

Impairment of Epidermal Growth Factor-Stimulated Cumulus Cell Expansion by 4-Nitrophenol in Bovines: Morphological, Genetic and Hormonal Assessment

^{1,2}Omnia A.H.A. El-Gendy, ³Satoshi Sugimura, ²Gen Watanabe,
¹Gamal A. Sosa, ¹Abd El-Salam I. El-Azab, ¹Mahmoud E.A. Abou-Elrous,
¹Mohamed M.M. Kandiel, ²Kentaro Nagaoka and ³Akiya Yajima

¹Department of Theriogenology, Faculty of Veterinary Medicine, Benha University, Kaliobia, Egypt

²Laboratory of Veterinary Physiology, Department of Veterinary Medicine,
Tokyo University of Agriculture and Technology, Tokyo, Japan

³Institute of Agriculture, Department of Biological Production,
Tokyo University of Agriculture and Technology, Tokyo, Japan

Abstract: The present investigation aimed to outline the effects of PNP on Amphiregulin (AREG)- induced oocytes maturation. Bovine COCs were aspirated from small antral follicles (2–6 mm in diameter) and incubated in IVM medium contained 1 µl /ml Amphiregulin of human origin supplemented with PNP at 0 (control), 10⁻⁷ M (LD), 10⁻⁶ M (MD), 10⁻⁵ M (HD) and 10⁻⁴ M (VHD) at 38.5°C in a humidified atmosphere of 5% CO₂ in air for 22 h. Oocytes were evaluated for cumulus cells' expansion, nuclear maturation and the expression of the expansion-related genes (HAS2, TNFIP6, StAR, CYP11A1 and PTGS2). Progesterone and prostaglandin-E2 concentrations were measured in the *in vitro* maturation media after 22 h using EIA. Data showed that the presence of VHD of PNP (10⁻⁴M), decreased the cumulus expansion. An exposure to PNP was found to alter the expansion-related gene expression in the bovine cumulus cell expansion, specifically PTGS2 (up-regulated with MD) and CYP11A1 (down-regulated with HD). The expression of HAS2, TNFIP6 and StAR genes, hormonal excretory activity and nuclear maturation did not show significant differences in PNP groups as compared with control. Concluding that the exposure to PNP during oocyte maturation may impair the epidermal growth factor-stimulated cumulus cell expansion mediated by altering of PTGS2 and/or CYP11A1 genes and this presents risk on acquisition of oocyte competence in mammals.

Key words: Amphiregulin • Bovine Oocyte • Cumulus Cell Expansion • Nuclear Maturation • 4-Nitrophenol • Progesterone • Prostaglandin E2

INTRODUCTION

Oocyte maturation is coordinated by granulosa and cumulus cells. Cumulus cells are a group of cells that in intimate contact with the oocyte and are connect with each other by the gap junction [1]. These cells essentially participate in the oocyte maturation [2] by keeping the oocyte under meiotic arrest, inducing meiotic resumption [3] and supporting cytoplasmic maturation [4].

Under the effect of LH, the cumulus cells form a compact mass surround the oocyte in pre-ovulatory follicle lead to the deposition of muco-elastic matrix,

hyaluronic acid, in the intracellular space between the cumulus cells [5]. The expansion of cumulus cell mass around the oocyte is considered a critical step between LH surge and ovulation [6].

Parker and Schimmer [7] demonstrated that the stimulation of LH induces the transient and sequential expression of the members of epidermal growth factor (EGF) family, including amphiregulin, epiregulin and beta-cellulin. Incubation of follicles with these EGF - like growth factors recapitulates the biochemical and morphological events induced by LH, including cumulus expansion and oocyte maturation. Thus, these

EGF-related growth factors considered to be paracrine mediators that convey the LH signal throughout the follicle. Sigumura *et al.* and others documented that amphiregulin (AREG) stimulates the expression of genes responsible for cumulus cell expansion (HAS2, TNFIP6 and PTGS2) and induces cumulus cell expansion in bovine porcines [8] and bovines [9].

Cumulus cells expansion induces a cascade of events leading to re-programming of gene expression, changes in the secretory property of the cumulus cells surrounding the oocyte, resumes meiotic division and finally ovulation [7].

These cascades of events can be altered by several factors, including environmental pollutants [10]. These chemicals may reach to the ovary, accumulate in the follicular fluid and destroy the oocyte or change the balance of feedback control of the hypothalamus-pituitary-ovarian system [11].

