE2 has low effect on cumulus expansion but significantly increased the nuclear maturation [133]. Marei et al [69] have shown using LA in maturation media significantly decreased cleavage and blastocyst rate in bovine. Whereas, the results from our current study have shown that there is no any different in cleavage and blastocyst rates. The reason of this difference could be explained because in our maturation conditions that we have supplemented maturation media with antioxidants (L-glutamine and pyruvic acid). Marei et al [69] have shown that antioxidants prevent the detrimental effects of LA on oocytes maturation and subsequently on embryo development. Marei et al [69] have resulted that these effects related to a reduction of glutathione peroxides and superoxide dismutase (SOD) mRNA expression in bovine oocytes. It has shown that LA stimulates production of reactive oxygen species (ROS) and oxidative stress that has induced by hydrogen peroxide during maturation of oocytes. There was a significant improvement in embryo quality by lowest concentration of LA (50µM). LA has been shown to decrease phosphorylation of intracellular cAMP and MAPK in bovine oocytes [69]. cAMP and MAPK are reported to regulate signalling of G-protein coupled receptors of gonadotrophins, EGF and PGE2 during the maturation period [315-318]. These molecules are known to stimulate hyaluronan synthase 2 expression [319] that is required for the production of HA, which is the main component of extracellular matrix of expanded cumulus cells [320-321]. In the current study we have used EGF and antioxidants that has supported oocytes to suffer the harmful effects of LA. We have measured E2 and P4 to evaluate the relationship of them with embryo development and embryo quality. In our study steroid concentrations have not affected in vitro fertilization, cleavage and blastocyst rates. As we observed in study, the concentration of E2 was reduced in groups compared to control group. Also, P4 concentration was higher but not statistically significant in LA groups than in control group.

The last aim of our work was supplementation of Hyaluronan in vitro culture media 48h after in vitro fertilization of sheep embryos in the stages of 4 to 8 cells. Accordingly we used large size hyaluronan (HA). We showed that the addition of large size HA to serum free culture medium significantly improves blastocyst percentage at day 9 after fertilization and this was also observed for embryo quality that it was evaluated by counting total cell, trophectoderm cell and inner cell mass and finally by freezing and thawing blastocysts at day 7. Previous reports have shown that HA

increases blastocyst percentages in combination with BSA in bovine [295]. HA has also shown to improve blastocyst quality leading to improving pregnancy rate in sheep [287]. Our results are in agreement with previous researches and show that supplementation with HA is beneficial for blastocyst development. Recently, Marei *et al* [67] have reported that Addition of hyal2 (the main receptor of HA) in culture medium 48h post insemination significantly increases blastocyst rate. It has been demonstrated that embryos produce HA during embryo development [298], even HA is releasing during mitosis [299].

in vitro produced embryos have low survival after freezing/thawing in comparison to *in vivo* derived embryos [300]. These differences appear to be exacerbated when embryos achieve IVM, IVF and IVC with darker ooplasm, lower density, swollen blastomeres, slower growth rate and sensitivity to increased temperature [300-303], making them more sensitive to freezing, resulting in a lower pregnancy rate than *in vivo* derived embryos [304]. Stojkovic *et al* [291] have shown that after freezing\thawing, the total percentage of 8-day embryos that re-expanded and hatched during culture with HA was higher than control BSA. These results are similar with the present study that survival rate of sheep vitrified blastocysts after thawing were better than control group. Also Stojkovic *et al* [291] have shown that embryos treated with HA have less ultra-structural deviation (formation of the blastocoels, number of organelles in the cytoplasm and irregular forms of embryonic cells) and de-differentiation (the differentiation in trophoblast and ICM cells was no longer visible) after freezing/thawing compared to the embryos cultured along by BSA [291].

Large size HA improved Day 7 blastocyst quality by higher total cells numbers and higher percentage of survival rate after freezing. Marei *et al* [67] have shown using hyal2 with 300 IU/ ml in culture media 48h after fertilization has improved the embryo quality significantly. Supposedly, embryos containing many cells have better embryo development and are important in embryo quality. Apparently, increasing the number of cells in embryos is in association to embryo development and embryo survival making embryos able to grow and survive subsequent freezing\thawing. On the other hand, higher survival and containing many trophectoderm cells will be useful in improving pregnancies; cells of trophectoderm produce interferon tau that is important in maternal recognition of pregnancy.

CHAPTER 7:

GENERAL CONCLUSION

7. General Conclusion

1. Using ALA in maturation media showed that ALA affects cumulus cell expansion and nuclear maturation in highest concentration of ALA (200µM). ALA showed increases in the concentration of PGs (PGE2 and PGF2α). Two pro-nuclei (2PN) increased by ALA, that might be implied to male nucleus formation and improvement of forming presumptive zygotes. ALA altered the reproductive hormones (E2 and P4) production, too. ALA had beneficial effect on embryo quality with decreasing the apoptotic cells. In conclusion, ALA may be used in maturation medium for supporting embryo quality in lower concentrations as higher concentrations showed can be harmful.

2. LA decreased cumulus cell expansion but nuclear maturation did not affect with LA concentrations (50, 100 and 200 µM). LA increased the concentration of PGs (PGE2 and PGF2α). Only the lowest concentration of LA could increase the two pro-nuclei (2PN). LA also had decreased the concentration of reproductive hormone (E2). The lowest concentration of LA improved embryo quality. In conclusion, LA in lower concentrations may be has beneficial effects for embryo quality as it has increased the embryo quality via increasing the total cell numbers in blastocysts.

3. Hyaluronan increased blastocyst rate at day nine of post insemination of in vitro. Embryo quality increased using HA in all concentrations (0.125, 0.25 and 0.50 mg/ml) with increasing the total cell and trophectoderm cells. But the ratio of inner cell mass to trophectoderm cells decreased. HA improved survival rate of vitrified-warmed blastocysts of day seven of in vitro produced embryos. After warming total cell number, trophectoderm and inner cell mass highly significant increased. In conclusion, HA highly might be useful to be used in improving the embryo quality.

CHAPTER 8:

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