Linoleic Acid Reduces Apoptosis via NF- κ B During the in Vitro Development of Porcine Embryos.

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Abstract

Background: Recent studies suggest that endogenous and exogenous free fatty acids play many important roles in mammalian oocyte and preimplantation embryo development. Among these fatty acids, linoleic acid has been reported to affect the apoptosis pathway via nuclear transcription factor- κ B. The transcription factor NF- κ B is a key modulator of apoptosis in a variety of cell types, but to date, this specific function of NF- κ B has not been demonstrated in porcine preimplantation embryos. To examine the effect of linoleic acid on parthenogenetic pig embryos produced *in vitro*, we treated these embryos with linoleic acid at various concentrations to examine the developmental rate, NF- κ B expression, IL-6 expression and apoptosis-related gene mRNA levels.

Results: Linoleic acid had a positive effect on embryo development and was not toxic at a certain concentration, but toxicity was observed at higher concentrations. Furthermore, it was confirmed that the concentration of NF- κ B increased, unlike that of IL-6, as the concentration of linoleic acid increased, and the concentration of NF- κ B was found to increase even at the concentration of linoleic acid at which embryo development decreased. We found that pro-apoptotic gene expression was downregulated in the linoleic acid-treated group. It was also found that *BCL-XL*, an anti-apoptotic gene, was not affected by linoleic acid, which appears to be an effect of IL-6. In contrast, *MCL-1*, an anti-apoptotic gene known to be unaffected by IL-6, was found to be increased at the mRNA level in linoleic acid-treated pig embryos. Furthermore, based on both apoptosis and immunocytochemistry staining, as the concentration of NF- κ B increased, the nuclear translocation of C-JUN, which is also related to apoptosis, gradually increased, which was dependent on the linoleic acid concentration. It was confirmed that NF- κ B is an important factor in the development of porcine embryos by confirming that treatment with a very low concentration of ammonium pyrrolidinedithiocarbamate (APDC, inhibitor of NF- κ B) affected NF- κ B protein expression, IL-6 protein expression and blastocyst production.

Conclusion: These data could suggest that porcine embryos can use exogenous linoleic acid as a metabolic energy source via NF- κ B. The data also demonstrate the important role of NF- κ B in porcine early embryo development.

Background

Evidence from recent studies suggests that individual fatty acids may affect normal oocyte maturation, fertility and embryo development [1-3]. We investigated the effects of oleic acids on embryo development and found that porcine embryos can use exogenous oleic acid as a metabolic energy source [3]. Khandoker et al. demonstrated that the content of oleic acid was the highest (21.90 to 36.24%) in both pigs and cows, followed by that of palmitic (18.61 to 31.90%) and stearic (10.34 to 20.39%) acids. Furthermore, three PUFAs, linoleic, linolenic and arachidonic acids, were detected in pig and cow reproductive fluid samples [4]. In addition, multiple authors' analyses of the fatty acid content of mammalian oocytes show that the greatest fraction of lipids in the majority of mammalian oocytes are saturated

fatty acids, which are composed of a simple acyl chain containing no carbon–carbon double bonds. Palmitic (16:0) and stearic (18:0) acids account for nearly half of these saturated fatty acids [5].

Monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) account for ~30% and ~15% of total fatty acids, respectively. The most abundant MUFA is consistently oleic acid (18:1 n-9), while linoleic acid (18:2 n-6) and arachidonic acid (20:4 n-6) primarily constitute the PUFA component [5, 6]. Interestingly, PUFAs account for more than double the amount total fatty acids in porcine oocytes compared with those in ruminants, predominantly containing linoleic acid [5]. Indeed, linoleic acid-mediated porcine embryo development has not been reported. Furthermore, linoleic acid activates nuclear factor-kappa B (NF- κ B) [7], which plays a key role in many cellular and developmental processes [8]. Only a few reports show the link between NF- κ B and gamete maturation and early embryonic development. In mouse embryos, NF- κ B activation is crucial to continue development beyond the 2-cell stage [9]. Furthermore, NF- κ B is a bifunctional nuclear transcription factor that has roles in inflammation, oxidative stress, and apoptosis [10]. The Rel/NF- κ B family of transcription factors has recently been demonstrated to play a crucial role in regulating apoptotic cell death by acting as inducers or inhibitors of apoptosis in a stimulus- and cell type-dependent manner [11]. The Rel/NF- κ B family of transcription factors comprises several related protein subunits, including RelA (p65), RelB, p50/p105, and p52/p100 [12]. Experiments in knockout mice addressing the functional role of NF- κ B proteins revealed that RelA^{-/-} embryos died on day 15 of gestation from substantial hepatocyte apoptosis [13]. Subsequent studies led to the conclusion that RelA acts in the embryonic liver as a protector against TNF α -induced physiological apoptosis [14-16]. The important effect of NF- κ B during embryonic development was determined based on the embryonic lethality of several knockout mice in which key components of the NF- κ B pathway were disrupted [9]. These lethal embryonic knockouts include the knockout of p65 [13]. Additionally, activated NF- κ B maintains not only the levels of anti-apoptotic proteins but also the levels of autocrine IL-6 [17]. IL-6 is a cytokine that targets a broad group of genes that plays many roles in cell signaling [18]. IL-6 and its receptor are both expressed and secreted in the endometrium of several species, including humans [19-23], and the IL-6 receptor expressed in preimplantation mouse embryos [24]. In some studies, the secretion of IL-6 by preimplantation embryos has also been observed [25, 26]. Furthermore, IL-6-supplemented mouse embryo culture media leads to an increase in blastocyst cell numbers and changes in signal transducer and activator of transcription 3 (STAT3) and anti-apoptotic gene expressions [27].

C-JUN N-terminal kinase (JNK) is a member of the mitogen-activated protein (MAP) kinase family and is stimulated by various extracellular stimuli via the MAP kinase cascade, which consists of JNK kinases (JNKK1/MKK4/SEK1 and JNKK2/MKK7) and several MAP kinase kinase kinases [28-30]. Activated JNK phosphorylates and activates C-JUN, a major component of the transcription factor AP-1, and other factors [28, 29]. It has been reported that the contribution of JNK activation to apoptosis depends on the type of cell and stimulation [28, 29]. Recently, studies have shown that negative regulation of TNF- α induced apoptosis [31]. Thus, the regulation of JNK activation by NF- κ B may play a critical role in embryo survival. In addition, continuous activation of JNK has been associated with apoptosis, while acute and transient activation of JNK has been reported to be involved in cell proliferation or survival pathways [32]. The activity of activator protein-1 (AP-1), a transcription factor consisting of homodimers and

heterodimers of members of the Jun family (C-JUN, JUNB, and JUND) and heterodimers of the Jun and FOS (C-FOS, FOSB, FRA1, and FRA2) families, is regulated by the activation of JNK [33]. It has also been suggested that the activation of NF- κ B is regulated by some upstream MAP kinases that also regulate JNK activation in cells [34]. Therefore, the aim of this study was to determine whether NF- κ B and IL-6 promote the growth of embryonic development and reduce apoptosis under more optimized culture conditions with linoleic acid. In addition, we confirmed the expression of C-JUN during the porcine blastocyst stage and determined whether JNK is expressed acutely or chronically in response to treatment with linoleic acid.

