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Assessing the Quality of Bovine Embryos Derived from Metabolically Stressed Oocyte during Maturation using TUNEL

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Authors' Contribution
MAMMS, SAA and KAA designed the study, analysed and interpreted the data and drafted the MS. MAMMS *in vitro* embryo production and TUNEL staining.

Key words
Bovine embryo quality, Metabolic stress, NEFA, Oocyte maturation, Apoptosis, TUNEL

ABSTRACT

The current research aimed to assess apoptotic cell ratio (ACR) via TUNEL staining in bovine embryos produced in vitro from oocytes matured in stressful conditions of elevated concentrations of non-esterified fatty acids (NEFAs). The maturation conditions in the current study were resemble the situation in the oocyte microenvironment of metabolically stressed lactating dairy cows during the post-partum period where the negative energy balance occurred. Oocytes were in vitro matured under elevated levels of NEFAs for 24 h in serum-free maturation media. Obtained zygotes were cultured in synthetic oviduct fluid with 5% FCS for eight days. Blastocyst stages from each treatment group were assessed and evaluated for their quality by determining the ACR by means of TUNEL staining. The presence of palmitic or stearic acid at high concentrations during oocyte maturation increased ACR, whereas oleic acid had no significant effects. The results of the present study concluded that compromised oocyte microenvironment by metabolic stressor such as high NEFAs concentrations which is the situation during the negative energy balance that are happened post-partum in dairy cows could decrease the quality of preimplantation embryos as indicated by the incidence of apoptosis.

INTRODUCTION

Metabolic stressors during the post-partum period in high yielding dairy cows are generated from the negative energy balance and it consequences that are occurred during this period. These metabolic stressors are vast and include a lot of biochemical and metabolic adaptation in blood and follicular fluid (FF). These metabolic stressors may include elevated concentrations of non-esterified fatty acids (NEFA) in growing oocyte microenvironment (i.e. follicular fluid) (Shehab-El-Deen *et al*., 2010). In an in vitro model when NEFAs, such as palmitic and stearic acids, added to oocyte maturation medium with similar concentrations associated with negative energy balance resulted embryo quality have been declined (Shehab-El-Deen *et al*., 2009). In the tropical and subtropical regions, high ambient temperature during summer is the main reason for postpartum dairy cattle subfertility (Ealy *et al*., 1993; Shehab-El-Deen *et al*., 2010). Summer heat stress worsen the situation of negative energy balance and its associated elevated NEFAs (Shehab-El-Deen *et al*., 2010). At early stage of lactation, high lactating cows are more susceptible to high ambient temperatures, because their accelerated metabolic heat production (Blackshaw and Blackshaw, 1994). Accordingly, if the growing oocyte is being subjected to stress conditions during its development processes, its quality will be negatively affected and subsequently embryonic development (Butler, 2003). Some of these stress conditions include biochemical changes in oocyte microenvironment associated with negative energy balance postpartum (Leroy *et al*., 2004; Shehab-El-Deen *et al*., 2010). The stress conditions that present in oocyte microenvironment have late effects on pregnancy, parturition and post-natal development (Greve and Callesen, 2005). The biochemical adaptations during the beginning of lactation in high lactating dairy cows can become morbid and therefore may interfere with cow fertility (Butler and Smith, 1989; Leroy *et al*., 2004; Shehab-El-Deen *et al*., 2010; Rodney *et al*., 2018). Altered

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levels of non-esterified fatty acids (NEFAs) in FF have already linked with changes in oocyte quality (Leroy *et al.* 2004; Shehab-El-Deen *et al*., 2010). High concentrations of NEFAs during the negative energy balance period (NEB) in high lactating dairy cows have already linked with extensively studied reduced fertility (Robinson 1999; Rukkwamsuk *et al*., 1999; Vanholder *et al*., 2005; Shehab-El-Deen *et al*., 2010). This assumption has been proved by addition of NEFAs, such as oleic acid (OA), palmitic acid (PA) and stearic acid (SA) at concentrations, which prevail in FF during NEB to oocyte maturation conditions of embryo production protocol *in vitro* in bovine. Inclusion of saturated fatty acids to bovine oocyte maturation medium had obvious deleterious effects on oocyte quality and developmental competence, whereas the monounsaturated OA had no effect (Leroy *et al*., 2005; Shehab-El-Deen *et al*., 2010). Assessing the competence of bovine embryos obtained from oocyte subjected to stress conditions has become crucial in embryo transfer program that are used to bypass the negative effects of heat stress in dairy cows and research purposes (Vandaele *et al*., 2007). The terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique is used to assess bovine preimplantation embryo quality by detecting apoptosis as apoptotic cell ratio (ACR) and hence DNA fragmentation (Spanos *et al*., 2000; Paula-Lopes and Hansen, 2002).

