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Effects of Nonylphenol on the Production of Progesterone on the Rats Granulosa Cells

Po-Ling Yu,[1,2] Han-Wei Lin,[2] Shyi-Wu Wang,[3] and Paulus S. Wang[2,4,5*]

[1] *Department of Surgery, Taipei City Hospital, Taipei 10431, Taiwan, Republic of China*

[2] *Department of Physiology, School of Medicine, National Yang-Ming University, Taipei 11221, Taiwan, Republic of China*

[3] *Department of Physiology and Pharmacology, College of Medicine, Chang-Gung University, Taoyuan 33333, Taiwan, Republic of China*

[4] *Department of Medical Research and Education, Taipei City Hospital, Taipei 10431, Taiwan, Republic of China*

[5] *Laboratory Animal Center, National Yang-Ming University, Taipei 11221, Taiwan, Republic of China*

ABSTRACT

We investigated the effects of nonylphenol (NP) on release of progesterone (PG) by granulosa cells (GCs) of rats in vitro and in vivo. First, GCs were treated with different doses of NP for 2–24 h alone or with human chorionic gonadotropin (hCG). Maximal PG secretion at 8 h noted, GCs were treated for 2 h with hCG, 8-bromo-adenosine 3′:5′-cyclic monophosphate (8-Br-cAMP), forskolin, A23187, nifedipine, and pregnelonone to evaluate the NP effects on PG steroidogenesis. Results indicated that all of chemicals except nifedipine stimulated the PG release compared to vehicle, but the stimulatory effects could not be enhanced by different doses of NP. Second, GCs were isolated to react with hCG, 8-Br-cAMP and PD98059 after the immature female rats gavaged with different doses of NP (ONP) for 7 days. PG released significantly when rats treated with oral NP 100 compared to 0 μg/kg/day. Third, GCs collected from the female offspring of mother rats which gavaged with NP 100 μg/kg/day for 21 days during pregnancy (MONP) reacted with different doses of chemicals. The results showed that PG release in the presence of chemicals was significantly higher in ONP and MONP groups; however, this stimulation was not noted by dose-dependent. The plasma concentration of PG was higher in ONP (100 μg/kg/day) and the offspring of MONP groups. The steroidogenic acute regulatory (StAR) protein expressed higher in all three groups by Western blotting. This study results indicated that low dose of NP stimulated PG release in rat GCs by activation of StAR protein. J. Cell. Biochem. 112: 2627–2636, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: NONYLPHENOL; PROGESTERONE; GRANULOSA CELLS; StAR

Nonylphenol (NP) is the final metabolite of nonylphenol polyethoxylate (NPE) which is a nonionic surfactant widely used as component of detergents, paints, herbicides, and many other synthetic products and is commonly found in wastewater discharges and in sewage effluents [Gong and Han, 2006]. NP (MW: 220.35), one of environmental hormones, has a structure mimicking the 17β-estradiol (Fig. 1) [Thiele et al., 1997] and has been reported to have xenoestrogenic effects, to affect the endocrine system [Soto et al., 1991; Blom et al., 1998]. High concentration of NP (250 mg/kg/day for 50 days by gavage) decreased the plasma testosterone concentration in rats [Han et al., 2004] and disrupted the immune and reproductive system [Yao et al., 2006]. Differential effects of NP on testosterone secretion in rat Leydig cells was reported [Wu et al., 2010]. In vitro, NP acted directly on rat zona fasciculata–reticularis cells to stimulate corticosterone release via the enzymes of cytochrome P450 side chain cleavage (P450scc) and 11β-hydroxylase [Chang et al., 2010]. NP had opposite effects on the development of mammary glands epithelium of the female offspring by altering the receptor levels of mammary PG and prolactin in pregnant rats, which were gavaged with NP on gestation days [Moon et al., 2007]. Besides, alkylphenols (including NP) incubated with rat pheochromocytoma cell line for 24 h significantly inhibited acetylcholinesterase activity in vitro [Talorete et al., 2001]. The incidences of adenomas and carcinomas in the lungs of rats treated with NP were significantly higher than in the control group [Seike et al., 2003]. However, dietary administration of 4-NP caused significant reduction in the incidence of ovarian adenocarcinoma induced by 7, 12-dimethylbenz(a)anthracene (DMBA) in rats [Tanaka et al., 2002].

*Correspondence to: Paulus S. Wang, Department of Physiology, School of Medicine, National Yang-Ming University, Shih-Pai, Taipei 11221, Taiwan, Republic of China. E-mail: pswang@ym.edu.tw

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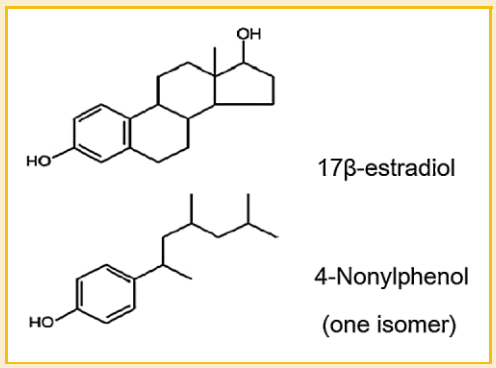

Fig. 1. Chemical structures of NP and estradiol (Thiele et al., 1997).

Above mentioned implicated the effects of NP on gonad, mammary gland, neurological disturbance, and carcinogenesis in animal models.

Cholesterol is transported into the outer membrane of mitochondrion via StAR protein after entering into the cytoplasm of granulosa cells (GCs) from blood. In inner membrane, the cholesterol is metabolized to pregnenolone by P450scc enzyme. After catalysis by 3β-hydroxysteroid dehydrogenase (3β-HSD), pregnenolone is metabolized into PG which is released into blood from cytoplasm of GCs under stimulations (Fig. 2). It has been noted that the effects of NP on PG secretion in GCs were seldom reported. Therefore, the aim of this study was to investigate the influences of NP on GCs in rats and the mechanisms involving PG steroidogenesis.