Exhaust from diesel engines is considered to contribute to more than 50% of ambient particulate matter, such particles smaller than 10 μm in a mass median aerodynamic diameter (PM10), greatly contributing to overall air pollution [12].

P-nitrophenol or 4-nitrophenol (PNP), a member of diesel exhausted particles, is a degradation product of parathion and methyl parathion insecticide [13] which is worldwide used as acaricides, fumigant, pre-harvest soil and foliage treatments, for a wide variety of crops both outdoors and in greenhouses [14]. PNP is commonly found in water and soil from agriculture and industrial manufacturing [15] which increases the chance of livestock exposure [16].

It was presented that phenolic compounds alter the follicular turnover in the ovary of sheep fetuses [17]. Nevertheless, there is a lack of information on the adverse effects of PNP on embryo development in farm animals. The objective of this study is to investigate the effect of exposure to different concentrations of 4-Nitrophenol (PNP) on AREG-induced *in vitro* maturation and developmental competence in the bovine model.

MATERIALS AND METHODS

This study was carried out during the period from October, 2014 to September, 2015 at the laboratories of Basic Physiology and Biotechnology, Tokyo University of Agriculture and Technology, Japan. Unless specified, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Chemicals and Culture Media: P-Nitrophenol crystal (PNP, CAS No. 100-02-7, $\text{C}_6\text{H}_5\text{NO}_3$, >99.9% purity, molecular weight at 139.11 g/ Mol) was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). The PNP stock solution (10 mM) was prepared in absolute ethanol, according to Miet *et al.* [18].

Oocyte *in vitro* maturation medium (IVM) was Tissue Culture Medium 199 (25 mM HEPES-buffered TCM 199, Gibco BRL, Grand Island, NY, USA) without phenol red (Gibco Grand Island®, NY, USA) supplemented with 5% BSA (Essentially globulin free, Lot. NO. SLBF5375V), Sodium pyruvate and penicillin/streptomycin mixture.

Oocyte *in Vitro* maturation: Bovine ovaries were collected from the local slaughterhouse, Kanagawa Prefecture, Japan and transported in an insulated thermos to the laboratory. Upon arrival, ovaries were washed several times using normal saline 0.9% at 37°C until obtaining clear saline free from blood and kept in a water bath at 37°C during oocyte collection. COCs were aspirated from small antral follicles (2–6 mm in diameter) and transferred to IVM medium supplemented with 1 μl /ml Amphiregulin (AREG) of human origin. PNP solution was added to IVM at 0 (Control), 10^{-7} M (LD), 10^{-6} M (MD), 10^{-5} M (HD) and 10^{-4} M (VHD). COCs were washed twice with IVM medium and only the oocyte with finely granulated cytoplasm surrounded by at least three compact layers of the cumulus cells were selected [19] and incubated in 100 μl IVM medium (n=30/droplet) covered with paraffin oil (Nacalai Tesque, Inc., Kyoto, Japan) in 35-mm Petri dishes (Nunc Multidishes; Nalge Nunc International, Roskilde, Denmark) in a humidified atmosphere of 5% CO_2 in air at 38.5°C for 22 h. Cumulus cells expansion was assessed at 22h of IVM according to Sugimura *et al.* [8] and the cumulus expansion index was calculated according to Fagbohun *et al.* [20]. After removal of cumulus cells by gentle pipetting, oocytes were stained by Hoechst 33254 for evaluation of nuclear configuration. The oocyte nuclear stage was evaluated under the fluorescent microscope and classified according to the configuration of the chromatin into GV, GVBD and MII. Oocytes at metaphase II (MII) with the first polar body were regarded as mature.

RNA Isolation and qRT-PCR: The expression of the expansion-related genes (HAS2, TNFIP6, StAR, CYP11A1 and PTGS2) was analyzed by real-time PCR as previously described [21]. Mature oocytes surrounded with expanded COCs were denuded in D-PBS using pulled

Table 1: Forward and reverse primers for the expansion-related genes designed from bovine sequences

