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Effect of stearic acid on *in-vitro* formation of sheep oocytes

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ABSTRACT

The present study was undertaken to study the effect of stearic acid (SA), a non esterified fatty acid (NEFA) on oocyte development of ewes (*Ovis aries*). Sheep oocytes were matured *in vitro* in the presence of different concentration of stearic acid (0 µM, 10 µM, 20 µM, and 30 µM) for 24h. After *in vitro* maturation, oocytes were *in vitro* inseminated with cauda epidydimus sperm processed with BO medium with concentration of 2 million sperms/ml and cultured on oviductal cell culture for 8 days. Oocytes were evaluated for cleavage and fertilization rates and after 7–8 days post insemination zygotes were evaluated for moruale /blastocyst stages. The maturation, cleavage and morulae/ blastocyst production rates were significantly lowered in media containing 20 µM stearic acid. Increment of stearic acid to 30 µM in media further reduced the maturation, cleavage and morulae/ blastocyst production. In conclusion, the metabolic stressors NEFA (SA) impaired the maturation, viability, cleavage and embryo production rates at the level 20 µM in ewes.

Key words: IVF, Metabolic stressors, NEFA, Stearic acid

India is a rich source of diverse ovine germplasm with 74 million sheep which is 6.8% of world sheep population (FAOSTAT 2010). There are 60 sheep breeds in India including well-recognized, lesser known and some wild species. Karnataka, an Indian state lying in its southern peninsular agro-economical zone has sheep as a socioeconomically important livestock reared primarily as a source of mutton in rural areas. A balanced nutrition exerts a very large influence on flock reproduction, milk production, and lambs. Feeding imbalance diet (energy deficit feeding) is accompanied by lipolysis and is typically featured by high non-esterified fatty acid (NEFA) in combination with low glucose concentrations in serum (Leroy *et al.* 2004). Not only the endocrine signaling is disturbed but also the quality of the oocyte and/or embryo may be adversely affected by these nutrient metabolites (van Knegsel *et al*. 2007). Oocyte quality, built upon a total maturation time in the ovary of around months, is very sensitive to negative influences such as nutritional deficiencies or over conditioning. Accumulation of NEFA derived from the adipose tissue during negative energy balance in the follicle fluid constrains the proliferation and health of the granulosa cells and thus jeopardizes oocyte development (Vanholder, et al. 2005). The early embryo losses might result from a malfunctional cytoplasm, which impairs further development of the fertilized oocyte. We observed that oleic acid, palmitic acid and stearic acid are the 3 predominant free fatty acids both in serum and in follicular in our laboratory (Nandi *et al*. 2013). The mean value of stearic acid in sheep follicular fluid was found to be 10.3 µM (Nandi *et al*. 2013). *In vitro* studies have demonstrated differences in the tolerance somatic cells have of different fatty acids, saturated fatty acids being toxic and unsaturated fatty acids being relatively harmless (Aardema *et al.* 2011). No report on effect of saturated fatty acid on oocyte development was reported. Hence, the present study was undertaken to study the effect of stearic acid (SA), a non esterified fatty acid (NEFA) on oocyte development of ewes (*Ovis aries*).

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MATERIALS AND METHODS

Collection of sheep ovary and testis: Ovaries from sheep of unknown reproductive status were collected from nearby Corporation slaughterhouse, Bengaluru. They were brought to the laboratory within 2 h in a thermo flask containing warm 0.9% normal saline. Likewise, testes from matured ram were collected from the same Corporation slaughterhouse, Bengaluru. The testes were brought to the laboratory within 1 h in an ice box containing cold 0.9% normal saline.

Oocytes collection and grading: The aspiration medium consisted of TCM- 199 + FBS (10%) + PBS + BSA (1%) in 1:1 ratio. Oocytes were graded by morphological appearance of the cumulus cells investments and homogeneity of ooplasm under zoom stereomicroscope. Oocytes with more than four layers of compact cumulus

cells and with granular homogenous ooplasm were chosen for our studies.

In vitro *maturation of oocytes:* The oocytes were washed once with the aspiration medium and twice in the medium in which they would be cultured. Oocytes were cultured in 50 ml droplets of TCM-199 + FBS (10%) + FSH-P (10 mg/ml) in a 35-mm petri dish in a CO_2 incubator (38.5 °C, 5% CO_2 in air, 90–95% relative humidity) for 24 h. Maturation of oocytes was assessed on the basis of cumulus cell expansion and first polar body extrusion after 24 h of incubation as described earlier (Nandi *et al.* 2002). Oocytes with expanded cumulus cell mass to at least 2 diameters away from the zona pellucida and with extruded first polar body in the perivitte line space were considered as matured and used for IVF.

