Original Article

Effect of nicotine on gene expression and developmental competence of bovine oocytes

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Abstract

Nicotine affected meiosis during oocyte maturation is associated with poor subsequent embryonic development. The aim of this study was to analyze the effects of nicotine exposure during in vitro maturation (IVM) on the outcomes of in vitro fertilization (IVF) and the expression of gene related development of oocytes and embryos. Immature (germinal vesicle, GV) oocytes were randomly cultured in IVM media containing nicotine at concentrations of 0.0 (control) 2.5, 5.0, and 10.0 mM. After culturing for 24 h, oocytes were assessed for their maturation rate, subjected to IVF and mRNA extraction for gene expression analysis using quantitative real time PCR (qRT-PCR). The maturation rates of oocytes exposed to nicotine at 5.0 (62%) and 10.0 mM (54%) was significantly ($P<0.05$) lower than oocytes exposed to nicotine at 2.5 mM (87%) and the control (95%). Following IVF, oocytes exposed to nicotine at 0, 2.5, 5 and 10 mM resulted in similar cleavage rates (96, 91, 91 and 89%, respectively). However, blastocyst rates developed from oocytes treated with nicotine were lower than the control (11, 14, 12 and 24%, respectively). The expressions of GDF9 and ZAR1 in oocytes treated with nicotine at 5 mM were upregulated significantly ($P<0.05$) than the control. The expression of BCL2 and DMNT in oocytes treated with nicotine at 2.5 and 5.0 mM was upregulated significantly ($P<0.05$) than the control. The expression of BAX in all nicotine treated groups was comparatively higher than the control but not statistically different ($P>0.05$). The present study indicated that nicotine negatively affected oocyte and blastocyst development in a dose dependent manner and altered the expression levels of genes associated with the development of oocytes and embryos.

Keywords: nicotine, gene expression, in vitro maturation, bovine, oocytes

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Introduction

Cigarette smoke is well accepted to contain more than 4,000 chemical compounds, including 43 carcinogens, more than 300 polycyclic aromatic hydrocarbons and the addictive chemical nicotine and its metabolites (Zenzes, 2000). Nicotine, an organic compound found in tobacco leaves is highly addictive, extremely toxic and known as a carcinogen. The detrimental effects of cigarette smoke on female fertility has been reported including on oocyte maturation, fertilization, and embryo development (Sharara et al., 1998; Zenzes, 2000). Meta-analysis has reported a 60% increase in the risk of infertility among cigarette smokers (Augood et al., 1998). A decrease of IVF outcomes has been reported including a response to hormonal stimulation, rate of fertilization and subsequent embryonic development and pregnancy rate in cigarette smokers as compared to non-smokers (Crha et al., 2001). Several studies have demonstrated that female smoking is associated with a decrease of oocytes numbers (Harrison et al., 1990; Barbieri et al., 2005; Lambert-Messerlian and Harlow, 2006), decreased rates of fertilization (Klonoff-Cohen et al., 2001; Neal et al., 2005; Hassa et al., 2007; Gruber et al., 2008) and pregnancy (Klonoff-Cohen et al., 2001; Neal et al., 2005) and increased miscarriage rates (Pattinson et al., 1991; Kinney et al., 2007). In humans, maternal smoking affects oocyte meiosis (Klonoff-Cohen et al., 2001), increases the zona pellucida thickness (Zenzes et al., 1995) and thereby decreases rates of fertilization and pregnancy (Shiloh et al., 2004). Previous studies in rodents have shown that nicotine interfered with oocyte meiosis and chromosome disjunction (Racowsky et al., 1989; Mailhes et al., 1999) and is associated with a shrink size and poor quality of oocytes (Mai et al., 2014). Moreover, chronic nicotine treatment has adversely affected the ultrastructure of oocytes (Rajikin et al., 2009) and has increased apoptosis in mouse oocytes (Asadi et al., 2012). Other studies in bovine oocytes have indicated that nicotine affects microtubule and microfilament poly-assembly, resulting in significantly decreased maturation of oocytes and early embryo development (Liu et al., 2007; 2008[a,b]). However, no data is available regarding the effect of nicotine on the expression of the genes associated with oocyte maturation and embryo development. GDF9 (growth/differentiation factor-9) was a growth factor derived from oocytes that prevents apoptosis of cumulus cells and associated with the oocyte development process (Hussein et al., 2006). Zygote arrest 1 (ZAR1) is an oocyte-specific maternal-effect gene that functions at the oocyte-to-embryo transition in many mammalian species including humans, pigs, cattle, sheep, mice and rats. BCL2 is an anti-apoptotic gene and inhibits the release of cytochrome c from mitochondria, consequently preventing apoptosis. BAX (apoptosis factor) is a member of the BCL2 gene family. DNMT, DNA methyltransferases is responsible for life-long maintenance of DNA methylation patterns during DNA replication (Tang et al., 2002). The aim of this study was to determine the effects of nicotine on developmental competence and the expression level of the gene related development of oocytes.