No	Gene	Sequence (5, -3)	GenBank Accession number
1	HAS2 F Bovine	GGATCTCCTTCCTCAGCAGTGT	NM_174079.2
2	HAS2 R Bovine	ATCCCCAGAGGTCCGCTAATG	
3	TNF AIP6 F Bovine	TGAAAGATGGGATGCATATTGC	NM_001007813.2
4	TNF AIP6 R Bovine	CATTGGGAAGCCTGGAGATT	
7	PTGS2 F Bovine	CTTAAACAAGAGCATCCAGAATGG	NM_174445.2
8	PTGS2R Bovine	GCTGTACGTAGTCTTCAATCACAATCF	
9	RPL 19 F Bovine	TGAGGCCCGCAGGTCTAAG	NM_001040516.1
10	RPL 19 R Bovine	CTTCTCCTTGGACAGAGTCTTG	
11	STAR F Bovine	CCCAGCAGAAAGGGTGTTCATC	NM_174189.2
12	STAR R Bovine	TGCGAGAGGACCTGGTTGA	
13	CYP11A1 F Bovine	GACGTGGACACCATATTCAACCTC	NM_176644.2
14	CYP11A1 R Bovine	CGTTTATTGCCTTCATGGGATGGG	

HAS2: HayluronanSynthesase 2, TNFAIP6: Tumor Necrosis Factor-Alpha-Induced Protein 6, PTGS2: Prostaglandin-Endoperoxidase 2, RPL: Ribosomal Protein L19 (housekeepings gene) StAR: Steroidogenic Acute Regulatory Protein and CYP11A1: Cholesterol Side-Chain Cleavage Enzyme.

glass Pasteur pipette, lyzed using 300 µl of RTL buffer containing 10 µl/ml of 2-mercaptoethanol and stored at -80 °C until RNA extraction. Total RNA from COCs (n=35/group) was extracted with the use of RNeasy Micro Kit (Qiagen, Cat. No. # 74004) following the manufacturer's instructions. Using the RPL 19 gene as a housekeeping gene, as this was found to be stably expressed, regardless of the experimental treatments [8]. RNA (200 ng) was reverse transcribed with random primers using Super-Script III (Invitrogen, Tokyo, Japan). Quantitative real-time PCR analysis was performed using the Step One Plus System (Applied Biosystems™, Thermofisher Scientific, Japan) in a 20.0 ml reaction volume containing 3.0 ml cDNA, 2.5 ml of forward and reverse primers designed from bovine sequences (Table 1), 2.0 ml nuclease free water and 10.0 ml SYBR Green PCR Master Mix (Invitrogen, Tokyo, Japan). Universal thermal cycling parameters (Initial step of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C) were used to quantify the expression of all genes. In every PCR run a standard curve was generated for the genes of interest and for the housekeeping gene (RPL19) by using a serial 5-fold dilution of the amplified cDNA prepared from the RNA extracted from cumulus cells. Final quantitative analysis of each gene was calculated using the standard curve method and the results were recorded relative to the endogenous control gene (RTL19).

Hormonal Assay: The concentration of the progesterone and prostaglandin E2 metabolite hormones were measured in the *in vitro* maturation media after 22h using EIA kits, item No.5826 and No. 514010 (Cayman Chemical, Michigan, USA), respectively, in accordance with the manufacturer's protocol. For each treatment, the assays were conducted in triplicates.

Statistical Analysis: Data were tabulated (mean±SEM) and the differences between means were analyzed by One-way ANOVA and Post-Hoc Fisher's least significant difference (LSD) test. The rate of COCs expansion in response to different doses of PNP in maturation media was analyzed using *chi* square test. All statistical analysis was done using SPSS program (Ver. 20). Differences were considered significant at $P < 0.05$.

RESULTS

Effect of Direct Exposure of 4-nitrophenol in Vitro on the Cumulus Cell Expansion: G0 stage (no expansion) of the bovine COCs was associated with VHD (10^{-4} M) of PNP (1.94%). The proportion of G1stage (Slight expansion) accompanied AREG (12.24%) increased with the presence of HD and VHD of PNP (16.48 and 23.3%, respectively). VHD of PNP (10^{-4} M) was associated with high rates of G2 stage (62.14%) and reduce rates of G3 stage (12.62%) as compared with other PNP (10^{-5} , 10^{-6} and 10^{-7} M) as well as the control groups (Table 2).

Chi-square analysis revealed highly significant differences in the COCs expansion rate in response to IVM-PNP exposure. VHD of PNP (10^{-4} M) upturned the stimulatory effect of AREG on cumulus cell expansion index (1.86 ± 0.09). Yet, LD (10^{-7} M) and MD (10^{-6} M) did not affect CCEI in comparison to AREG (2.43 ± 0.07 , 2.41 ± 0.08 Vs. 2.43 ± 0.08 , respectively) as shown in Fig. 1A.