Viability of oocytes: Oocytes were checked for viability of oocytes (Gupta *et al*. 2002). Oocytes whose ooplasm get stained were considered as dead and unstained oocytes ooplasm were considered as alive.

Sperm preparation for IVF: Semen obtained from cauda epidydimus of ram testis was spinned with BO medium for 6 min at 300×g. The supernatant was discarded. The process was repeated again and pellet was dissolved in BO medium and sperm concentration was adjusted to 2 million concentrations per ml (2X 10^6 /ml) before inseminating the oocytes. The processed semen was kept in 5% CO_2 incubator at 38.5°C for 5–10 min for swim up.

IVF of oocytes: After IVM, the matured oocytes were washed twice with BO medium and then transferred to 50µl fertilization droplet; 10 µl of swim up sperm (2 ×10^6 /ml) was added and the dishes were incubated in CO_2 incubator for 3 h. The 20 µl of BO medium was removed from upper part of the droplet (containing unattached sperm and detached cumulus cells) and same quantity of TCM-199 supplemented with 10% FBS and gentamycin were added into sperm oocyte droplets. The dishes were again placed in a CO_2 incubator for 48 h.

Following 40–42 h after inseminating the oocytes, the presumptive zygotes were evaluated under stereo zoom microscope at 110 × magnification for evidence of cleavage. Results were recorded in terms of cleavage rate (percentage of oocytes inseminated and that were cleaved to 2 cell stage. The cleaved embryos were further cultured for 7 days for production of morulae /blastocysts.

Statistical analysis: Results were expressed as means ± SEM. A value of $P < 0.05$ was considered statistically significant. The maturation rates, fertilization rates and embryo yield were analysed by ANOVA followed by Tukey's multiple comparison tests (the percentage values were transformed to arcsine values before analysis). The statistical package of Graph Pad Prism, San Diego, USA was used for analyzing the data.

RESULTS AND DISCUSSION

The effect of different concentration of stearic acid on *in-vitro* maturation, viability, cleavage and morulae / blastocyst formation is presented in Table 1. Exposure of oocytes to 20 µM concentration of stearic acid in oocyte maturation medium significantly decreased maturation and cleavage rates compared to those observed in oocytes culture in media containing 0 µM (control group) or 10 µM concentration of stearic acid. Further increase of stearic acid concentration to 30 µM significantly decreased the maturation and cleavage rates. The viability rates were not significantly affected in oocytes cultured in media containing 0, 10 µM and 20 µM stearic acid concentrations. However the viability rate significantly decreased in oocyte culture in media containing 30µM stearic acid compared to lower concentration group. No significant changes in the morulae/ blastocyst yield were observed in oocytes cultured in 0 or 10 µM stearic acid. However, the morulae/ blastocyst yield was significantly decreased in oocytes cultured in 20 µM or 30 µM group (being significantly lowest in 30 µM group) compared to lower concentration groups.

Non-esterified fatty acids (NEFA) are the major component of triglycerides (the fat stores in the body), which consist of 3 fatty acids linked to a glycerol backbone. Hydrolysis of stored triglycerides (fat) in adipose tissue by hormone sensitive lipase liberates NEFA and glycerol. Lipolysis of triglycerides releases NEFAs (which are usually long-chain fatty acids) and glycerol. Glycerol is taken up by cells and used for glucose production or can be used to re-form triglycerides. NEFAs are water-insoluble and are transported bound to albumin.

The high circulating NEFA levels, associated with NEB, are indeed reflected in the follicular fluid of dominant follicles in ruminants early postpartum (Roth *et al*. 2001, Leroy *et al*. 2008). Furthermore, this knowledge was applied in an *in vitro* model, in which the oocyte maturation medium was supplemented with the predominant fatty acids at concentrations observed in follicular fluid (FF) of dairy cows during an episode of NEB. Results revealed that the saturated long chain fatty acids in particular (such as:

Table 1. Effect of stearic acid on *in-vitro* maturation, viability, cleavage rate and morulae/blastocyst formation of sheep oocytes

Concentration	No of oocyte	Maturation rate, %	Viability rate, %	Cleavage rate, %	Morulae/blastocyst yield, %
0 µM	111	84.6±1.9^a	94.1 ±1.9^a	55.1±3.9^a	24.6 ±2.1^a
10 µM	114	82.7±1.8^a	93.9±1.8^a	55.2±1.4^a	20.4 ±1.6^a
20 µM	120	70.6±1.2^b	88.1 ±4.1^a	40.4±1.7^b	11.6±1.0^b
30 µM	118	55.6±1.1^c	70.4 ±1.9^b	20.9±1.7^c	2.1±0.2^c

Superscripts bearing different letters in the same row differ significantly ($P< 0.05$).