Methods

***In vitro* embryo production** Mature oocytes were obtained using the method described in our previous study [35]. Briefly, prepubertal gilt ovaries collected in Anseong, Kyunggi Province, Korea, were used in this experiment. The follicular fluid and cumulus-oocyte complexes (COCs) were aspirated using an 18-gauge needle and then pooled to obtain sediments. The sediments were washed with TL-HEPES-PVA medium, and the oocytes with compact cumulus cells and granulated cytoplasm were selected for *in vitro* maturation. The washed COCs were cultured in tissue culture medium (TCM-199; Life Technologies, Carlsbad, CA, USA) containing 10 ng/ml epidermal growth factor, 1 mg/ml insulin, and 10% porcine follicular fluid for 44 h at 39.8°C at 5% CO₂ and 100% humidity. The COCs were treated with a 4 IU/ml solution of the hormones Q6 equine chorionic gonadotropin and human chorionic gonadotropin (Intervet, Cambridge, UK) for the first 22 h. The COCs were then matured under hormone-free conditions. To generate parthenotes, the cumulus-free oocytes were activated with an electric pulse (1.0 kV/cm for 60 ms) in activation medium (280 mM mannitol, 0.01 mM CaCl₂, 0.05 mM MgCl₂) using a BTX Electro-cell Manipulator (BTX, CA, USA), followed by 4 h of incubation in porcine zygote medium-3 (PZM-3) medium containing 2 mmol/l 6-dimethylaminopurine. Subsequently, the zygotes were transferred in groups of 25–50 to wells with 500 μ l PZM-3 medium for 7 days with or without linoleic acid (L-9530) or APDC (P8765) from Sigma.

Immunocytochemistry

Embryos without zona pellucida at each stage were fixed in 4% paraformaldehyde for 30 min at 4°C. The fixed samples were permeabilized using 1% Triton X-100 for 5 min at room temperature and washed three times with phosphate-buffered saline (PBS). The embryos were blocked using 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature, and primary antibodies targeting NF- κ B p65 (SC-8008, mouse IgG; Santa Cruz), IL-6 (AHP-2391, rabbit IgG; Bio-Rad) and C-JUN (PA5-17890, rabbit IgG; Invitrogen) were added overnight at 4°C. The primary antibodies were diluted 1:500 in PBS containing 1% BSA. The embryos were washed three times with PBS containing 0.1% Tween-20 before incubation with the fluorescent-conjugated secondary antibodies anti-mouse IgG (green, 1:500, A11008; Invitrogen, Carlsbad, CA, USA) and anti-rabbit IgG (red, 1:500, A11012; Invitrogen), which were diluted in a blocking solution, for 2 h at room temperature. The samples were washed three times with PBS containing 0.1% Tween-20, and the nuclei were stained using 0.1% Hoechst 33342 (Molecular Probes, Eugene, OR, USA) for 10 min. After

washing three times with PBS, the samples were mounted on a slide glass. The stained samples were visualized under a microscope (Eclipse TE2000-U; Nikon), and the captured images were processed using a Nikon digital sight DS-L1.

Measurement of apoptosis

Terminal deoxynucleotidyltransferase (TdT)-mediated deoxy-uridine nick-end labeling (TUNEL) is the enzymatic addition of fluorescently labeled nucleotides to the free 3'-ends of DNA strands made available by DNA fragmentation that typically accompanies programmed cell death or apoptosis. To visualize apoptotic nuclei, the embryos were washed four times at room temperature in phosphate buffered saline (PBS) supplemented with 1 mg polyvinyl pyrrolidone (PVP) per milliliter PBS (PBS-PVP). The embryos were then fixed for 1 h at room temperature in 4% (w/v) paraformaldehyde in PBS (pH 7.4). After fixation, the embryos were washed an additional two times in PBS-PVP. The embryos were then permeabilized for 10 min at room temperature in a 0.5% (v/v) Triton X-100 solution in PBS. After permeabilization, the embryos were washed one time in PBS-PVP in preparation for the TUNEL procedure. The fixed and permeabilized embryos were then subjected to the TUNEL assay procedure using an in situ cell death detection kit (Roche; Mannheim, Germany) according to the manufacturer's instructions. Briefly, the embryos were incubated with terminal transferase enzyme and labeled nucleotide solution (mixed in a 1:10 ratio) in a humidified, sealed chamber in the dark at 37°C for 1 h. After completion of the TUNEL reaction procedure, the embryos were washed three times for 5 min in PBS-PVP and then transferred to a solution containing 10 mg/ml Hoechst 33342. Nuclear staining by 0.1% Hoechst 33342 was allowed to proceed for approximately 10 min at room temperature in the dark. The nuclei displaying distinct labeling and condensed or pyknotic morphology were considered to be TUNEL-positive. The average embryo cell numbers were determined by counting the number of nuclei stained with the fluorescent nuclear dye Hoechst 33342.

