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Effect of Non-Esterified Fatty Acids on Oocyte and Granulosa Cell Growth in Vitro

S. Nandi[1], Shiv Kumar Tripathi[2], M. Farman[3], P.S.P. Gupta[4], S. Mondal[5]

National Institute of Animal Nutrition and Physiology (NIANP), Adugodi, Bangalore-560030

Abstract: **The occurrence of a negative energy balance (NEB) and concurrent metabolic changes may be responsible for impaired reproductive performance. A characteristic metabolic feature of NEB is the mobilization of body fat, reflected in an increased plasma non-esterified fatty acids (NEFA) concentration. High plasma concentration of NEFA is reflected in elevated NEFA follicular concentrations. The aim of the study was to examine the effects of different non-esterified fatty acids on oocyte and granulosa cell growth. Oocytes and granulosa cells were exposed to physiological and supra-physiological concentrations (in follicular fluid) of non-esterified fatty acids (Stearic acids:10, 20, 30 and 40μM or Palmitic acid: 20 40, 60 and 80μM or Oleic acid 40, 80, 120 and 160μM). The oocyte growth was measured in terms of the viability, maturation, cleavage, morulae/ blastocysts yield while granulosa cells growth was measured in terms of viability, cell number increment, cell metabolism, monolayer formation, support to oocyte growth and reversibility of functions after elimination of metabolic stressors (non-esterified fatty acids). We found that Oleic acid (40 and 80 μM) was beneficial to the oocyte and granulosa cell growth, Palmitic acids had no significant effect and stearic acid (30μM) was most inhibitory to oocyte and granulosa cell growth. The effect of different non-esterified fatty acids in combo levels on ovarian cell functions are in progress.**

1. INTRODUCTION

Oocyte quality, built upon a total maturation time in the ovary of around 3 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 [1]. The early embryo losses might result from a malfunctioned cytoplasm that impairs further development of the fertilized oocyte. These metabolites can adversely affect uterine function and indirectly cause early embryonic death. It is now widely accepted that negative energy balance (NEB) in early lactation is associated with reduced fertility performance [2].Follicles grown during the period of negative energy balance (NEB) affected by non-significant metabolic changes and responsible for developmentally incompetent oocyte [3]. High NEFA concentration is toxic for bovine [1] and human [4] granulosa cell growth and function in vitro along with these some cytotoxic effects were studied in pancreatic b-cells, Leydig cells and blood mononuclear cells. Hence, the present study was undertaken with the objectives to examine the consequences of different non-esterified fatty acid (NEFA) on oocytes and granulose cell developments.

2. MATERIALS AND METHODS

Ovaries were transported to the laboratory in 0.9% normal saline supplemented with gentamicin (50μg/ml) within one h of slaughter. The IVM was performed as follows. The aspirated and sliced fluid was transferred to a dish and the oocytes were searched under stereo zoom microscope (Olympus, Japan) at 110x magnification. All the oocytes were picked up by pipette and placed in 35 mm culture petridishes containing 4 ml of washing medium for grading. Only good quality COCs were used for further culture following selection under a stereomicroscope. After several washings in medium the COCs were cultured in groups of 30–40 for 24 h at 38.5 ºC in 500ml of maturation medium in a humidified 5% CO2 incubator. Oocytes viability was examined by Trypan blue staining. After IVM, the Degree 1 and Degree 2 oocytes were washed twice with fertilization medium and then transferred to 50μl fertilization droplet. Then added 10 μl of swim up sperm with 2x 10^6 /ml and the dishes were incubated in CO_2 incubator for 3 hrs. The 20μl 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 gentamicin were added in to sperm oocyte droplets. The dishes again placed in a CO_2 incubator for 48 hrs. Oleic acid (OA, cis C18: 1), Palmitic acid (PA, C16:0) and stearic acid (SA, C18:0), were dissolved in pure ethanol (Vel/Merck Eurolab, Zaventem, Belgium) and are used for the study. After aspirating follicular fluid, the light brown sheets of compact granulosa cells were picked up and suspended in TCM-199 supplemented with 0.3% BSA and centrifuged at 500 g for 5 min. Then washed for two times in washing medium, followed by the final pellet of granulosa cell suspension in the medium in which they were to be cultured. The GC ($1x10^5$/droplet), were cultured in a 100 μl droplet of culture medium supplemented with different concentration of

NEFA. The cells were cultured for 5 days. Media were refreshed once on day 2 of culture. The monolayer formation in GC was evaluated for 5 day and necrotic and apoptotic cells in granulosa cells were examined by HE staining.