palmitic and stearic acid) provoked an inhibition of maturation rate, leading to relatively low fertilization, cleavage and blastocyst formation rates. Furthermore, it has been shown that stearic acid and palmitic acid induce apoptotic changes in the cumulus cells (Roth *et al*. 2001, Leroy *et al*. 2008), which in turn influence oocyte maturation and probably also embryo development in a negative way. The metabolic imbalance of high NEFA concentrations during oocyte maturation increased the cryosensitivity of the resulting embryos. Increased cryosensitivity is due to increased fatty acid concentrations in the oocytes micro environment during maturation may lead to accumulation of these fatty acids in the oocyte that may alter their lipid content and composition (Kim *et al*. 2001, Leroy *et al.* 2005, Leroy *et al*. 2008). Exposing immune cells *in vitro* to NEFA at concentrations compatible with those observed in high producing postpartum ruminants (0.12 to 1 mM) has been shown to reduce function and viability. Increasing the concentration of NEFA in the culture media prevented the synthesis of interferon-ã and IgM by peripheral blood mononuclear cells (Carson, 2008).

In the present study it was hypothesized that possible toxic effects of NEFA on oocyte quality may be a partial explanation for the fertility decline. The stearic acid in the FF of the dominant follicle, were added in an IVM model at concentrations observed *in vivo*, to investigate their effect on the developmental capacity of the oocyte. NEFA concentrations in serum and in FF had also been described in heifers and lactating cows that were subjected to an acute dietary restriction (Comin *et al*. 2002, Jorritsma *et a*l. 2003). In the presence of high NEFA levels, a substantial portion of the NEFA could be partitioned to low density lipoproteins (LDL) (Chung *et al.* 1995). Especially, since the saturated fatty acids are bound on LDL, while the unsaturated ones are preferably bound on albumin (Chung *et al.* 1995). The fact that LDL was absent in FF (Brantmeier *et al*. 1987, Wehrman *et al*. 1991), might explain the observed differences early postpartum in the concentration and composition of NEFA in FF compared with serum in our study. Active transport, desaturating enzymes and selective uptake or metabolisation by intrafollicular cells (Yao *et al.* 1980) could be responsible for the observed differences in NEFA concentration and composition in the 2 compartments.

Oocytes having a considerably lesser cell number, augmented apoptotic cell index, unusual transcriptional activities, distorted amino acid turnover and tainted metabolism, with particular reference to glucose intolerance and reduced oxidative activity. All these are indicators of lower embryo quality and viability (Van Hoeck *et al.* 2011, 2013).Embryonic exposures to elevated NEFA concentrations not only reduce the embryo development but also alter the DNA methylation profile in embryos. Particularly genes associated with metabolism and cell fate are affected which may lead to an altered embryonic or fetal development or even postnatal health. The developmental stage during and/or the duration of the NEFA exposure could influence the number of genes that were differentially methylated (Desmet *et al*. 2014).

Conclusively, it can be stated that mimicking NEB associated NEFA concentrations in IVM models should be based on the intrafollicular rather than on the serum concentrations. Supplementation of the medium with elevated concentrations of SA resulted in a negative effect on the progression of meiosis. The 2 other studies which have investigated the effect of fatty acids on oocyte maturation differ from the others study in the fact that they added fetal calf serum and applied albumin bound fatty acids in supra-physiological concentrations (Homa and Brown 1992, Jorritsma *et al.* 2004). The reduced fertilization rate and hampered *in vitro* development are most likely carry-over effects of the delayed or blocked maturation. Parallel with the results of the present study, it has been shown earlier in other laboratory that PA and SA and not OA exert a toxic effect on bovine granulosa cell growth and function *in vitro* (Vanholder *et al.* 2005). Jorritsma *et al*. (2004), suggested that changes in membrane properties of the oocyte could be responsible for the observed negative effects of albumin bound OA in the IVM medium. Whatever the mechanisms, our results clearly indicated that exposure of oocytes to SA during 24 h has a deleterious effect on cumulus cell health and survival. Because a healthy cumulus investment is indispensable for correct oocyte maturation (Tanghe *et al*. 2002), the oocyte is most likely indirectly affected by these fatty acids. The findings of the present study support the hypothesis of Britt (1994) confirming that metabolic changes during a period of NEB (in case of high NEFA concentrations) may have detrimental effects on the developmental capacity of the oocyte. Elevated serum NEFA concentrations, arising from up-regulated lipolysis, have been implicated as a key factor in the association between metabolic imbalances, cellular dysfunction and related pathologies (Lu *et al*. 2003, Ulloth *et al*. 2003).

In this context, this study demonstrated, using an ovine model, that elevated stearic acid concentrations during oocyte maturation have a profound negative impact on embryo quality. The maturation, cleavage and morulae/ blastocyst production rates were impaired in media with 20 µM stearic acid. Increment of stearic acid to 30 µM in media further reduced the maturation, cleavage and morulae/ blastocyst production.

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