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Shanthi Ganesan
Iowa State University, shanthig@iastate.edu

Aileen F. Keating
Iowa State University, akeating@iastate.edu

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7,12-dimethylbenz[a]anthracene (DMBA) destroys ovarian follicles in a concentration-dependent manner. The impact of DMBA on connexin (CX) proteins that mediate communication between follicular cell types along with pro-apoptotic factors p53 and Bax were investigated. Postnatal day (PND) 4 Fisher 344 rat ovaries were cultured for 4 days in vehicle medium (1% DMSO) followed by a single exposure to vehicle control (1% DMSO) or DMBA (12.5 nM or 75 nM) and cultured for 4 or 8 days. RT-PCR was performed to quantify Cx37, Cx43, p53 and Bax mRNA level. Western blotting and immunofluorescence staining were performed to determine CX37 or CX43 level and/or localization. Cx37 mRNA and protein increased ($P < 0.05$) at 4 days of 12.5 nM DMBA exposure. Relative to vehicle control-treated ovaries, mRNA encoding Cx43 decreased ($P < 0.05$) but CX43 protein increased ($P < 0.05$) at 4 days by both DMBA exposures. mRNA expression of pro-apoptotic p53 was decreased ($P < 0.05$) but no changes in Bax expression were observed after 4 days of DMBA exposures. In contrast, after 8 days, DMBA decreased Cx37 and Cx43 mRNA and protein but increased both p53 and Bax mRNA levels. CX43 protein was located between granulosa cells, while CX37 was located at the oocyte cell surface of all follicle stages. These findings support that DMBA exposure impacts ovarian Cx37 and Cx43 mRNA and protein prior to both observed changes in pro-apoptotic p53 and Bax and follicle loss. It is possible that such interference in follicular cell communication is detrimental to follicle viability, and may play a role in DMBA-induced follicular atresia.

Keywords
Ovary, DMBA, Ovotoxicity, Connexins, Gap Junctions, Follicle

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Shanthi Ganesan and **Aileen F. Keating***
Department of Animal Science, Iowa State University, Ames, IA 50011, USA

Abstract

7,12-dimethylbenz[a]anthracene (DMBA) destroys ovarian follicles in a concentration-dependent manner. The impact of DMBA on connexin (CX) proteins that mediate communication between follicular cell types along with pro-apoptotic factors *p53* and *Bax* were investigated. Postnatal day (PND) 4 Fisher 344 rat ovaries were cultured for 4 days in vehicle medium (1% DMSO) followed by a single exposure to vehicle control (1% DMSO) or DMBA (12.5 nM or 75 nM) and cultured for 4 or 8 days. RT-PCR was performed to quantify *Cx37*, *Cx43*, *p53* and *Bax* mRNA level. Western blotting and immunofluorescence staining were performed to determine CX37 or CX43 level and/or localization. *Cx37* mRNA and protein increased ($P < 0.05$) at 4 days of 12.5 nM DMBA exposure. Relative to vehicle control-treated ovaries, mRNA encoding *Cx43* decreased ($P < 0.05$) but CX43 protein increased ($P < 0.05$) at 4 days by both DMBA exposures. mRNA expression of pro-apoptotic *p53* was decreased ($P < 0.05$) but no changes in *Bax* expression were observed after 4 days of DMBA exposures. In contrast, after 8 days, DMBA decreased *Cx37* and *Cx43* mRNA and protein but increased both *p53* and *Bax* mRNA levels. CX43 protein was located between granulosa cells, while CX37 was located at the oocyte cell surface of all follicle stages. These findings support that DMBA exposure impacts ovarian *Cx37* and *Cx43* mRNA and protein prior to both observed changes in pro-apoptotic *p53* and *Bax* and follicle loss. It is possible that such interference in follicular cell communication is detrimental to follicle viability, and may play a role in DMBA-induced follicular atresia.

Keywords

Ovary; DMBA; Ovotoxicity; Connexins; Gap Junctions; Follicle

Introduction

The female gamete, the oocyte, is encased in a follicular structure surrounded by granulosa cells and, as the follicle matures, also by theca cells. Primordial follicles are maintained in a dormant state until activation into the growing follicular pool (Hirshfield, 1991). In women,

***Corresponding author:** Department of Animal Science, Iowa State University, Ames, IA 50011. Telephone number 1-515-294-3849; Fax number: 1-515-294-4471. akeating@iastate.edu.
shanthig@iastate.edu