MATERIALS AND METHODS

REAGENTS

Chemicals and reagents including collagenase, hyluronidase, pregnant mares' serum gonadotropin (PMSG), Dulbecco's modified Eagle medium (DMEM)/F12, fatty acid-free bovine serum albumin (BSA), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), penicillin G, streptomycin sulphate, insulin, medium-199 (MED-199), L-glutamine, 8-bromo-adenosine 3′:5′-cyclic monophosphate (8-Br-cAMP), forskolin, A23187, nifedipine, pregneno-

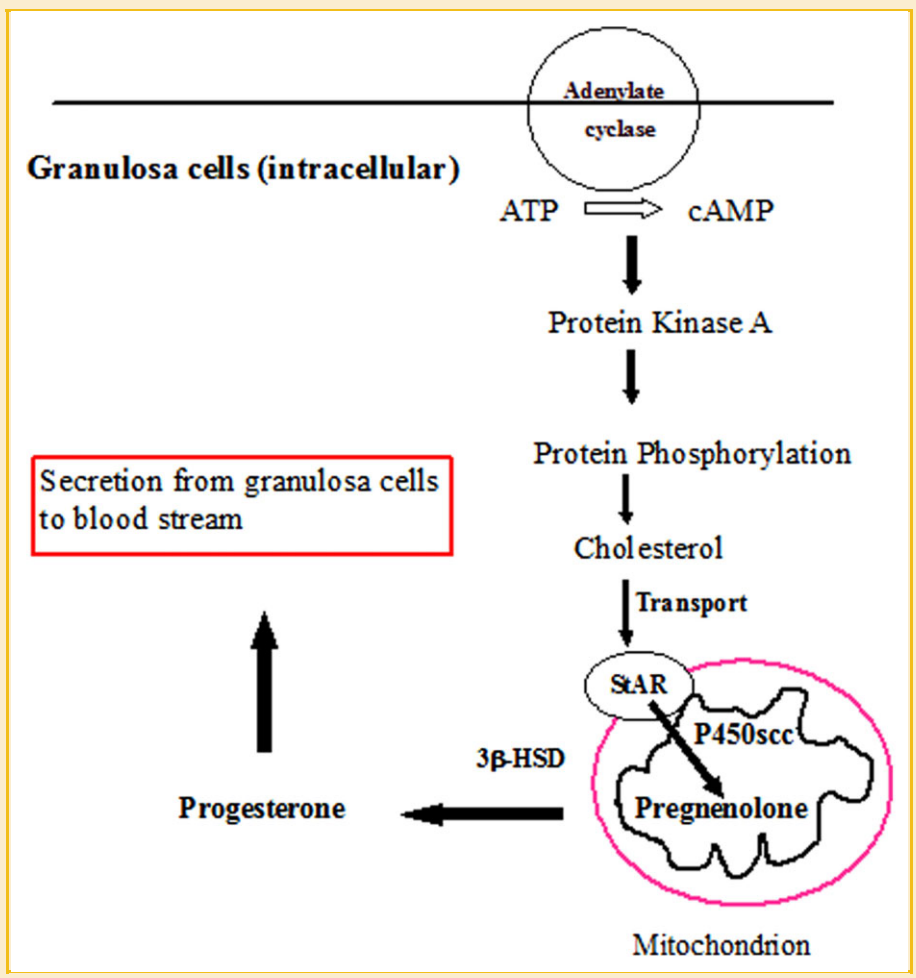

Fig. 2. Cholesterol to progesterone pathway in GCs.

lone, and phenylmethylsulphonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO), sodium dodecyl sulphate (SDS), bromophenol blue, and dithiothreitol were purchased from Research Organics Inc. (Cleveland, OH). Proteinase inhibitor cocktail tablets were purchased from Boehringer Mannheim (Mannheim, Germany). Cell culture plastic ware was obtained from Falcon Labware (Lincoln Park, NJ). The anti-pregnenolone antiserum was purchased from Biogenesis (Poole, England, UK). The peroxidase-conjugated IgG fraction to mouse IgG and peroxidase-conjugated IgG fraction to rabbit IgG were purchased from ICN Pharmaceuticals, Inc. (Aurora, OH). Anti-StAR antibody was kindly provided by Dr. Chung [Hu et al., 1991] and Dr. Stocco [Lin et al., 1998], respectively.

ISOLATION AND CULTURE OF GCs

Immature female Sprague–Dawley rats 21–25 days of age were housed in a temperature-controlled room ($22 \pm 1^{\circ}C$) with 14 h (0600–2000) of artificial illumination daily and were given food and water ad libitum. The preparation of GCs was modified from the method described elsewhere [Tsai et al., 1999]. To obtain the homogeneous and same staged GCs, rats were injected subcutaneously with PMSG (for stimulating growth and maturation of follicles) 20 IU per rat. Forty-eight hours later, rats were killed by cervical dislocation. Ovaries were excised and transferred into the sterile harvest medium (mixture of DMEM/HEPES, 100 IU/ml penicillin G and 50 μg/ml streptomycin sulphate, pH 7.4). After trimming free fat and connective tissues, the large- and medium-sized follicles were punctured with a 26-gauge needle to release GCs. The harvested cells were pelleted and resuspended in growth medium (DMEM/F12 containing 10% bovine calf serum, insulin 2 μg/ml, penicillin G 100 IU/ml, and streptomycin sulphate 100 μg/ml, pH 7.4). Cell viability was greater than 90% as determined using a hemocytometer and trypan blue method. GCs were aliquoted in 24-well plates at approximately 1×10^5 cells/well and incubated at $37^{\circ}C$ with 5% CO_2–95% air for 2 days before reaction [Chen et al., 2001].

EFFECTS OF DIFFERENT DOSES AND DURATIONS OF NP ON THE BASAL AND hCG-STIMULATED PG RELEASE BY RAT GCs

The GCs were washed and incubated with 0.5 ml BSA-medium 199 (mixture of MED-199 and HEPES, 0.3% BSA, pH 7.4) per well, containing different doses of NP in the presence of human chorionic gonadotropin (hCG, 0.05 IU/ml) at $37^{\circ}C$ with 5% CO_2–95% air for 2 h or more. The medium was collected and stored at $-20^{\circ}C$ until further analysis for PG by radioimmunoassay (RIA).

EFFECTS OF DIFFERENT DOSES OF NP ON THE BASAL, hCG-, 8-Br-cAMP-, AND FORSKOLIN-STIMULATED PG RELEASE BY RAT GCs

Similarly, GCs were treated with different doses of NP in the presence of hCG (0.05 IU/ml), 8-Br-cAMP (a permeable cAMP analog, 10^{-4} M) or forskolin (an adenylyl cyclase activator, 10^{-6} M) and analyzed by RIA.

EFFECTS OF DIFFERENT DOSES OF NP ON THE BASAL, A23187-, AND NIFEDIPINE-STIMULATED PG RELEASE BY RAT GCs

GCs were treated with different doses of NP in the presence of A23187 (a calcium ionophore, 10^{-5} M) or nifedipine (L-type calcium channel blocker, 10^{-5} M) and analyzed by RIA.