Quantitative RT-PCR

Pooled embryos, each stage of the *in vitro*-produced embryos (2–3-cell, n = 20; 4-cell, n = 20; 6–8-cell, n = 20; morula, n = 10; and BL, n = 5), and single blastocysts were processed with a Dynabeads® mRNA DIRECT™ Kit (Invitrogen) following the manufacturer's instructions. The zona pellucida was removed using Tyrodé's acid before mRNA extraction. cDNA was synthesized using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). The extracted cDNA samples were amplified using a DyNAmo HS SYBR Green qPCR Kit (Thermo Fisher Scientific, MA, USA) containing 1–2 pmol of each primer set listed in Table 1 in a 10-μl reaction volume. The amplification and detection were conducted using the ABI 7300 Real-Time PCR system (Applied Biosystems) under the following conditions: one cycle of 50°C for 2 min and 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension for 1 min (the annealing/extension temperatures were dependent on each primer set). The dissociation curves were analyzed, and the amplified products were loaded onto gels to confirm the specificity of the PCR products. The relative expression levels were calculated by normalizing the

threshold cycle (Ct) values of each gene to that of the reference gene GAPDH via the delta-delta Ct method.

Table 1: Primer sequences used for real-time PCR.

Genes	Sequences	Size
<i>RelA</i>	5'- CGGGGACTACGACCTGAATG -3'	158
	5'- CTTTCTGCACCTTGTCGCAC-3'	
<i>C-JUN</i>	5'- AGCAGAGCATGACCCTGAAC-3'	132
	5'- ACTGGATTATCAGGCGCTCG-3'	
<i>C-FOS</i>	5'- CAAGCGGAGACAGACCAACT-3'	105
	5'- GTGAGCTGCCAGGATGAACT-3'	
<i>BAK1</i>	5'- CAGCCGACAGCGGAAAAC-3'	109
	5'- GGTAGCCAAAGCCCAGAAGA-3'	
<i>TP53</i>	5'- CCCATCCTCACCATCATCAC-3'	80
	5'- GCACAAACACGCACCTCAA-3'	
<i>CASP3</i>	5'- GAACTCTAACTGGCAAACCCAA-3'	84
	5'- CACTGTCCGTCTCAATCCCA-3'	
<i>BCL-XL</i>	5'- ACTGTGCGTGGAGAGCGTAG-3'	87
	5'- AGGTGGTCATTCAAGTAAGTGG-3'	
<i>MCL1</i>	5'- GTTTGGCCTCCAGAGAAACG-3'	120
	5'- TTTCCCGTAGCCAAGAGACG-3'	
<i>IL-6</i>	5'- GCAGTCACAGAACGAGTGGA-3'	146
	5'- CTCAGGCTGAACTGCAGGAA-3'	
<i>ACTB</i>	5'-GTGGACATCAGGAAGGACCTCTA-3'	131
	5'- ATGATCTTGATCTTCATGGTGCT -3'	

Statistical analysis

The data obtained in this study were analyzed using the GraphPad Prism statistical program (GraphPad Software, San Diego, CA, USA). The data on the developmental rates were arcsine-transformed and then analyzed using analysis of variance and the Newman–Keuls multiple comparison test. The relative transcription levels in the embryos were analyzed using the unpaired Student's *t*-test. All the data are expressed as the means $\pm$ standard error of the mean. A probability of $P < 0.05$ was considered statistically significant.