The current study aimed to assess the quality of bovine preimplantation embryos obtained from oocyte exposed to high NEFAs concentrations during in vitro maturation by means of TUNEL staining to determine the incidence of apoptosis and hence embryo quality.

MATERIALS AND METHODS

In vitro *production of embryos*

Bovine embryos were produced in vitro as described earlier (Shehab-El-Deen *et al*., 2009). Briefly, the ovaries of slaughtered bovine were collected and processed within 2 h from slaughtering. The ovaries, after removing of mesovaria, were rinsed many times in warm physiological saline containing kanamycin (25mg/m). only follicles of diamtere ranged between 4 and 8 were aspirated

In vitro *maturation*

Good oocyte based on appearance and cumulus cells layers were cultured in serum free maturation conditions in groups 60 oocyte per well in 500 µL modified bicarbonate buffered TCM199 with Earle's and glutamine and supplemented with murine epidermal growth factor (EGF) (20 ng/mL) for 22 h at 38.5°C in 5% CO_2 in air.

In vitro *fertilization*

Oocytes were washed and transferred to in vitro fertilization medium 500 µL IVF-TALP (60 oocytes/well) consisting of bicarbonate buffered Tyrode solution, with BSA (6 mg/mL) and heparin (25 µg/mL). Frozen-thawed bovine semen from the same bull was separated over a Percoll-density gradient (45 and 90%, Pharmacia, Uppsala, Sweden) and washed. Sperm concentration was adjusted in IVF-TALP. Bovine spermatozoa were added to the well containing oocyte in IVF-TALP at a final concentration of 10^6 spermatozoa/mL. Inseminated oocytes were incubated for 20-24 h at 38.5°C in 5% CO_2 in air.

In vitro *culture*

The probable zygotes were subjected to vortex to get rid of cumulus cells and spermatozoa. Fertilized oocytes were washed several times and moved on to SOFaa supplemented with 5% FCS and were cultured in 50 µL droplets of SOFaa in groups of 25 zygotes per well under mineral oil for 8 days under low oxygen conditions; 5% O_2, 5% CO_2 and 90% N_2.

Addition of non-esterified fatty acids (NEFAs) to maturation medium

The studied non-esterified fatty acids; oleic acid (OA), palmitic acid (PA) or stearic acid (SA) were dissolved in absolute ethanol. The three fatty acids were tested in 3 replicates (1800 oocytes), in addition to negative and positive control groups. Control media consisted of normal maturation medium without NEFAs or ehtanol (negative control) or with absolute ethanol (positive control) (Leroy *et al*., 2005).

Detection of apoptosis

Bovine *in vitro* produced blastocysts at day eight after insemination from each treatment group were subjected to TUNEL staining (In Situ Cell Detection kit, Boehringer, Mannheim, Germany) for detection of apoptosis according to Gjørret *et al*. (2003). Breifly, blastocysts yielded in each group were washed in phosphate buffer saline (PBS) twice for 2 min at 37°C and then were fixed in 4% paraformaldehyde and kept until staining. Blastocysts were permeabilized in Triton X-100 (0.5% in PBS) for 1 h at room temperature in the dark. TUNEL positive and TUNEL negative blasotcysts were incubated for 1 h in the dark at 37°C in DNase (50 Units/mL in PBS). Durig DNase step, the blastocysts from other groups were kept in polyvinyl pyrrolidone (PVP) in PBS at room temperature. For TUNEL solutions, blastocysts from all treatment groups and TUNEL positive control were incubated in fluorescein dUTP and terminal deoxynucleotidyl transferase in the dark for one h (37°C). Meantime, the

TUNEL-negative control blastocysts were not subjected to transferase and was incubated in nucleotide mixture only. Thereafter, all blastocysts were incubated in RNase (50µg/ml in PBS) for 1 h in the dark at room temperature. Subsequently, all blasotcysts were subjected to 0.5 % propidium iodide (PI) for nuclei stainimg during one h at room temperature. Finally, blastocysts were washed quickly in PVP in PBS and mounted in 1, 4-diazabicyclo (2.2.2) octane (DABCO) droplet on slides with vaseline bridges. Blastomeres that are TUNEL Positive were read by fluorescence microscopy. Propidium iodide helped to indentify normal, condensed or fragmented nuclei and count the total cell number, while TUNEL-positive nuclei appeared yellowish-green, condensed or fragmented.

Apoptotic cell ratio was assessed as percentage of TUNEL-positive blastomeres either in inner cell mass or in trophectoderm.