EFFECTS OF DIFFERENT DOSES OF NP ON THE 3β-HSD ACTIVITY IN RAT GCs

GCs were treated with different doses of NP in the presence of pregnelonone (10^{-8} or 10^{-7} M) and analyzed by RIA.

EFFECTS OF DIFFERENT DOSES OF NP ON THE EXPRESSION OF StAR PROTEIN IN RAT GCs

GCs were treated with different doses of NP at $37^{\circ}C$ with 5% CO_2–95% air for 2 h before Western blotting analysis.

EFFECTS OF DIFFERENT DOSES OF ONP ON THE BASAL, hCG-, AND 8-Br-cAMP-STIMULATED PG RELEASE BY 16 DAYS' RAT GCs

Immature 16 days' female rats were gavaged with NP (ONP) by daily doses of 0, 50, 100, and 200 μg/kg for 7 days. Similarly, GCs were treated with different doses of NP in the presence of hCG (0.05 IU/ml) or 8-Br-cAMP (10^{-4} M) and analyzed by RIA.

EFFECTS OF DIFFERENT DOSES OF ONP ON THE BASAL AND PD98059-STIMULATED PG RELEASE BY 16 DAYS' RAT GCs

GCs were treated with different doses of NP in the presence of PD98059, that is, an extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) inhibitor (10^{-5} M) and analyzed by RIA.

EFFECTS OF DIFFERENT DOSES OF ONP ON THE EXPRESSION OF StAR PROTEIN IN 16 DAYS' RAT GCs

GCs coming from different doses of ONP were treated at $37^{\circ}C$ with 5% CO_2–95% air for 2 h before Western blotting analysis.

EFFECTS OF DIFFERENT DOSES OF ONP ON THE PLASMA CONCENTRATION OF PG IN 16 DAYS' RATS

The plasma concentrations of PG in 16 days' rats with different ONP were measured by RIA.

EFFECTS OF DIFFERENT DOSES OF hCG ON THE VEHICLE, MONP-, AND ONP-STIMULATED PG RELEASE BY GCs

GCs of vehicle, MONP (the 23 days' female offspring of maternal rats which were gavaged with NP 100 μg/kg/day for 21 days during pregnancy) or ONP were treated with different doses of hCG (0–0.05 IU/ml) and analyzed by RIA.

EFFECTS OF DIFFERENT DOSES OF 8-Br-cAMP ON THE VEHICLE, MONP-, AND ONP-STIMULATED PG RELEASE BY GCs

GCs of vehicle, MONP or ONP were treated with different doses of 8-Br-cAMP (0–10^{-4} M) and analyzed by RIA.

EFFECTS OF DIFFERENT DOSES OF FORSKOLIN ON THE VEHICLE, MONP-, AND ONP-STIMULATED PG RELEASE BY GCs

GCs of vehicle, MONP or ONP were treated with different doses of forskolin (0–10^{-6} M) and analyzed by RIA.

EFFECTS OF DIFFERENT DOSES OF SQ22536 ON THE VEHICLE, MONP-, AND ONP-STIMULATED PG RELEASE BY GCs

GCs of vehicle, MONP or ONP were treated with different doses of SQ22536 (an adenylyl cyclase inhibitor, 0–10^{-5} M) and analyzed by RIA.

EFFECTS OF DIFFERENT DOSES OF PREGNENOLONE ON THE VEHICLE, MONP-, AND ONP-STIMULATED PG RELEASE BY GCs

GCs of vehicle, MONP or ONP were treated with different doses of pregnenolone (0–10^{-7} M) and analyzed by RIA.

EFFECTS OF MONP ON THE EXPRESSION OF StAR PROTEIN IN GCs OF OFFSPRING

GCs in offspring of MONP group were treated at 37°C with 5% CO_2 − 95% air for 2 h before Western blotting analysis.

EFFECTS OF MONP ON THE PLASMA CONCENTRATION OF PG IN OFFSPRING

The plasma concentration of PG in offspring of MONP group was measured by RIA.

SURVIVAL RATES OF THE OFFSPRING OF VEHICLE AND MONP

The survival rate of the offspring of MONP was examined and compared to the vehicle.

RIA OF PG

The concentrations of PG in culture medium or plasma were determined by RIA as described elsewhere using anti-progesterone serum W-5 [Lu et al., 1996]. With anti-progesterone W-5, the sensitivity of progesterone (PG) RIA was 5 pg per assay tube. The cross-reactivities were 8% with testosterone, androstenedione, and pregnenolone; 2.5% with 5-dihydrotestosterone, 2% with 17α-hydroxyprogesterone, and <0.3% with estrone, 17α-estradiol, 17β-estradiol, estriol, cortisone, hydrocortisone, and cholesterol. The intra- and interassay coefficients of variability (CV) were 4.8% (n = 5) and 9.5% (n = 4), respectively.

GEL ELECTROPHORESIS AND WESTERN BLOTTING

GCs were washed twice with ice-cold 0.9% NaCl, followed by extraction for 30–60 s on ice with homogenization buffer, pH 8.0, containing 1.5% Na-Lauroylsacrosine, 2.5 mM Tris-base, 1 mM EDTA, 0.68% PMSF, and 2% proteinase inhibitors using an ultrasonic sonicator (model XL 2020, Heat Systems, Inc, Farmingdale, NY). Cell mixtures were centrifuged for 12 min at 13,000 rpm. The protein concentration in the supernatant was determined by the Bradford method [Bradford, 1976]. Extracted proteins were denatured by boiling for 10 min in SDS buffer (0.125 M Tris-base, 4% SDS), 0.001% bromophenol blue, 12% sucrose, and 0.15 M dithiothreitol) [Hu et al., 1991]. The proteins in the samples were separated using 12% SDS–PAGE at 50 V for 20 min and then at 100 V for 60 min using a running buffer. The proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (NEN Life Science Products, Boston, MA) using a Trans-Blot SD semi-dry-transfer cell (170-3940; Bio-Rad, Hercules, CA) at 60 mA (for 8 10 mm membrane) for 40 min in a transfer solution. The membranes were washed in TBS-T buffer (0.8% NaCl, 0.02 M Tris-base, and 0.3% Tween-20, pH 7.6) for 5 min and then blocked by 120 min incubation in blocking buffer (TBS-T buffer containing 5% nonfat dry milk). These membranes were incubated with anti-StAR protein antibody (1:2,000, rabbit) in 5% nonfat dry milk of TBS-T buffer overnight at 4°C. After three washes with TBS-T buffer that were 15, 5, and 5 min respectively, the membranes were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG, 1:8,000 dilution) in 5% nonfat dry milk of TBS-T buffer. The membranes were washed three times with TBS-T buffer, then the band for StAR protein was visualized by chemiluminescence (ECL reagent Kit, Amersham, UK). The StAR protein signals were corrected by β-actin signal [Yu et al., 2009].