Effect of the 4-nitrophenol on the Expression of Cumulus Cell Expansion Genes: The effect of PNP exposure *in vitro* on the expression of cumulus cell expansion related genes is presented in Fig 2B-F. Although the relative expression of PTGS2 and CYP11A1 genes varied considerably in PNP groups ($p = 0.08$ and $p < 0.05$,

Table 2: Effect of 4-nitrophenol on bovine oocytes cumulus cell expansion

PNP groups	Number of oocyte	Cumulus cell expansion							
		G0		G1		G2		G3	
		n	%	n	%	n	%	n	%
Control (AREG)	98	0	0.00	12	12.24	34	34.69	52	53.06
LD	95	0	0.00	11	11.57	30	31.57	53	55.78
MD	94	0	0.00	11	11.7	33	35.11	50	53.19
HD	91	0	0.00	15	16.48	31	34.06	45	49.45
VHD	103	2	1.94	24	23.30	64	62.14	13	12.62

LD, MD, HD and VHD designated 4-nitrophenol (PNP) at levels of 10^{-7} , 10^{-6} , 10^{-5} and 10^{-4} M, respectively in in vitro maturation media

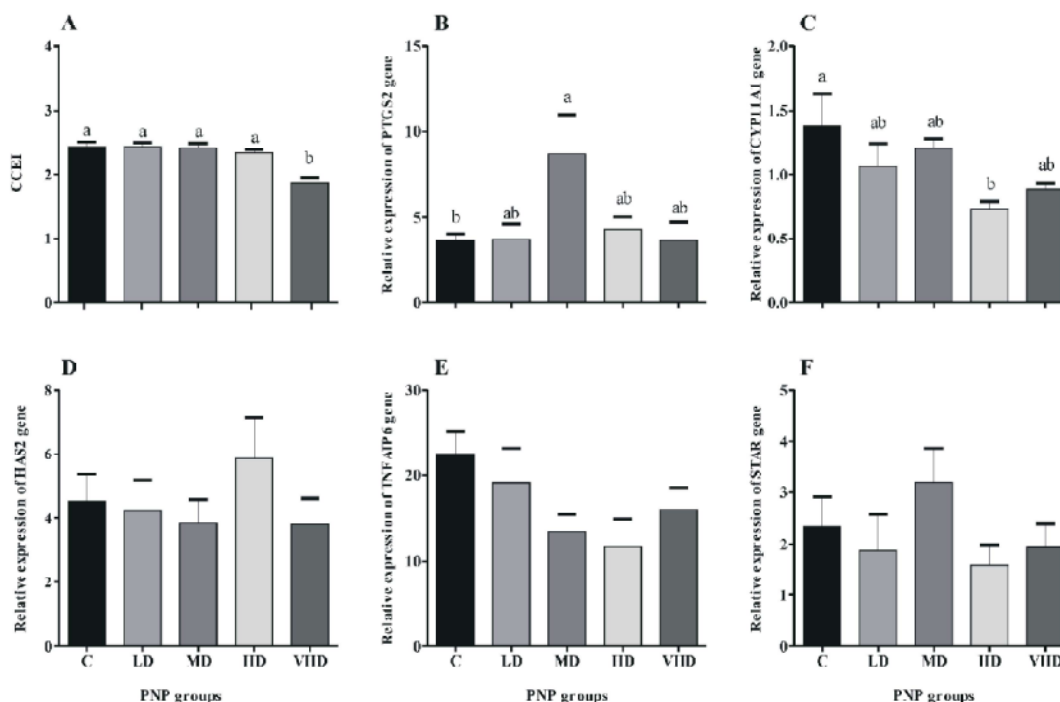


Fig. 1: Effect of the exposure to PNP in vitro maturation media on the cumulus cell expansion index and genes responsible for cumulus cell expansion. A) Cumulus Cell Expansion Index (CCEI), B) Prostaglandin-Endoperoxidase2 (PTGS2), C) Cholesterol Side-Chain Cleavage Enzyme (CYP11A1), D) HyaluronanSynthesase 2 (HAS2), E) Tumor Necrosis Factor-Alpha-Induced Protein 6 (TNFAIP6) and F) SteroidogenicAcute Regulatory Protein (StAR). Means ($\pm$ SEM, n=5). Columns with different superscript letters were significantly different at $p<0.05$.

respectively), the expression of HAS2, TNFAIP6 and StAR genes did not show significant variation upon exposure to different doses of PNP.