Materials and Methods

Chemicals: Unless otherwise noted, all chemicals used in this study were purchased from Sigma Chemical Co. (Sigma, St. Louis, MO, USA). Nicotine (N 3876, Sigma) solution was diluted to a 100 mM stock in phosphate buffered saline (PBS) and the pH adjusted to 7.4 before being added to the culture medium. All culture media were freshly prepared, filtered, and then kept in sterile bottles at 4°C.

Oocyte collection and in vitro maturation: Bovine ovaries were collected from a local slaughterhouse and cumulus oocyte complexes (COCs) were retrieved by aspirating the antral follicles (2-6 mm) using an 18-gauge needle containing TALP-HEPES. The COCs which had at least five layers of cumulus cells and homogenous cytoplasm were washed three times in oocytes maturation medium (TCM 199) supplemented with 10% FCS, 0.2 mM pyruvate, 0.2 mM HEPES and 5µg/ml FSH and submitted to IVM. They were cultured in 50-µl drops of maturation medium in a humidified atmosphere of 5% CO_2 at 39°C for 22-24 h.

Assessment of nuclear maturation: After IVM, COCs were denuded by exposure to 0.2% hyaluronidase for 5 min and the pipette gently pressed to remove cumulus cells. To analyze the stages of oocytes, the denuded oocytes were characterized as being in the metaphase II-arrest (MII) stage according to the presence of the first polar body within the perivitelline space under a stereomicroscope.

In vitro fertilization (IVF): Frozen bovine semen samples were thawed at 37°C for 30 sec. The motile spermatozoa were prepared in sperm-TALP medium using the swim-up method. After centrifugation, the sperm pellet was resuspended in Fert-TALP medium supplemented with 10 µg/ml heparin. Sperm cells at a final concentration of 1×10^6 (cells/mL) were added in 30 µl drops of Fert-TALP containing 10 matured oocytes. Sperm and oocytes were co-incubated for 18-20 h at 39 °C under 5% CO_2 in air.

In vitro culture (IVC): After fertilization for18-20 h, the presumptive zygotes were washed and partially denuded in TCM-HEPES medium using small pipettes. They were then transferred to synthetic oviductal fluid (SOF) supplemented with 4 mg/ml BSA and cultured at 39 °C under an atmosphere of 5% CO_2. The rates of cleavage and blastocyst development were assessed at days 2 and 7, respectively. The number of cells in the blastocyst was determined using Hoechst 33342 staining.

Quantitative real time polymerase chain reaction (qRT-PCR): Briefly, following IVM, fifty denuded oocytes with 1st polar body extruded (MII oocytes) from the control and experimental groups were collected to determine gene expression using qRT-PCR. RNA was preserved in TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and RNA was isolated using Direct-zol columns (Zymo Research, Irvine, CA, USA), following the manufacturer's instructions. RNA quantification