STATISTIC ANALYSIS

All data were expressed as the mean ± standard error of the mean (SEM). Treatment means were tested for homogeneity using the analysis of variance (ANOVA), and the differences between the specific means were tested for the significance by Duncan's multiple range test. The level of significance chosen was $P < 0.05$.

RESULTS

EFFECTS OF DIFFERENT DOSES AND DURATIONS OF NP ON THE BASAL AND hCG-STIMULATED PG RELEASE BY RAT GCs

The administration of different doses of NP (0–43 μM) showed that high concentration of NP (13 and 43 μM) treatment resulted in a significant increase of PG release compared to 0 μM ($^{*}P < 0.05$, $^{**}P < 0.01$; Fig. 3). The stimulatory effects reached the maximum 8 h later, then declined significantly esp. 24 h later ($^{++}P < 0.01$). Similar phenomenon was also noted in the presence of hCG (0.05 IU/ml), however, this event declined significantly after 24 h ($^{+}P < 0.05$, $^{++}P < 0.01$).

EFFECTS OF DIFFERENT DOSES OF NP ON THE BASAL, hCG-, 8-Br-cAMP-, FORSKOLIN-, A23187-, NIFEDIPINE-STIMULATED PG RELEASE AND 3β-HSD ACTIVITY IN RAT GCs

The administration of different doses of NP (0–43 μM) showed that high concentration of NP (13 and 43 μM) treatment resulted in a significant increase of PG release ($^{*}P < 0.05$, $^{**}P < 0.01$; Fig. 4, upper left). Administration of hCG, 8-Br-cAMP or forskolin alone stimulated PG release significantly ($^{++}P < 0.01$) as compared with the vehicle group. Moreover, the stimulatory effects of 8-Br-cAMP or forskolin increased in the presence of NP (43 μM, $^{*}P < 0.05$) compared with 0 μM.

A23187 alone stimulated PG release significantly as compared with the vehicle group ($^{++}P < 0.01$, Fig. 4, upper right). However, the stimulatory effects did not increase in the presence of NP. In contrast, nifedipine in the presence of NP (13 and 43 μM) stimulated the PG release significantly as compared with the 0 μM NP ($^{**}P < 0.01$).

Different doses of pregnelonone (10^{-7} or 10^{-8} M) stimulated PG release significantly as compared with vehicle group ($^{++}P < 0.01$, Fig. 4, lower left). However, the stimulatory effects of pregnelonone decreased in the presence of NP (43 μM).

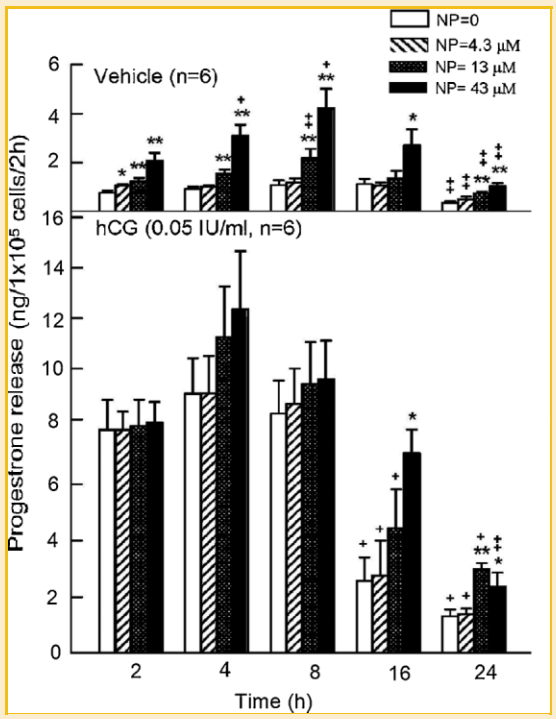

Fig. 3. Time course of NP effects on PG release by rat GCs alone or in the presence of hCG. $^{*}P<0.05$ and $^{**}P<0.01$ compared with 0 μM. $^{+}P<0.05$ and $^{++}P<0.01$ compared with 2 h. Each value represents mean ± SEM.

EFFECTS OF DIFFERENT DOSES OF ONP ON THE BASAL, hCG-, 8-Br-cAMP-, AND PD98059-STIMULATED PG RELEASE BY 16 DAYS' RAT GCs

The PG release by GCs in ONP (100 μg/kg/day) alone or in the presence of hCG or 8-Br-cAMP increased significantly compared to 0 μg/kg/day ($^{*}P<0.05$, $^{**}P<0.01$, Fig. 5, left). However, the stimulatory effects of both chemicals were not significant compared to the vehicle. PG release by GCs in ONP (100 μg/kg/day) alone or in the presence of PD98059 increased significantly compared to 0 μg/kg/day ($^{**}P<0.01$, Fig. 5, right). However, the stimulatory effect was not significant compared to the vehicle.

EFFECTS OF DIFFERENT DOSES OF hCG, 8-Br-cAMP, FORSKOLIN, SQ22536, AND PREGNENOLONE ON THE VEHICLE-, MONP-, AND ONP-STIMULATED PG RELEASE BY GCs

Administration of different concentrations of hCG stimulated the PG release significantly in MONP and ONP group compared to the vehicle ($^{+}P<0.05$, $^{++}P<0.01$, Fig. 6). Moreover, the stimulatory effects of hCG increased significantly in high concentrations (0.001 IU/ml, $^{*}P<0.05$; 0.005 and 0.05 IU/ml, $^{**}P<0.01$) compared to 0 IU/ml.

Administration of different concentrations of 8-Br-cAMP stimulated the PG release significantly in MONP and ONP group compared to the vehicle ($^{++}P<0.01$, Fig. 6). Moreover, the stimulatory effects of 8-Br-cAMP increased significantly in high concentration (10^{-4} M, $^{**}P<0.01$) compared to 0 M.