Statistical analyses

The percentages of apoptotic cells in blastocyst from different treatment groups were analyzed using mixed model analyses of variance with group as fixed factor and replicate as random factor. The same model was used to evaluate the percentage of blastocyst at 8 dpi. For all analyses, differences were considered to be statistically significant at the 5% level. Statistical analyses were performed in SPSS version 14.00

RESULTS

No significant differences could be detected in the blastocyst yield or expanded blastocyst yield at 8 dpi (Table I). However, the addition of the three fatty acids to maturation medium significantly decreased total cell number and trophectoderm cell number of the resulting embryos (P<0.01) (Fig. 1). The same results were found in inner cell mass for oleic and palmitic acids but not for stearic acid comparing to negative control group. When apoptosis in expanded blastocysts was assessed (Fig. 2), it was found that stearic and palmitic acids during maturation increased ACR in inner cell mass in the resulting embryos (P<0.01). However, in trophectoderm, palmitic acid only increased the incidence of apoptosis (P<0.01). The same effect of palmitic acid was found in total cell number (P<0.01). Whereas the addition of ethanol during *in vitro* maturation did not affect ACR in resulting embryos in the current study.

DISCUSSION

Current study tests the effects of oocyte maturation conditions on subsequent embryo quality. It is well known that oocyte of low quality led to slow developing embryos and high incidence of apoptosis during preimplantation embryonic development (Vandaele *et al*., 2007). Non-esterified fatty acids have been widely reported to induce apoptosis in granulosa and cumulus cells of developing ovarian follicles. Furthermore, NEFA present in oocyte micro-environment or in maturation medium could negatively affect oocyte quality and developmental competence. Moreover, high levels of NEFA are determine for follicular cells as they adversely affect steroidogenesis and proliferation processes (Leroy *et al*., 2004; 2005; Vanholder *et al*., 2005; Shehab-El-Deen *et al*., 2010; Baddela *et al*., 2020). Additionally, NEFAs may also contribute to the problem of the polycystic ovaries as they might upregulate Sertoli cell marker-9, an androgenic transcription factor and downregulating its estrogenic counterpart in grnaulosa cells (Yenuganti and Vanselow, 2017). Accordingly, assessing the quality of bovine embryos resulted from oocytes subjected to high NEFAs levels during maturation becomes an important approach for determining fertility outcomes in high yielding dairy cows during negative energy balance- or heat stress-associated metabolic stress. The occurrence of apoptosis in in vitro produced bovine embryos can be detected at the time of embryonic genome activation (i.e. around the eight-cell stage) using TUNEL (Byrne *et al*., 1999; Matwee *et al*., 2000; Gjørret *et al*., 2003). It is well demonstrated that increased non-esterified fatty acids concentrations in oocyte micro-environment hinder oocyte quality and have consequences on presumptive embryos quality, as well. At morula and blastocyst stages, certain blasotmeres undergo apoptosis as a physiological mechanism to eliminate weak or

Table I. Blastocyst and expanded blastocyst yield (mean±SE) at day 8 post insemination of bovine oocyte matured in the presence of palmitic acid (C16:0), stearic acid (C18:0) or oleic acid (C18:1). Negative control was maturation medium without fatty acids or ethanol and positive control group was maturation medium with the addition of absolute ethanol.

Treatment group	No. cultured oocytes	% blastocyst	% Expanded blastocyst
Palmitic acid	318	28.15±4.34	17.02±2.58
Stearic acid	318	27.2±3.89	16.02±2.3
Oleic acid	333	32.73±3.9	18.15±2.4
Negative control	314	30.46±2.75	15.76±1.63
Positive control	300	29.89±2.62	16.46±1.55

Blastocyst and expanded blastocyst percentages were calculated from cultured oocytes.

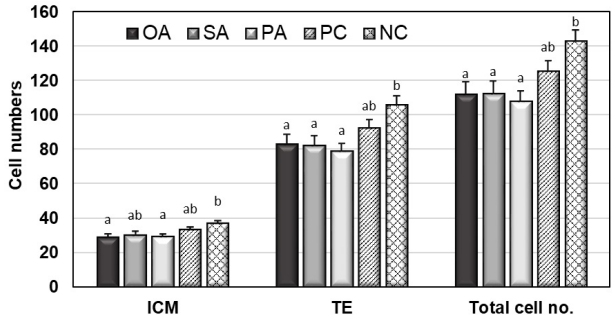

Fig. 1. Cell number (mean±SE) of inner cell mass (ICM), trophectoderm (TE) and total cell number in bovine embryos (8 dpi) derived from oocytes matured either in oleic acid (OA) (C18:1), stearic acid (SA) (C18:0) or palmitic acid (PA) (C16:0). Negative control (NC) was maturation medium and positive control group (PC) was maturation medium with the addition of pure ethanol. [a, b] Bars bearing different superscripts differ within each category; inner cell mass (ICM), trophectoderm (TE) or total blastocyst cell number *(P<0.01)*.