Results

Effect of linoleic acid on embryo development and NF- κ B (p65) expression

To determine the effect of linoleic acid on porcine embryo development, embryos were cultured either with or without linoleic acid during the entire culture period. Linoleic acid had a positive effect on embryo development without toxicity at low concentrations (25 μ M) (Fig. 1A-B). At linoleic acid concentrations above 25 μ M, embryonic development and total cell numbers were similar to those of the control group. We investigated the effect of linoleic acid on NF- κ B and IL-6 protein expression. As the concentration of linoleic acid increased, the expression of NF- κ B also increased (Fig. 1C, D). To confirm the effect of linoleic acid on the porcine embryo expression of NF- κ B, we determined the mRNA level of RelA. The RelA mRNA expression level was significantly higher in the linoleic acid-treated group (Fig. 3A). These results indicate that linoleic acid increases NF- κ B signaling. The expression of IL-6 was not the same as that of NF- κ B (Fig. 1C, E). It was confirmed that the protein expression of IL-6 did not depend on the concentration of linoleic acid and remained constant (Fig. 2C, E). However, at the transcriptional level, the IL-6 mRNA level was increased by linoleic acid (Fig. 3A).

Reduction of apoptosis by linoleic acid

To observe the effect of linoleic acid on porcine embryo apoptosis, the embryos were treated with different concentrations of linoleic acid, and the blastocysts were examined under a fluorescence microscope. In the 25 μ M linoleic acid-treated groups, 5% apoptotic cells were observed based on staining with pink spots (Fig. 2A, B), while 15% apoptotic cells were detected in the control blastocysts. In addition, continuously increasing the linoleic acid concentration did not decrease the number of apoptotic cells (Fig. 2B). These results indicate that linoleic acid reduces apoptosis at low concentrations but not at high concentrations because of toxicity. Similar results could be obtained at the mRNA level (Fig. 3B). The expression of some apoptotic genes, known as pro-apoptotic genes (BAK1, TP53, and CASPASE3), decreased at certain low linoleic acid concentrations (25 μ M) (Fig. 3B). To identify which of the anti-apoptotic genes are involved in porcine embryo cell survival, we examined two anti-apoptotic genes. The results showed that the MCL-1 gene was significantly upregulated with IL-6 in the in porcine blastocysts in the linoleic acid-treated group (Fig. 3B). In particular, it was found that BCL-XL was not upregulated by linoleic acid. BCL-XL is known to be a gene that is influenced by the expression of IL-6, and a similar pattern was observed in this experiment. In the case of MCL-1, which was not affected by IL-6, it was confirmed that its mRNA level was increased due to treatment with linoleic acid. For this reason, it was

found that the anti-apoptotic gene related to the decreased apoptosis in linoleic acid-treated embryos is MCL-1, not BCL-XL (Fig. 3B).

Effect of linoleic acid on C-JUN nuclear translocation

We investigated the ability of exogenous linoleic acid to activate *C-JUN* in porcine embryos. Exogenous linoleic acid increased C-JUN phosphorylation (p-C-JUN) and transcription at a certain low concentration (Fig. 4A, B). The transcription levels of *C-JUN* and *C-FOS* indicated that *AP-1* activation was increased by exogenous linoleic acid (Fig. 4C). However, it was confirmed that the mRNA expression of JNK, known as the regulator of C-JUN, decreased in the linoleic acid-treated group (Fig. 3A).

Correlation between C-JUN and apoptotic cells

To investigate if there was a correlation between C-JUN activity and cell viability, apoptosis and C-JUN activation were determined in blastocysts after treatment with linoleic acid (Fig. 5A). A scatter graph showing a significant negative correlation between C-JUN phosphorylation and blastocyst apoptosis ($R^2 = -0.781$, $y = 60e-0.046x$, Fig. 5B).