Administration of different concentrations of forskolin stimulated the PG release significantly in MONP and ONP group compared to the vehicle ($^{++}P<0.01$, Fig. 6). Moreover, the stimulatory effects of forskolin increased significantly in high concentrations (3×10^{-7} and 10^{-6} M; $^{*}P<0.05$ and $^{**}P<0.01$) compared to 0 M.

Administration of different concentrations of SQ22536 stimulated the PG release significantly in MONP and ONP group (10^{-5} M) compared to the vehicle ($^{++}P<0.01$, Fig. 6). Moreover, the stimulatory effects of SQ22536 increased significantly in high concentration (10^{-5} M; $^{*}P<0.05$ in MONP and $^{**}P<0.01$ in ONP) compared to 0 M.

Administration of different concentrations of pregnenolone stimulated the PG release significantly in MONP and ONP group compared to the vehicle ($^{++}P<0.01$, Fig. 6). Moreover, the stimulatory effects of pregnenolone increased significantly in high concentrations (3×10^{-8} and 10^{-7} M, $^{**}P<0.01$) compared to 0 M.

EFFECTS OF DIFFERENT DOSES OF ONP AND MONP ON THE PLASMA CONCENTRATION OF PG IN RAT GCs

In vivo, the plasma concentration of PG in ONP rats increased significantly compared to 0 μg/kg/day ($^{*}P<0.05$, Fig. 7, left). Also, the plasma concentration of PG in offspring of MONP increased significantly compared to control ($^{*}P<0.05$, Fig. 7, right).

EFFECTS OF DIFFERENT DOSES OF NP, ONP, AND MONP ON THE EXPRESSION OF StAR PROTEIN IN RAT GCs

Administration of NP (13 and 43 μM) increased StAR protein expression significantly compared to 0 μM in rat GCs by Western blotting ($^{*}P<0.05$, $^{**}P<0.01$, Fig. 8, upper left). The expression of StAR protein in ONP (100 μg/kg/day) increased significantly compared to 0 μg/kg/day ($^{*}P<0.05$, Fig. 8, upper right). Also, the expression of StAR protein in GCs of the offspring in MONP was significantly higher than control group ($^{**}P<0.01$, Fig. 8, lower left).

SURVIVAL RATES OF THE OFFSPRING OF VEHICLE AND MONP

The survival rate of the offspring of MONP decreased compared to the vehicle and only 70% of offspring survived 3 weeks after birth (Fig. 9).

DISCUSSION

This study characterized the NP effects on PG release in GCs by interacting with chemicals involving steroidogenesis in three animal models, that is, mature female rats, immature female rats gavaged with NP (ONP) and mature female offspring of maternal rats gavaged with NP during pregnancy (MONP). We found that low dose of NP stimulated PG release in GCs by activating StAR protein expression and decreasing survival of offspring in MONP rats.

Because of the similarity of bioactivity (stimulating the adenylyl cyclase) and amino acid sequence between luteinizing hormone (LH) and hCG, the hCG was used as stimulatory hormone for rat GCs in this study [Yu et al., 2010]. The stimulatory effects of hCG on the release of PG from the GCs was not significant even in the presence of high concentrations of NP (43 μM), which stimulated the PG