was determined by a Nanodrop 2000 ultraviolet-vis spectrophotometer (Thermo Fisher Scientific, Inc.). First-strand cDNA was prepared by RNA (40 µg/sample) using cDNA Synthesis Kit, (Biotechrabbit, Berlin, Germany) and was performed at 53°C for 60 min. To study gene expression, real-time qRT-PCR was performed with KAPA SYBR® Fast qPCR Kit (cat no. KK4600; KAPABIOSYSTEMS, Massachusetts USA), according to the manufacturer's instructions via CFX96 Real-Time PCR platform (Bio-Rad Laboratories, Inc.). The PCRs were performed in quantitate conditions as follows: 95°C for 3 min; followed by 40 cycles of 95°C for 3 sec (denaturation), 60°C for 30 sec (annealing temperature) and 72°C for 45 sec (elongation); and 72°C for 5 min. The primers used for gene expression analysis are shown in Table 1 (Chen et al., 2014; Zhao et al., 2016; 2018). As an internal control, β-actin were quantified in parallel with target genes. Normalization and fold changes were calculated using the ΔΔCt method. The level of each gene was analyzed using Bio-Rad CFX Manager version 3.1 (Bio-Rad Laboratories, Inc.) (Livak and Schmittgen, 2001).

Experimental design: In experiment 1, the developmental competence of oocytes cultured in maturation media containing various doses of nicotine was evaluated. A total of 400 COCs were selected and randomly incubated in IVM media containing 0.0 (control), 2.5, 5.0, and 10.0 mM. After culturing for 24 h, oocytes were subjected to IVF. Cleavage and blastocyst rates were determined at days 2 and 7 of the culture, respectively. Blastocysts were stained with Hoechst 33342 and the cell numbers of blastocysts was counted.

In experiment 2, bovine COCs were randomly allocated into the control and experimental groups which were treated with nicotine by the same method as described in the experiment 1. After culturing for 24 h, oocytes were denuded and subjected to gene expression analysis using qRT-PCR as described above.

Statistical analysis: All experiments were repeated three times. Statistical analysis was performed using PASW Statistics version 18 (IBM Corp., Armonk, NY, USA). The percentage data of oocyte maturation, embryo development and cell numbers of blastocysts were analyzed by one-way ANOVA. The relative expression levels of different genes were analyzed by one-way ANOVA followed by Duncan's test to analyze differences between groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Maturation and subsequent embryo development of oocytes treated with nicotine: The effects of various concentrations of nicotine added to IVM media on nuclear maturation of bovine oocytes is shown in Table 2. Nicotine at a concentration of 2.5 mM resulted in maturation rates similar to the control (87 and 95%, respectively). Nicotine at concentrations of 5.0 and 10.0 mM resulted in significantly ($P<0.05$) lower maturation rates (62 and 54%, respectively) than nicotine at concentrations of 2.5 mM and the control. Following IVF, oocytes matured in IVM media containing nicotine at all concentrations (2.5-10 mM resulted in similar cleavage rates compared to the control group (89, 91, 91 and 96%, respectively). Blastocyst developmental rates of oocytes treated with different concentrations of nicotine at 2.5,5.0 and 10 mM were 11, 14 and 12%, respectively, which was significantly ($P<0.05$) lower than the control (24%, Table 3). However, the cell of blastocysts was not statistically different between the nicotine treated and control groups.

Table 1 Primer sequences used for quantitative RT-PCR analysis.

Gene	Primer sequences (5'-3')	Product size (bp)
β-actin	F: GCGGCATTCACGAAACTA R: TAGAAGCATTTGCGGTGG	313
GDF9	F: CTCAGTGCCAAGACCATC R: AGATGCCACAGAATACGC	294
BAX	F: AGTGTCTGAAGCGCATCG R: GCCTTGAGCACCAGTTTG	190
ZAR1	F: AAGTGCCTATGTGTGGTGTG R: GTTTGCCTTTGCATCTCC	212
BCL2	F: TCAATTGTCGTGGCATCAA R: CCCCCGACACCTGTTAGCTT	73
DNMT	F: TGCAGAGTGGTAATGGCAGA R: TCAGGTTTTCGTTTGGCAGG	191

Table 2 Nuclear maturation of oocytes following culture in IVM media containing various doses of nicotine.

Nicotine (mM)	No. of oocytes	No. of MII oocytes (%)
0	100	95[a]
2.5	100	87[a]
5	100	62[b]
10	100	54[b]

Table 3 The in vitro development IVF embryos derived from oocytes cultured in IVM media containing various doses of nicotine.