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nearly 99% of ovarian follicles undergo degenerative changes by a process known as atresia (Hirshfield, 1991) and ovarian senescence (menopause) occurs when the finite pool of primordial follicles has become exhausted (Mattison and Nightingale, 1982; Broekmans *et al.*, 2007; Hansen *et al.*, 2008). Since primordial follicles cannot be regenerated (Hirshfield, 1991), chemical-induced depletion of this follicle pool can lead to infertility and premature ovarian failure (POF). A number of chemical classes can deplete follicles causing ovotoxicity, including the polycyclic aromatic hydrocarbon, 7,12-dimethylbenz[a]anthracene (DMBA) (Mattison, 1980; Hoyer *et al.*, 2009; Igawa *et al.*, 2009). Since DMBA can induce DNA double strand break (DSBs) damage in oocytes and granulosa cells (Ganesan *et al*., 2013), there is concern that exposure could lead to negative consequences for offspring should this damage be improperly repaired.

DSBs activate ataxia-telangiectasia mutated (ATM) protein which phosphorylates p53, a tumor suppressor protein. p53 can then activate DNA repair proteins, induce cell cycle arrest or initiate apoptosis if the DNA damage proves to be irreparable. Phosphorylated p53 activates a number of downstream pro-apoptotic molecules including *Bax* (Lane, 1992). BAX promotes apoptosis by binding to and antagonizing the action of BCL-2 protein, resulting in release of cytochrome c and activation of caspases to induce apoptosis (Weng *et al*., 2005). We and others have previously demonstrated that ovaries exposed to DMBA have increased levels of caspase 3 protein (Tsai-Turton *et al.*, 2007; Ganesan *et al*., 2013). Additionally, BAX-deficient mice ovaries are resistant to DMBA-induced primordial follicle destruction (Matikainen *et al.*, 2001).

Granulosa:granulosa and granulosa:oocyte cell to cell communication are necessary for maintenance of follicular viability. Communication between these cells occurs through gap junction intra-cellular channels, which directly connect the cytoplasmic compartments of neighboring cells and allow exchange of ions, metabolites and second messenger such as Ca^{2+} and inositol phosphates (Goldberg *et al.*, 2004). Gap junctions are involved in regulation of cellular growth, metabolism and differentiation (Sohl and Willecke, 2003; Wei *et al.*, 2004) and ovarian folliculogenesis (Simon *et al.*, 2006). The major ovarian gap junction proteins are connexin (CX) 37 and 43 (Kidder and Mhawi, 2002a). CX37 communicates from the oocyte to granulosa cell (Simon *et al.*, 1997), while CX43 functions in intra-granulosa cell communication (Granot *et al.*, 2002; Kidder and Mhawi, 2002a). CX37 is thought to be involved in follicular development and ovulation as well as luteal tissue growth, differentiation, and regression (Borowczyk *et al.*, 2006). *Cx37*-null mouse oocytes suffer growth retardation and do not survive to become meiotically competent (Carabatsos *et al.*, 2000). Follicle growth is also interrupted: $Cx37^{-/-}$ granulosa cells form structures resembling corpora lutea in the absence of ovulation. CX43 levels are increased in granulosa cells following activation of follicular growth and maturation (Melton *et al.*, 2001), while reduced granulosa cell expression of CX43 is linked to elevated apoptosis in porcine, bovine (Johnson *et al.*, 1999; Cheng *et al.*, 2005) and avian (Krysko *et al.*, 2004) species. Thus, CX37 and CX43 play important roles in the ovary to maintain follicular and oocyte viability and quality.

Little is known about the impact of ovotoxicant exposures on ovarian function, thus in this study we investigated any impact of DMBA on ovarian gap junction genes *Cx37* and *Cx43* along with the pro-apoptotic cellular components, *p53* and *Bax*. We utilized a neonatal rat whole ovary culture method to determine the effect of a single DMBA exposure at two concentrations – 12.5 nM and 75 nM, since we have previously shown that these concentrations induce DNA damage and increased caspase 3 levels 8 days after exposure (Ganesan *et al*., 2013). Also, we have observed that these DMBA concentrations induced large primary and secondary follicle loss (unpublished data). Our hypothesis was that DMBA would alter *Cx37* and *Cx43* at time points prior to changes in pro-apoptotic genes

(*p53* and *Bax*) and observed follicle loss supporting that they are targets of DMBA's mechanism of ovotoxicity.