The data displayed in Fig. 2B and C, showed that PTGS2 gene expression eminently increased with MD (8.67 ± 2.33), yet CYP11A1 gene expression markedly decreased with HD (0.73 ± 0.06) as compared with control (3.64 ± 0.41 and 1.37 ± 0.26 , respectively).

The HAS2 gene expression increased with MD of PNP (5.86 ± 1.30), but decreased with a VHD dose of PNP (3.81 ± 0.80) as compared with control (4.51 ± 0.85) (Fig. 1D).

The relative expression of TNFAIP6, that increased in the presence of AREG (22.57 ± 2.58), is down-regulated with PNP in a dose dependent manner (Fig. 1E). The relative expression of StAR gene was the higher with HD dose (2.34 ± 0.57) than control (1.59 ± 0.39) (Fig. 1F).

Effect of 4-Nitrophenol on Nuclear Maturation of Bovine Oocyte in Vitro: The effect of exposure to different levels of PNP in the IVM media on the nuclear maturation of bovine oocytes data is presented in Fig. 2A & B. One critical event in meiotic maturation is germinal vesicle

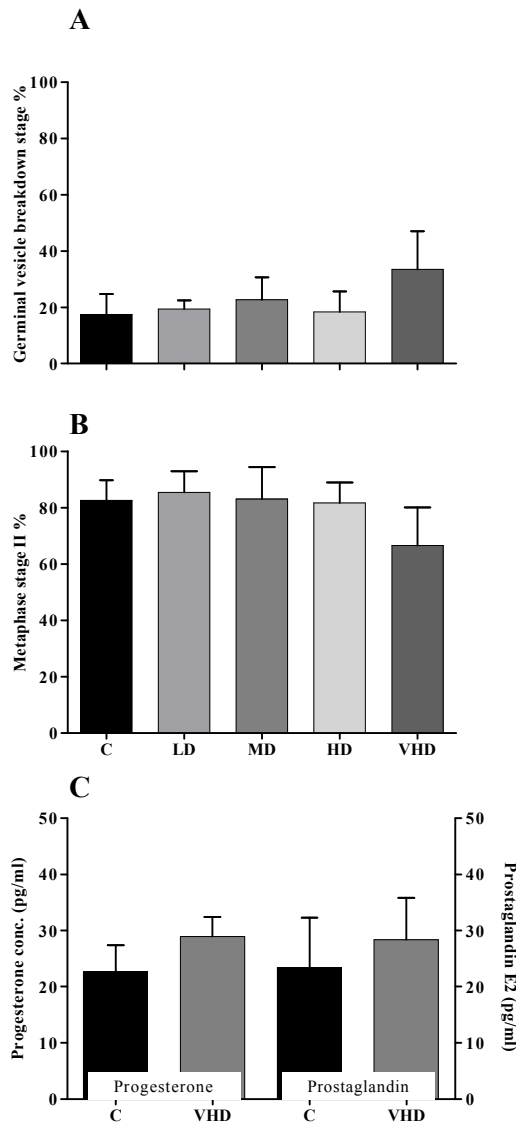


Fig. 2: Effect of direct exposure to 4-nitrophenol *in vitro* on the nuclear maturation and hormonal excretory levels of progesterone and prostaglandin E2 concentration in the IVM medium. A) Germinal Vesicle Breakdown, B) Metaphase II, C) Hormonal levels. Data presented as means ($\pm$ SEM, n=5).

breakdown (GVBD), which marks the onset of meiosis I. GVBD was 2.67 ± 0.88 , 2.00 ± 1.00 , 2.00 ± 1.53 , 2.67 ± 0.88 and 3.33 ± 2.44 in control, LD, MD, HD and VHD dose of PNP, respectively (Fig. 2A).

Meiosis II is the second meiotic division and usually involves equational segregation, or separation of sister chromatids. The proportion of oocytes at MII stage was

13.67 ± 2.33 , 13.33 ± 2.6 , 9.67 ± 2.19 , 13.00 ± 2.31 and 9.33 ± 3.84 in control, LD, MD, HD and VHD dose of PNP, respectively (Fig. 2B).

Statistical analysis revealed that there was no significant ($p=0.67$) difference between the control and different PNP groups. Nonetheless, data showed that higher rate of oocytes arrested at GVBD (Immature) stage and consequently the lower rate of MII (Mature) stage was in association with the VHD dose of PNP.