Nicotine (mM)	No. of zygotes	2-cell n (%)	Blastocyst n (%)	Cell number of blastocysts (mean ± SD)
0.0	100	96	24[a]	123.1 ± 4.5
2.5	100	89	11[b]	102.0 ± 8.6
5.0	100	91	14[b]	119.5 ± 11.2
10.0	100	91	12[b]	116.6 ± 9.3

Gene expression in oocyte treated with nicotine: The results of quantitative real-time RT-PCR assessed the expression of GDF9, BAX, BCL2, DMNT and ZAR1 mRNA in oocytes treated with various doses of nicotine are shown in Figure 1. After normalization to β-actin expression, real-time quantitative PCR analysis indicated that the expression of GDF9 and ZAR1 in oocytes treated with nicotine at concentration of 5 mM was upregulated significantly (P<0.05) compared with the control. However, there was no significant difference in oocytes treated with various doses of nicotine (P>0.05). The expression of BCL2 and DMNT in oocytes treated with nicotine at concentrations of 2.5 and 5.0 mM was upregulated significantly (P<0.05) compared with the control. In addition, the expression of both genes in oocytes treated with nicotine at concentration of 5 mM was significantly (P<0.05) higher than those treated with nicotine at concentration of 2.5 mM. Although the expression of BAX in oocytes treated with nicotine at concentrations of 5.0 mM was comparatively higher than the control and other treatment groups, the difference was not statistically significant (P>0.05).

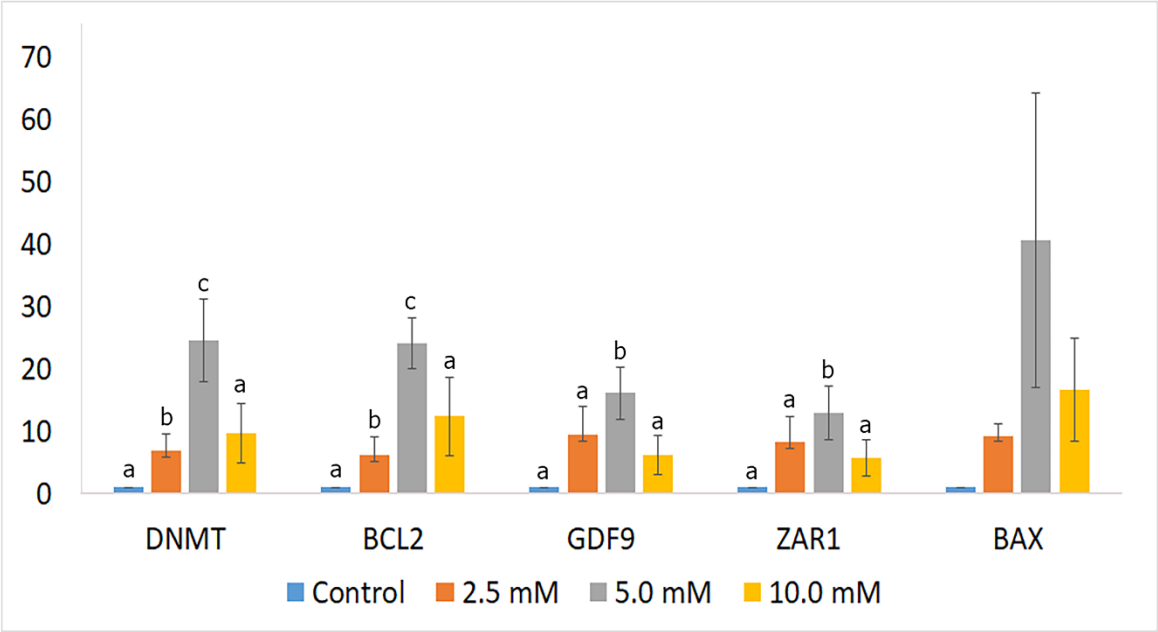

Figure 1 Relative expression levels of DNMT, BCL2, GDF9, ZAR1 and BAX in oocytes exposure to IVM containing nicotine at concentrations of 0 (control), 2.5, 5.0 and 10.0 mM. Different lowercase letters show a significant difference (P < 0.05).