The maturation rates, fertilization rates and embryos yield were analyzed by ANOVA followed by Tukey's multiple comparison test (the percentage values were transformed to arcsine values before analysis). Granulosa cell growth parameters were analyzed by unpaired't' test. The statistical package of Graph Pad Prism, San Diego, USA was used for analyzing the data.

3. RESULT

Maturation in the presence of OA had no significant effect on the oocyte developmental capacity in terms of cleavage or blastocyst yield.However, addition of SA resulted in a significantly lower cleavage rate and subsequent blastocyst yield. Similarly, there was a significantly decrement for a reduced cleavage rate and blastocyst yield relative to the number of cultured oocytes or to the number of cleaved zygotes after maturation in the presence of PA. The fertilization rate was significantly reduced for the oocyte mature in PA and SA ($P<0.05$). In granulosa cell growth parameters there was no any significant value was observed in different concentration of NEFA but in cell number increment there was significant decrement was observed in SA when compare with control. Furthermore with respect to monolayer formation there was significant decrement was observed in SA compare to control as well as with OA and PA. With respect to support to oocyte growth there was significant decrement was observed in SA compared with OA, PA and control. However there were no any significant values was observed in OA and PA compared with control and between OA and PA.

4. DISCUSSION

The studies described here were to examine the effects of NEFA on the oocyte growth which was measured in terms of the viability, maturation, cleavage, morulae/ blastocysts yield while granulosa cells growth was measured in terms of viability, cell number increment, cell metabolism, monolayer formation, support to oocyte growth and reversibility of functions after elimination of metabolic stressors (non-esterified fatty acids).The concentrations of NEFA chosen were based on concentrations in follicular fluid observed in a previous study in our laboratory.

Negative effect on ovarian activity and fertility of high-yielding dairy cows has been observed in case of NEB. *In vivo* NEFAs are mainly bound to albumin, dissolved unbound fatty acids were used [5] to avoid counteracting effects between albumin and the fatty acids. It has been shown that both forms of fatty acids are taken up by the cell [6]. The high circulating NEFA levels, associated with NEB, are indeed reflected in the follicular fluid of dominant follicles in ruminants early postpartum [7]. Furthermore, it has been shown that stearic acid and palmitic acid induce apoptotic changes in the cumulus cells [7], which in turn influence oocyte maturation and probably also embryo development in a negative way. Medium with elevated concentrations of PA or SA showed a negative effect on the progression of meiosis. The subsequent fertilization and cleavage rates and blastocyst formation were significantly reduced. OA had no effect on any on the outcome of the variables, which confirms that maturation, and fertilization preceded normally [8] which in agreement with our findings. The reduced fertilization rate and hampered in vitro development are most likely carry-over effects of the delayed or blocked maturation. PA and SA had a negative effect on rate of Blastocyst formation relative. In our finding PA and SA and not OA exert a toxic effect on bovine oocyte maturation cleavage and blastocyst yield granulosa cell growth and function in vitro which is in agreement with the finding of Van holder et al. 2005 [1] The possible mechanism behind this may be due to the induction of apoptosis by PA and SA, probably through by down-regulation of the apoptosis inhibitor Bcl-2 and the up-regulation of an apoptosis mediator such as Bax. However our results clearly indicate that exposure of COC to PA or SA during 24 h has non–significant effect on cumulus and granulosa cell health and survival because a healthy cumulus solely responsible for correct oocyte maturation [9].

5. CONCLUSION

In vitro oocyte maturation in the presence of various concentrations of PA and SA is hampered, leading to reduced fertilization rate and developmental competence. The data of the present study suggest that toxic effects of elevated NEFA concentrations on oocyte quality may be one of the factors through which NEB exerts its negative effects on fertility in animals.

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