Effect of 4-Nitrophenol Exposure in Vitro on Progesterone and Prostaglandin E2 concentration: The concentration of progesterone (P4) and prostaglandin E2 (PGE2) in the IVM media after COCS maturation for 22 hr (Fig. 2C) did not significantly vary between VHD of PNP in respect to control contain AREG (28.94 ± 2.01 and 28.33 ± 7.50 vs. 22.55 ± 2.78 and 23.42 ± 8.83 pg/ml, respectively).

DISCUSSION

Sixty years ago, there was a huge number of natural and synthetic chemicals released to the environment and had adverse health effects including reproductive disorders in mammals. *In vivo* studies support the idea that PNP and related nitrophenols impair the reproductive performance in male and female [22, 23].

Oocyte competence is the ability of the oocyte to complete maturation, undergo successful fertilization and reach the blastocyst stage [24]. The present data showed that the cumulus cells expansion of bovine oocytes was significantly inhibited in association with very high dose of PNP (10^{-4} M). The oocyte maturation is a critical prerequisite for the subsequent fertilization and development. Pocar *et al.* [10] reported that exposure of bovine oocyte top-tert-Octylphenol during *in vitro* maturation inhibited cumulus cell expansion and negatively affect nuclear maturation. Multivariate analysis showed a negative dose-dependent effect of DEP on early embryo development and hatching process, blastocyst cell allocation and ICM morphology [2]. These findings further support the idea that the increased risk of failure of IVF outcomes of cows exposed to air pollution could be attributed to DEP concentrations specifically PNP in urban air.

The cumulus cells were thought to express some of the signals that are crucial to the oocyte maturation fulfillment [21]. Identification of potential candidates expressed in CCs associated with the oocyte quality is a key step to clarify the complex pathway of oocyte final

maturation and competence acquisition [24]. The current study investigated the effect of PNP on modulation of the expression of cumulus cell expansion-related genes and verified that PNP at MD (10^{-6} M) and HD (10^{-4} M) influenced cumulus cells' gene expression mainly CYP11A1 (Positive impact) and PGS2 (Negative impact), respectively. Altered genes expression in COCs in response to PNP might accuse for disrupting the cumulus cell expansion and consequently failure of oocyte cytoplasmic maturation. The obvious (Even nonsignificant) decrease of StAR noticed herein might provoke lower expression of CYP11A1 in bovine oocytes. In the present data, high proportion of oocytes arrested at GVBD (Immature) stage at VHD of PNP was coupled with low expression of StAR and CYP11A1 as compared with control, even though this was not statistically verified. Direct exposure of the antral follicle to PBA causes a reduction in the expression on CYP11A1 and consequently reduces progesterone synthesis [25]. *De novo* synthesis of all steroid hormones starts with the conversion of cholesterol (Its transport is regulated by the StAR protein) to pregnenolone by CYP11A [7]. In bovine COCs, CYP11A1 is expressed as early as the immature stage and is regarded as a rate-limiting step in steroidogenesis and helps in the cumulus cell expansion and ovulation [26].

PGS2 Prostaglandin-Endoperoxidase 2 plays a critical role in oocyte maturation, ovulation and fertilization [27]. Present data showed a tendency for PTGS2 gene expression to be higher in PNP-MD group than control, this accompanied relatively high proportion of G4 expanded oocytes. The exposure to environmental pollutants has been found to block the COX activity [28] or alter the mRNA expression level of COX [29]. It has been found that TCDD down regulates the expression of PGS2 [30].

Exposure of the oocyte to the endocrine disruptors during different stages of nuclear maturation results in an increased incidence of crossing over (i.e. Interference with pairing synapsis and recombination) and higher rates of oocyte degeneration [31]. Current results presented that PNP did not affect the oocyte nuclear maturation, even at very high dose. These findings are concomitant with Pocar *et al.* [10], who showed that the direct exposure of the *in vitro* matured oocyte to p-tert-octylphenol impaired meiotic progression and developmental competence of bovine oocyte. Early studies reported that PCB has an adverse effect on bovine oocytes and developmental competence [32]. Rossi *et al.* [33] reported that the exposure of mouse oocyte to environmental pollutants as mancozeb during IVM did not affect the percentage of

oocytes reached to the MII stage, but interfered with the formation of the normal meiotic spindle.

In conclusions, the current data demonstrated for the first time the deleterious effects of PNP exposure *in vitro* on bovine oocyte developmental competence. VHD of PNP (10^{-4} M) has been found to upset the stimulatory effect of AREG on bovine cumulus cell expansion and this may be mediated by alternation of PTGS2 (Positive impact with MD) and/or CYP11A1 (Negative impact with HD) gene expression.

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