Discussion

The effect of nicotine on oocytes maturation and embryo development has been reported in humans and other mammalian species. In humans, nicotine affected meiotic maturation of oocytes (Zenzes et al., 1995), decreases the number of mature oocytes (Zenzes et al., 1997) and decreases the rate of fertilization (Rosevear et al., 1992; El-Nemr et al., 1998; Gruber et al., 2008). In hamsters, oocytes cultured for 24 h in media containing nicotine at or below 0.5 mM did not negatively affect meiosis but at 5 mM there was impaired meiotic division that resulted in chromatin degeneration (Racowsky et al., 1989). Mice exposed to cigarette smoke extract (CSE) were associated with a shrunk size and poor morphology of oocytes (Mai et al., 2014).

The present study showed that bovine oocytes treated with nicotine at concentrations of 5.0 and 10 mM significantly reduced maturation rates compared with nicotine at 2.5 mM and the control. The cleavage rate of oocytes treated with nicotine at all concentrations (2.5-10 mM) was similar to the control. Blastocyst developmental rates in nicotine at concentrations 2.5, 5.0 and 10 mM were significantly (P<0.05) lower than the control but cell numbers of blastocysts were not different between treatment and control groups. It has been reported that nicotine affects microtubule and disfigures spindle structures during the first and second meiosis of bovine oocytes. Nicotine also affected the organization of microfilament and inhibits the movement of anaphase or telophase chromosomes to the cortical area (Liu et al., 2007; 2008a). In addition, nicotine treatment in mice causes apoptosis of oocytes (Asadi et al., 2012) and deleteriously effects the ultrastructure of oocytes including a loosening of the boundary and a tearing of the ZP, widened perivitelline space, abundant rough

endoplasmic reticulum (rER) with numerous vesicles and highly dense mitochondria with no cristae (Rajikin et al., 2009). Our results indicated that nicotine did not affect fertilization and cleavage rates but decreased the development of embryos to the blastocyst stage. It has been reported that bovine oocytes treated with nicotine significantly decreased cleavage rate and the development to blastocyst following parthenogenetic activation (Liu et al., 2007). Female mice exposed to cigarette smoke had decreased fertilization and cleavage rates following IVF (Mai et al., 2014). This data indicates the deleterious effect of nicotine on oocyte maturation and subsequent embryo development.

The expression of genes which relate to the development of oocytes and early embryos is important for further embryonic development. In the present study, we used β-actin as an internal control for the normalization of gene expression in bovine oocytes (Chen et al., 2014; Varshney et al., 2012). Our results demonstrated that GDF9 and ZAR1 were significantly upregulated in oocytes treated with nicotine compared with the control. But a decrease in the rates of oocyte maturation and embryo development in the blastocyst stage was found. This may imply that upregulation of GDF9 and ZAR1 was not sufficient to maintain oocyte viability and development after exposure to nicotine. Previous studies have demonstrated that GDF9 plays autocrine and paracrine roles in the regulation of oocyte maturation via proliferation and differentiation of the cumulus cell phenotypes (Thomas and Vanderhyden 2006). ZAR1 was shown to be essential for early embryogenesis, therefore, loss of ZAR1 results in two-cell arrest (Wu et al., 2003).

The relative expression of BCL2 in oocytes seems to be related to the competence of their development (Yang and Rajamahendran, 2002). In the present study, BCL2 and DNMT were significantly upregulated in oocytes treated with nicotine at concentrations of 2.5 and 5.0 mM compared with the control. It has been reported that cigarette smoke is one of the most powerful environmental modifiers of DNA methylation (Breitling et al., 2011). Nicotine in cigarettes affects DNA methylation (Lee and Pausova, 2013) and causes DNA damage by causing double-stranded breaks (Huang et al., 2012). This may imply that nicotine added during IVM alters the expression of genes which may be related to low oocyte maturation rate and developmental competence to the blastocyst stage.

In conclusion, nicotine impairs the rates of oocyte maturation and embryo development and alters the expression levels of genes associated with the development of oocytes and embryos.

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