Methods and Materials

Reagents

7,12-Dimethylbenz[a]anthracene (DMBA; CAS # 57-97-6), bovine serum albumin (BSA), ascorbic acid, transferrin, 2-β-mercaptoethanol, 30% acrylamide/0.8% bisacrylamide, ammonium persulphate, glycerol, N'N'N'N'-Tetramethylethylenediamine (TEMED), Tris base, Tris HCL, Sodium chloride, Tween-20 were purchased from Sigma-Aldrich Inc. (St Louis, MO). Dulbecco's Modified Eagle Medium:nutrient mixture F-12 (Ham) 1x (DMEM/Ham's F12), Albumax, penicillin (5000U/ml), Hanks' Balanced Salt Solution (without $CaCl_2$, $MgCl_2$ or$MgSO_4$) from Invitrogen Co. (Carlsbad, CA). Millicell-CM filter inserts and 48 well cell culture plates were obtained from Millipore (Bedford, MA) and Corning Inc. (Corning, NY) respectively. RNeasy Mini kit, QIA shredder kit, RNeasy Min Elute kit, and Quantitect TM SYBR Green PCR kit were purchased from Qiagen Inc (Valencia, CA). All primers were purchased from the Iowa State University DNA facility. All primary antibodies were purchased from Abcam (Cambridge, MA). RNA later was obtained from Ambion Inc. (Austin, TX). Goat anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology, (Santa Cruz, CA). Ponceau S was from Fisher Scientific. ECL plus chemical luminescence detection kit was obtained from GE Healthcare, Amersham (Buckinghamshire, UK). Primers were obtained from the DNA facility of the Office of Biotechnology at Iowa State University (Ames, IA).

Ovary culture

Ovaries were collected from PND4 Fisher 344 female rats and cultured as described previously (Devine *et al.*, 2002). Briefly, PND4 female F344 rat pups were euthanized by CO_2 inhalation followed by decapitation. Ovaries were removed, trimmed of oviduct and other excess tissues and placed onto membrane floating on 250 μl of DMEM/Ham's F12 medium containing 1 mg/ml BSA, 1 mg/ml Albumax, 50 μg/ml ascorbic acid, 5 U/ml penicillin and 27.5 μg/ml transferrin per well in a 48 well plate that had previously been equilibrated to 37 °C. A drop of medium was placed on top of each ovary to prevent it from drying. Ovaries were cultured for 4 days in control medium to allow development of large primary and secondary follicles, and were then treated once with medium containing vehicle control (1% DMSO) ± DMBA (12.5 nM or 75 nM) and the culture was maintained for four or eight days at 37 °C and 5% CO_2. These exposures have been found in our laboratory to induce large primary and/or secondary follicles after 8 days (unpublished data). Additionally, 75 nM DMBA was shown to cause primordial follicle loss after 15 days of exposure on alternate days, while the 12.5 nM exposure did not affect primordial follicle number (Igawa et al., 2009). Thus, the purpose of these experiments was to investigate the impact of DMBA exposures on large primary and secondary follicles. The medium was replaced every two days. One ovary per animal was placed in control medium, while the contralateral ovary was exposed to the experimental treatment.

RNA isolation and Quantitative RT-PCR

RNA was isolated using an RNeasy Mini kit (Qiagen) and the concentration was determined using an ND-1000 Spectrophotometer (λ = 260/280nm; NanoDrop technologies, Inc., Wilmington, DE) (n=3; 10 ovaries per pool). Total RNA (200 ng) was reverse transcribed to cDNA utilizing the Superscript III One-Step RT-PCR (Qiagen). cDNA was diluted (1:20) in RNase-free water. Diluted cDNA (2μl) were amplified on an Eppendorf PCR Master cycler using Quantitect SYBR Green PCR kit (Qiagen). Primers were designed by Primer 3 Input Version (0.4.0); *Cx37* forward - tgatcacaggtggttctgga; *Cx37* reverse - aggagaagtggggtgtgatg:

Cx43 forward - gagcgaggtttcaacagtgc; *Cx43* reverse - ccgaacacgacagcagttta: *Gapdh* forward - gtggacctcatggcctacat; *Gapdh* reverse – ggatggaattgtgagggaga: *p53* forward – tggtccagcaaatcctatc; *p53* reverse – gagtggaggaaatgggtcct: *Bax* forward – cgagctgatcagaaccatca; *Bax* reverse – ctcagcccatcttcttccag. The regular cycling program consisted of a 15-min hold at 95°C and 45 cycles of denaturing at 95°C for 15s, annealing at 58°C for 15s, and extension at 72°C for 20s at which point data were acquired. There was no difference in *Gapdh* mRNA expression between treatments, thus each sample was normalized to *Gapdh* before quantification. Quantification of fold-change in gene expression was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001). Expression level for control was set at 1, and treatment gene changes were expressed as fold-change relative to the vehicle control treated ovaries. Thus, fold-changes presented are increases above the control value of 1.