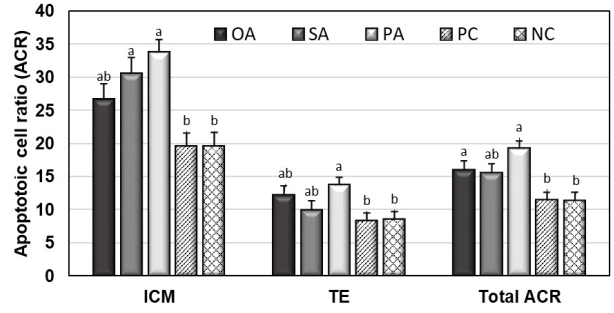

Fig. 2. Apoptotic cell ratio (ACR) (mean±SE) in inner cell mass (ICM) and trophectoderm (TE) of bovine embryos (8 dpi) derived from oocytes matured either in palmitic acid (PA) (C16:0), stearic acid (SA) (C18:0) or oleic acid (OA) (C18:1). Negative control (NC) was maturation medium and positive control group (PC) was maturation medium with the addition of pure ethanol. [a, b] Bars bearing different superscripts differ within each category; inner cell mass (ICM), trophectoderm (TE) or total blastocyst cell number *(P<0.01)*.

damaged cell to balance cell propagation and death, furthermore, programed cell death plays a critical role to sweep the balstomeres with altered genome from further development, which is crucial in *in vitro* produced blastocyst subjected to stressful conditions (Ramos-Ibeas *et al*., 2020). Accordingly, the incidence of apoptosis in embryos resulted from oocyte subjected to stressful condition seems to be an adaptive mechanism to ensure that unhealthy cells and early embryos do not progress in development, avoiding long-term detrimental effects.

Actually, both PA and SA caused a significant increase in ACR in inner cell mass compared to control groups either positive (ethanol) control group or negative control group. However, there was no significant difference among the three studied fatty acids in ACR either in ICM or in trophobalst. Increased ACR in ICM, may be due to qualitative and⁄or quantitative changes in the cytoplasmic lipids or the cell membrane. When intracellular lipids were removed from porcine embryos, they became more tolerance to cryogenic injury, which is indicating that these lipids may hinder embryo quality and development (Nagashima *et al*., 1995). Moreover, Abe *et al*. (2002) proved that lipid accumulated in embryos could impair their quality and cryotolerance (Abe *et al*., 2002).

Fatty acids in oocyte micro-environment are able to accumulate inside the oocytes and potentially altering oocyte lipid content and composition (Kim *et al*., 2001; Adamiak *et al*., 2005; Leroy *et al*., 2008). Kim *et al*. (2001) stated that PA, OA and SA are the three predominant fatty acids in bovine oocytes. Furthermore, in vitro maturation of bovine oocyte in the presence of serum altered triglyceride and total cholesterol contents, which is evident that lipids and fatty acids may be incorporated into the oocyte cytoplasm (Kim *et al*., 2001). Their findings suggest that both maturation conditions, oocyte morphology and cryopreservation, can affect fatty acid composition in bovine oocytes. Shehab-El-Deen *et al*. (2009) confirmed that exposure of bovine oocyte during maturation to stressful concentrations of NEFAs had a carry-over effects on embryo quality. However, it is still not known whether apoptosis is involved of the observed inferior embryo quality. More specifically, Palmitic acid may be an important negative element in this regard (Van Soom *et al*., 2001). It is well demonstrated that Fatty acids in culture medium can be up taken by the embryo to be used for the renewal of its membrane lipids (Pratt, 1980); accordingly, any changes in cell membrane during oocyte maturation could affect developing embryos. However, Matwee *et al*. (2000) concluded that as embryonic development proceed the bovine embryos develop resistance to apoptosis. Moreover, it has been previously shown that SA and PA induce apoptosis in the cumulus cells, which definitely affect oocyte maturation through gap junction and probably also embryo development may be affected in a negative way. Possible pathway by which fatty acids induce apoptosis is through affecting signal transduction, because they are implicated in several signal transduction pathways. Both stearic acid and linoleic acid have been implicated in stimulation of protein kinase C leading to apoptosis (Yu *et al*., 2001; Eitel *et al*., 2003). The results of the current study concluded that in vitro maturation of bovine oocytes in stressful levels of PA and SA can have

carry-over effects on embryo quality, leading to increased apoptosis in inner cell mass. However, PA only increased apoptosis in trophectoderm. However, more studies are still needed to elucidate the mechanism through which NEFA induced apoptosis.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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