Inhibition of NF-κB by APDC

To determine whether NF-κB plays a role in porcine embryo development, the NF-κB expression levels and developmental rate were examined after the inhibition of NF-κB. There was no significant effect on cleavage development at low concentrations of APDC. However, the inhibition of NF-κB with a low concentration of APDC decreased NF-κB expression levels and blastocyst formation rates (Fig. 6A-C). Thus, NF-κB appears to be a critical factor in porcine embryo development. Furthermore, the addition of the NF-κB inhibitor reduced IL-6 expression (Fig. 6D-F), suggesting the involvement of NF-κB in IL-6 regulation. However, IL-6 was observed to increase at a high concentration (0.2 μM) of APDC, which led to inhibition of embryo development (Fig. 6A).

Discussion

Exogenous fatty acids play many roles in developing mammalian oocytes and early embryos. PUFAs constitute a major portion of the fatty acid content of follicular and uterine fluid. Among these PUFAs, linoleic acid is the most abundant fatty acid, constituting approximately 30% of the total fatty acids [5]. Evidence from studies in other species suggests that individual fatty acids may affect normal oocyte maturation, fertility and embryo development [1, 36]. In addition, cows and mice have an inhibitory effect on oocyte maturation *in vitro* [37-39]. Conversely, linoleic acids stimulate oocyte development, but higher levels of these FAs may also inhibit the negative effects of saturated FAs [40]. Recently, the effect of various concentrations of nonesterified fatty acids (NEFAs) on *in vitro* maturation and *in vitro* embryo culture procedures in cattle was investigated. However, data about the dependence of porcine embryos on linoleic acid are rather scarce. In this study, we demonstrated that blastocyst development was promoted by the addition of linoleic acid to the culture medium (PZM-3). The results also demonstrated that linoleic

acid, which is found predominantly in pig oviducts and uterine fluids, has a considerably beneficial effect on pig embryos. This finding is consistent with the results of a previous study [5].

The importance of NF- κ B during embryonic development is highlighted by the embryonic lethality of several knockout mice in which key components of the NF- κ B pathway are disrupted. These lethal embryonic knockouts include the knockouts of p65 [13], I κ Ba [41, 42], IKKa [43, 44], and IKKb [15]. All these mutant mice show developmental defects, dying at early stages of embryonic development. These data show that NF- κ B is an important cellular survival factor, and this is probably because NF- κ B regulates many different anti-apoptotic factors, such as inhibitors of apoptosis in the cellular organism, caspase inhibitors, and Bcl-2 family members (Bcl-2, Bfl-1, and Bcl-xL) [45]. Despite this importance, there are only a few reports that link NF- κ B to gamete maturation and early embryo development. In our study, which was performed in porcine embryos, we have shown for the first time that NF- κ B is a gene that is involved in maintaining embryo development and reducing apoptosis and that NF- κ B and C-JUN nuclear translocation occur together in the early embryo. C-JUN is related to NF- κ B and is also associated with apoptosis. C-JUN is a transcription factor with a protective role in the cellular response to DNA damage [46]. C-JUN-deficient cells undergo premature senescence due to spontaneous DNA damage [47], suggesting a role for C-JUN in DNA repair [48]. While continuous activation of JNK is associated with apoptosis, acute and transient activation of JNK has been reported to be involved in cell proliferation or survival pathways, so we confirmed the expression of JNK according to the differences in apoptosis in porcine embryos. In the blastocyst stage, both the nuclear translocation and mRNA level of C-JUN were increased, but the mRNA of JNK was decreased compared with the control group (Fig. 4). This finding suggests that JNK expression in the linoleic acid-treated group became acute and transient at the cleavage stage compared to that in the control group, and apoptosis may have been affected. It was also confirmed that an increase in AP-1 in the linoleic acid-treated group, which is known to affect embryo apoptosis [49], was observed with the increased expression of C-JUN and C-FOS.