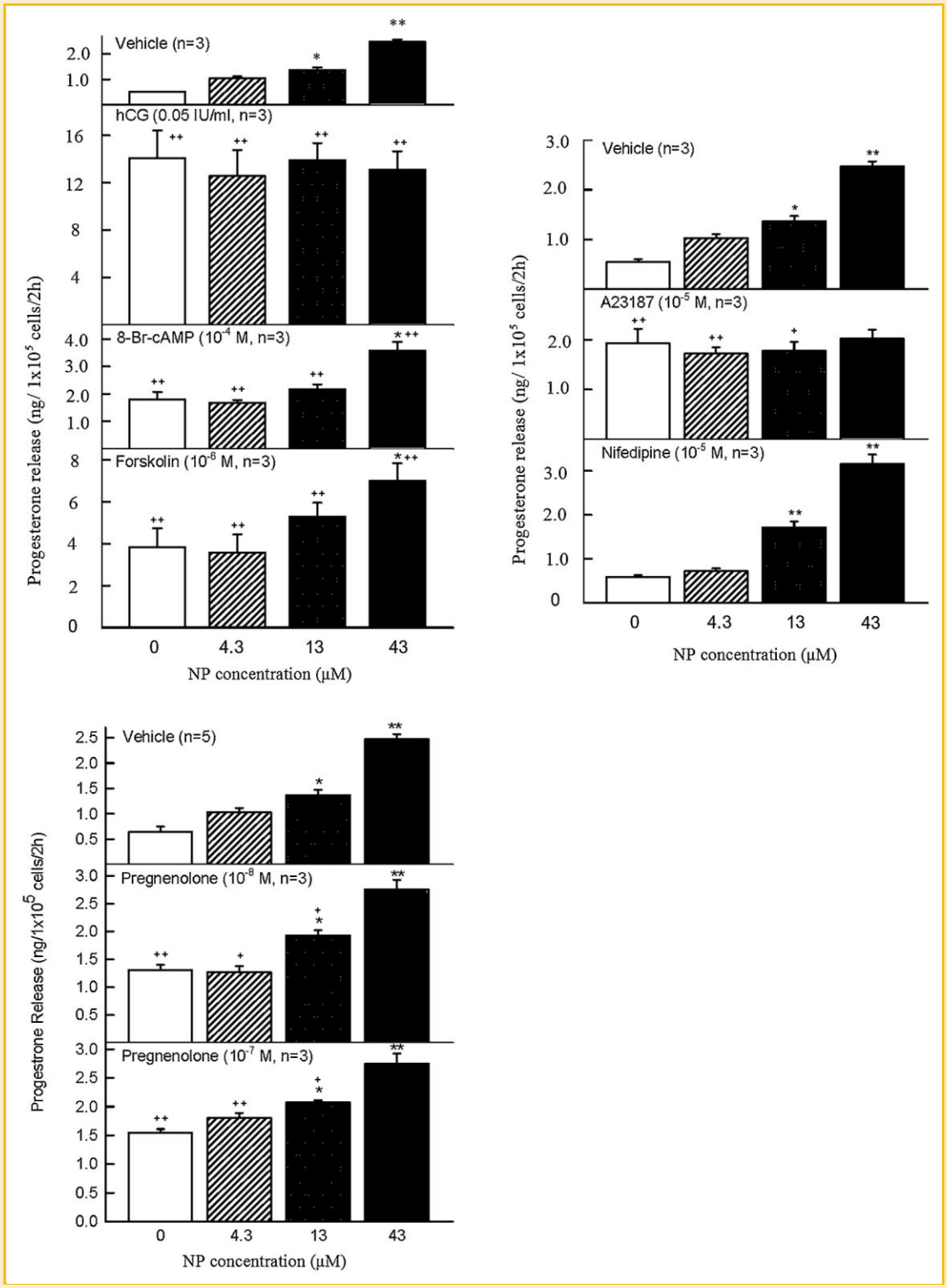

Fig. 4. *Upper left*: Effects of different doses of NP on the basal hCG-, 8-Br-cAMP-, and forskolin-; *Upper right*: effects of different doses of NP on the basal, A23187-, and nifedipine-stimulated PG release by rat GCs. *Lower left*: Effects of different doses of NP on the 3β-HSD activity in rat GCs. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with 0 μM. $^{+}P < 0.05$ and $^{++}P < 0.01$ compared with vehicle. Each value represents mean ± SEM.

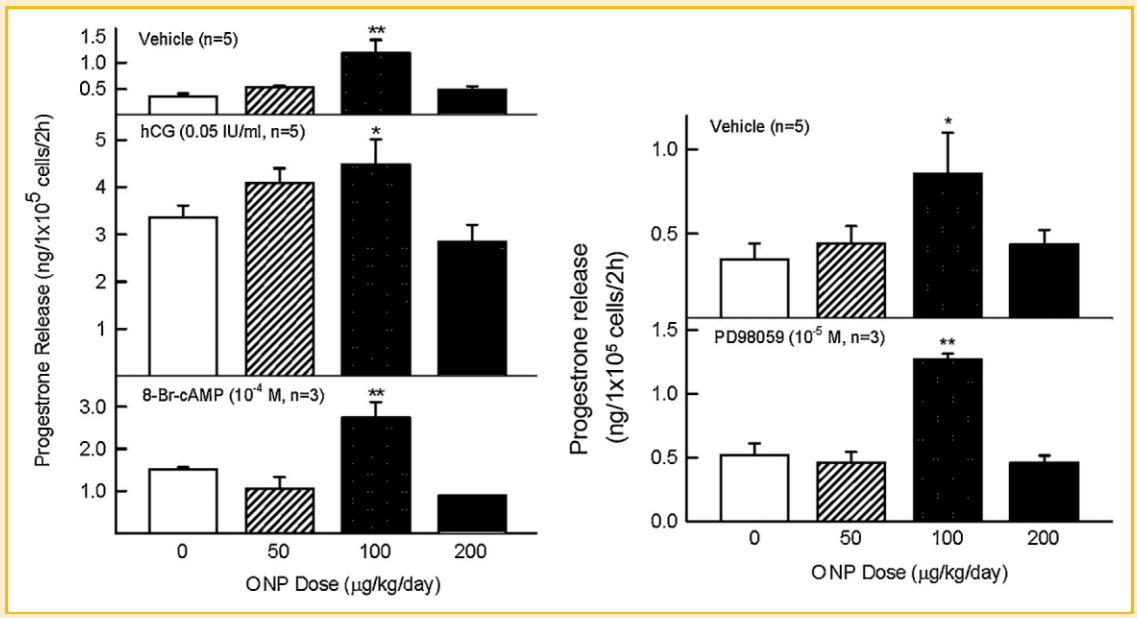

Fig. 5. *Left*: Effects of different doses of ONP on the basal hCG- and 8-Br-cAMP-; *Right*: effects of different doses of ONP on the basal, and PD98059-stimulated PG release by rat GCs. $^{*}P<0.05$ and $^{**}P<0.01$ compared with 0 μg/kg/day. Each value represents mean ± SEM.

release dominantly in this study. The stimulatory effect of NP or hCG on GCs reached maximum when incubated for 8 h, then decreased gradually and significantly 24 h later (Fig. 3). Because of the declination in time to effect, the GCs were treated with chemicals for 2 h in this study to obtain the optimal reactions.

In vitro, high concentration of NP increased the release of PG from GCs; this effect was not enhanced by dose-dependent in the presence of hCG, 8-Br-cAMP, or forskolin (Fig. 4). Similarly, the release of PG did not increase significantly compared to vehicle in the presence of hCG or 8-Br-cAMP in ONP rats (Fig. 5, left). In