NF- κ B can regulate the transcription of many genes, including cytokines, chemokines, adhesion molecules, and anti-apoptotic genes. Among these anti-apoptotic genes, interleukin-6 (IL-6) is known as one of the autocrine cytokines transcribed beginning in the blastocyst stage and plays an important role in proliferation and implantation [15]. It has previously been reported that the addition of 10 or 100 ng/mL recombinant IL-6 to the culture medium did not affect the development of 2-cell stage embryos into blastocysts [27]. However, the total cell number was significantly increased and apoptosis was reduced in blastocyst-stage embryos cultured in the presence of 100 ng/mL recombinant IL-6 [27]. There is considerable evidence to show that dietary fat can modulate different immune functions [50-52]. These data were supported by several reports that demonstrate specific *in vitro* and *in vivo* effects of saturated and unsaturated fatty acids on lymphocyte function, although the effects of specific forms of dietary fat on the immune system remain controversial [52-55]. Furthermore, the enhanced expression of IL-6 was also demonstrated after exposure to long-chain fatty acids in intestinal epithelial cells [56]. However, in pig embryos, the increase in IL-6 by linoleic acid in pig embryos could not be confirmed. (Fig. 1C, E), and it was confirmed that IL-6 decreased in the embryos in which NF- κ B was inhibited (Fig. 6C, D). Interestingly, IL-6 tends to increase at a high concentration of APDC, which adversely affects embryonic development

(Fig. 6). These results show that the abnormal expression of NF-κB may cause the aberrant expression of inflammation-related genes. Alternatively, it may be an immunological defense mechanism against embryo lethality, but more studies are needed to confirm this idea. Furthermore, IL-6 showed different patterns of mRNA and protein expression. This difference may have occurred because only the protein expression levels were measured or it could be possible that as shown in individual genetic studies, mRNA and protein expression are sometimes inconsistent [57, 58].

Conclusion

Changes in the microenvironment embryo culture conditions due to exogenous linoleic acids have a variety of effects on embryo development. These changes have a positive or negative effect on embryo development, depending on the timing and concentration of FFA treatment. Preimplantation porcine embryos require specific fatty acids with accurate concentrations at different stages of development. Collectively, our data suggest that linoleic acid modulates a regulatory pathway that reduces apoptosis in porcine embryos, which could serve as a model for the study of fertility in other species.

Abbreviations

AP-1	Activator protein-1
APDC	Ammonium pyrrolidinedithiocarbamate
BSA	Bovine serum albumin
COCs	Cumulus-oocyte complexes
IL	Interleukin
JNK	C-JUN N-terminal kinase
MAP	Mitogen-activated protein
MUFA	Monounsaturated fatty acid
NF-κB	Nuclear factor kappa-B
PBS	Phosphate-buffered saline
PUFA	Polyunsaturated fatty acid
PVP	polyvinyl pyrrolidone
PZM-3	Porcine zygote medium-3
STAT	Signal transducer and activator of transcription
TUNEL	Terminal deoxynucleotidyltransferase mediated deoxy-uridine nick-end labeling

Declarations

Author`s contribution

DK Lee and CK Lee designed the experiment. DK Lee and KH Choi verified the analytical methods. DK Lee wrote the manuscript with support from KH Choi. DK Lee and M Lee produced embryos for the experiment. DK Lee, JN Oh, SH Kim, M Lee, J Jeong and GC Choe produced matured oocytes. All authors discussed the results and contributed to the final manuscript.

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Availability of data and materials

The data analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The experimental use of pigs was approved by the Institutional Animal Care and Use Committee, Seoul National University (SNU-140328-2).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Not applicable

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Figures

Figure 1.

Figure 1

The effect of linoleic acid during the early development of porcine parthenogenetic embryos. The percentage of the parthenogenetic embryo blastocyst rate was counted at day 7 of culture of embryos simultaneously exposed to linoleic acid for 7 days. The dose-dependent effect of linoleic acid on the blastocyst formation rate (A) and the total blastocyst cell number (B). Immunocytochemistry for NF- κ B and IL-6 expression in blastocyst stages of in vitro-produced porcine embryos (C). The mean fluorescence intensity of NF- κ B with different doses of linoleic acid (D). The mean fluorescence intensity of IL-6 with different doses of linoleic acid (E). The 25 μ M linoleic acid group showed an increase in the embryo development rate without toxicity (A and B), but toxicity was confirmed at higher (50–100 μ M) concentrations (A). Each group includes six replicates. The different superscript letters indicate significant differences between the control (0 μ M) and linoleic acid-treated groups. ($P < 0.05$) scale bar: 100 μ m.

Figure 2.

Figure 2

Reduction of apoptosis by linoleic acid in in vitro produced blastocysts. To verify the effects of linoleic acid on apoptosis, cultured parthenotes were separated into the untreated (0 μM, control) and linoleic acid-treated groups (25 μM, 50 μM and 100 μM). The blastocyst apoptosis rate was measured through an in situ cell death terminal deoxynucleotidyltransferase (TdT)-mediated deoxyuridine nick-end labeling (TUNEL, red) assay. Blastocysts cultured for 7 days were stained by nuclear staining (blue). Arrows (A) indicate the simultaneous expression of blue and red. The rate of apoptosis in blastocyst cells was significantly decreased in the treated groups (25 μM) compared to that in the control group (B). The different superscript letters indicate significant differences between the control (CON), linoleic acid-treated (25 μM, 50 μM, 100 μM), ($P < 0.05$), Scale bar: 100 μm. Each group includes three replicates.

Figure 3.

Figure 3

Effects of linoleic acids on the expression of apoptosis-associated genes at the mRNA level. The transcription levels of apoptotic-related genes compared between the control- and linoleic acid-treated blastocyst groups. Each group of blastocysts was harvested at day 7 after parthenogenesis. Each sample includes three replicates. An asterisk indicates statistical significance ($p<0.05$).

Figure 4.

Figure 4

Linoleic acid increases the nuclear translocation of C-JUN. Immunocytochemistry for c-Jun nuclear translocation (Red) in the blastocyst stages of in vitro-produced porcine embryos. The nuclei of embryos were counterstained with Hoechst 33342 (blue) (A). The nuclear translocation rate of c-Jun with different doses of linoleic acid (B). The transcription levels of C-JUN-related genes compared between control- and linoleic acid-treated blastocyst groups (C). An asterisk indicates statistical significance ($p<0.05$). The different superscript letters indicate significant differences between the control- (Con) and linoleic acid-treated (LA) groups ($p<0.05$). Scale bar = 100 μ m.

Figure 5.

Figure 5

Translocation of c-Jun protects the early embryo from apoptosis. Immunocytochemistry for c-Jun nuclear translocation (green) in the blastocyst stages of in vitro-produced porcine embryos. The apoptosis of blastocysts was determined by the TUNEL assay (red). Simultaneous blue and red staining is shown in orange (A). Behavioral correlation between C-JUN nuclear translocation and apoptosis (B, n=4). Scale bar=100 μ m

Figure 6.

Figure 6

NF- κ B inhibition by APDC The dose-dependent effect of APDC (0.1-0.3 μ M) on cleavage (A) and blastocyst formation rate (B). The protein levels of NF- κ B in each group (control and APDC 0.1 μ M) (C) and the mean fluorescence intensity of NF- κ B (D). The protein levels of IL-6 in each group (E) and the mean fluorescence intensity of NF- κ B (F). Each group of blastocysts was harvested at day seven after parthenogenesis. Each sample includes four replicates. The different superscript letters indicate significant differences between the control and APDC groups ($P < 0.05$). Scale bar: 100 μ m.

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