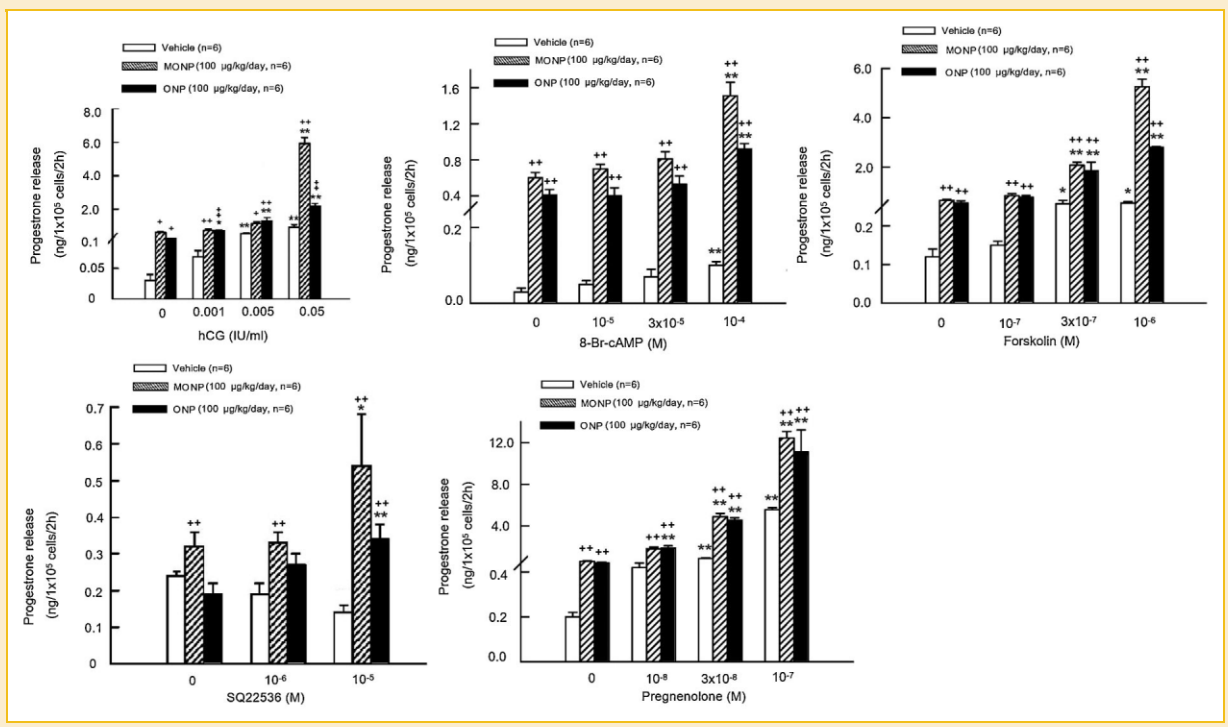

Fig. 6. Effects of different doses of hCG. 8-Br-cAMP forskolin. SQ22536 and pregnenolone on the vehicle-, MONP-, or ONP-stimulated PG release by GCs. $^{*}P<0.05$ and $^{**}P<0.01$ compared with 0 M or 1 IU/ml. $^{+}P<0.05$ and $^{++}P<0.01$ compared with vehicle. Each value represents mean ± SEM.

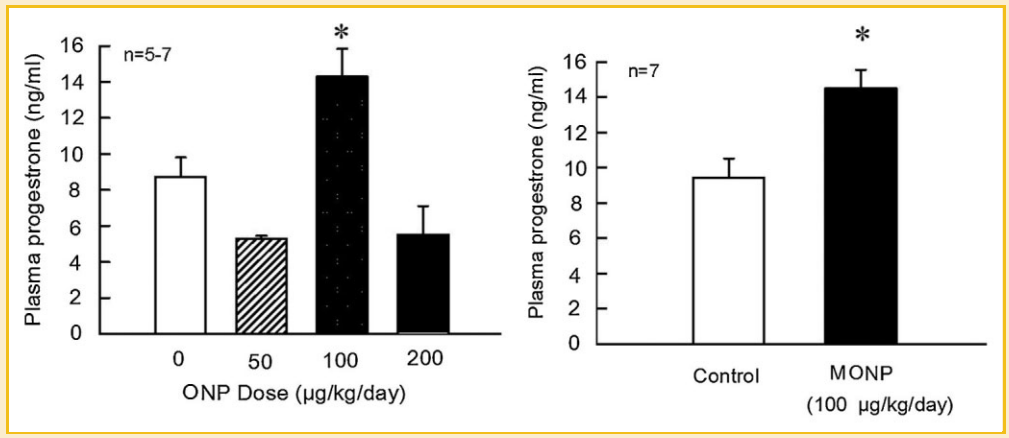

Fig. 7. In vivo study. *Left*: Effects of different doses of ONP on the plasma concentration of PG in 16 days' rats. *Right*: Effects of MONP on the plasma concentration of PG in offspring. $^{*}P < 0.05$ compared with oral 0 μg/kg/day. Each value represents mean ± SEM.

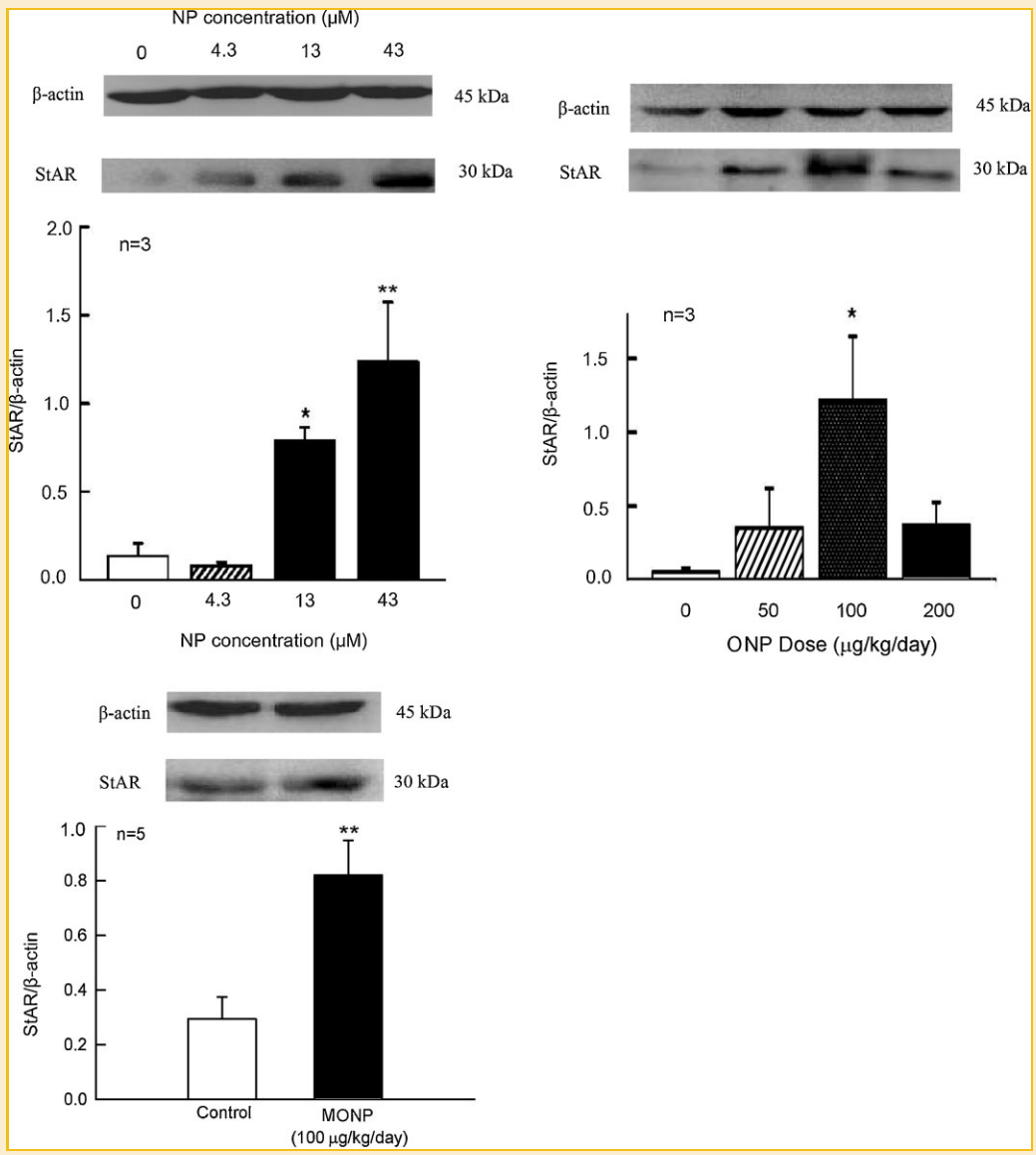

Fig. 8. Western blotting analysis. *Upper left*: Effects of different doses of NP on the expression of StAR protein in rat GCs. *Upper right*: Effects of different doses ONP on the expression of StAR protein in rat GCs. *Lower left*: Effects of MONP on the expression of StAR protein in GCs of offspring. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with 0 μM. Each value represents mean ± SEM.

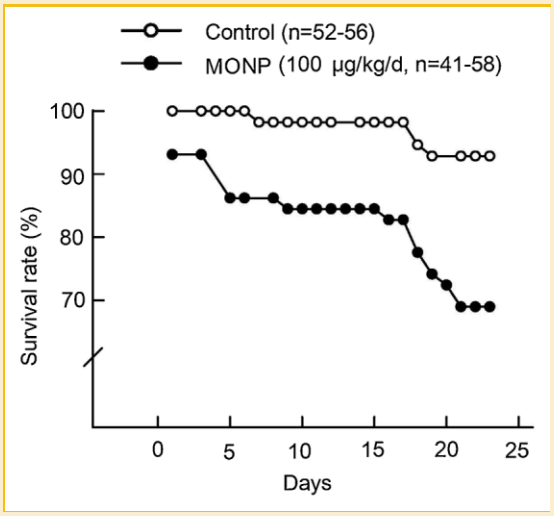

Fig. 9. Survival rates of the offspring of vehicle and MONP.

comparison of vehicle, MONP and ONP groups, the PG release did not increase significantly by dose-dependent in low concentrations of hCG, 8-Br-cAMP, forskolin, or SQ22536 (Fig. 6). These data suggested that the cAMP pathway might not directly involve in the mechanisms of NP-PG reaction in GCs of rats; while, combination of genistein and NP significantly accelerated capacitation and acrosome loss in human and mouse spermatozoa by stimulating cAMP production [Fraser et al., 2006].

A23187 increased the release of PG in GCs and counteracted with the stimulatory effect of high concentration of NP in vitro; in other words, NP (43 μM) inhibited the stimulatory effect of A23187. Also, administration of calcium channel blocker did not affect the NP effect in this study (Fig. 4, right upper). Although, alkylphenol endocrine disrupters, for example, NP disrupted endocrine function by inhibition of calcium channel in porcine cerebellum and rat testicular membranes [Khan et al., 2003], our results indicated that the calcium channel did not involve in NP-PG reaction of rats GCs.

In GCs, the cholesterol is transported into the mitochondrion via StAR protein and converted to pregnenolone by P450scc. The PG is released by mitochondrion after converting from pregnenolone under 3β-HSD (Fig. 2). It seemed that 3β-HSD enzyme did not play a major role in the mechanism of NP-PG reaction in GCs by ONP and MONP groups (Fig. 6). However, high concentration of NP might inhibit the activity of 3β-HSD (Fig. 4, left lower). The expression of 3β-HSD mRNA in the testes was downregulated in rats gavaged with NP (10, 50, 100, and 250 mg/kg/day BW) for 3 weeks [Kim et al., 2007]. Nevertheless, the fact that dosage used in our study (100 μg/kg/day for 7 days) was lower than those of Kim's might explain the different results between both.

NP was reported to induce proliferation of estrogen receptor-positive ovarian cancer cells, but this environmental hormones-activated MAPK was unlikely to be involved [Park et al., 2009]. The stimulatory effect of NP-PG in GCs was not affected consistently in the presence of PD 98059 (Fig. 5, right) in ONP model, ERK/MAPK pathway involvement was not confirmed.

Unlike cells that produce polypeptide hormones, which store large amounts of hormone in secretory vesicles ready for rapid release, steroidogenic cells store very little steroids. Thus a rapid steroidogenic response requires rapid synthesis of new steroids. The acute regulation of steroidogenesis (e.g., the rapid rise in serum cortisol following severe injury) is at the level of substrate access to P450scc, which is regulated at the level of cholesterol transport into the mitochondria [Miller, 1988; Stocco and Clark, 1996]. Orme-Johnson first showed that this acute steroidogenic response was accompanied by the rapid synthesis of a 37 kDa phosphoprotein [Pon and Orme-Johnson, 1986; Pon et al., 1986]. Stocco et al. cloned this factor and named the steroidogenic acute regulatory protein (StAR) [Clark et al., 1994]. StAR is principally expressed in the adrenal cortex and in the steroidogenic cells of the gonads. NP was reported to stimulate testosterone release in rat Leydig cells by activation of StAR [Wu et al., 2010]. In this study, Western blot analysis disclosed that the stimulatory effect of NP-PG in GCs was via activation of StAR protein in NP alone, ONP, and MONP, respectively (Fig. 8).

Mortality of fetuses/embryos was increased considerably in the maternal rats with subcutaneous injection of NP 1/100 (male) and NP 1/10 (female) of the LD_{50} [Kimura et al., 2006]. Human trophoblast was reported to be highly responsive to para-NP than 17β-estradiol in early gestation [Bechi et al., 2006]. Even low dose of para-NP was able to affect cytokine secretion in human placenta and might result in implantation failure, pregnancy loss [Bechi et al., 2010]. Five percent of the offspring of MONP was dead at birth and 70% left survived at 3 weeks after delivery; meanwhile, the plasma concentration of PG in this group was significantly higher than control. The PG concentration decreased both in vitro and in vivo when ONP with 200 compared to 100 μg/kg/day (Figs. 5 and 7). The exposure of NP during pregnancy causing the failure of birth, early death of rats by changing the plasma PG concentration in offspring was noted and could be applied to the human being in consequence of limited reports about NP effects on women's reproductivities.

In conclusion, the present study demonstrated that low dose of NP stimulated PG release in rat GCs by activation of StAR protein expression; while, this stimulatory effect was inhibited by high dose of NP. NP affected the pregnant rats and decreased the survival rate of offspring by increasing plasma concentration of PG in offspring rats. These findings provide more insights into the role of environmental hormone affecting PG release in GCs and offspring via pregnant mother in rats. Further investigations should be conducted in women.

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