Protein isolation and Western blotting

Protein was isolated from cultured ovaries (n=3; 10 ovaries per pool). Homogenates were prepared from cultured ovaries via homogenization in tissue lysis buffer containing protease and phosphatase inhibitors as previously described (Thompson *et al.*, 2005). Briefly, homogenized samples were placed on ice for 30 min, followed by two rounds of centrifugation at 10,000 rpm for 15 min and protein concentration was measured using a BCA protocol. Protein was stored at −80 °C until further use. SDS-PAGE was used to separate protein homogenates which were then transferred to a nitrocellulose membrane. Membranes were blocked for 1 hour in 5 % milk in Tris-buffered saline containing tween 20, followed by incubation with anti-rabbit CX37 or CX43 antibody (1:100) for 36 h at 4°C. Following three washes in TTBS (1X), membranes were incubated with species-specific secondary antibodies (1:2000) for 1 h at room temperature. Membranes were washed 3X in TTBS and incubated in chemiluminescence detection substrate (ECL plus) for 5 min followed by X-ray film exposure. Densitometry of the appropriate bands was performed using ImageJ software (NCBI). Equal protein loading was confirmed by Ponceau S staining of membranes and protein level was normalized to Ponceau S densitometry values.

Immunofluorescence Staining

Ovaries were fixed in 4% paraformaldehyde for 2 h, transferred to 70% ethanol, embedded in paraffin, serially sectioned (5 μM thick), and every 10th section was mounted. Slides were deparaffinized in xylene and rehydrated with subsequent washes in ethanol. Antigen retrieval was carried out by microwaving sections for 7 min in sodium citrate buffer (1M, pH 6.1). Sections were then blocked in 5% BSA for 1 h at room temperature. Sections were incubated with primary antibody CX37 (1:200) and CX43 (1:200) overnight at 4°C. After washing in 1% PBS, sections were incubated with the appropriate goat anti-rabbit IgG-FITC secondary antibodies for 1 h. Slides were then counterstained with 4-6-diamidino-2-phenylindole (DAPI) or Hoechst for 5 min. Images were taken using a Leica fluorescent microscope and protein expression were analyzed using ImageJ software (NCBI). 10-15 follicles per ovary (n=3) were analyzed for CX37 protein quantification and 3 ovaries were used to quantify CX 43 protein level by counting the number of CX43 positive foci in each ovary.

Statistical analysis

Raw data were analyzed by paired t-tests where two treatments were compared or ANOVA for comparison of more than two treatments, comparing treatment with control using Graphpad Prism 5.04 software. Values are expressed as mean ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = $P < 0.05$

Results

Effect of DMBA on *Cx37* and *Cx43* mRNA level

DMBA exposure increased ($P < 0.05$) *Cx37* mRNA (12.5 nM: 3.4-fold ± 0.9; 75 nM: 1.4-fold ± 0.9) at 4 days of exposure. In contrast, DMBA decreased ($P < 0.05$) *Cx43* mRNA (12.5 nM: 0.5-fold ± 0.004; 75 nM: 0.7-fold ± 0.2), compared to vehicle-treated ovaries at 4 days of exposure (Figure 1A). At 8 days, both *Cx37* and *Cx43* mRNA expression were decreased ($P < 0.05$) by 12.5 nM (*Cx37*: 0.9-fold ± 0.007; *Cx43*: 0.85-fold ± 0.008) and 75 nM (*Cx37*: 0.95-fold ± 0.04; *Cx43*: 0.95-fold ± 0.06) DMBA compared to vehicle-treated ovaries (Figure 1B).

Impact of DMBA on CX37 and CX43 protein level

CX37 protein level was increased ($P < 0.05$) by 12.5 nM and decreased ($P < 0.05$) by 75 nM DMBA at 4 days, relative to vehicle-treated ovaries. CX43 protein level was increased ($P < 0.05$) by 12.5 nM and by 75 nM DMBA at 4 days, relative to vehicle-treated ovaries (Figure 2A).

At 8 days, CX37 was decreased ($P < 0.05$) by 12.5 nM compared to vehicle-treated ovaries, but there was no impact of 75 nM DMBA on CX37. CX43 protein level was decreased ($P < 0.05$) by 12.5 nM and by 75 nM DMBA compared to vehicle-treated ovaries (Figure 2B).

Localization and amount of ovarian CX37 and CX43 proteins and impact of DMBA thereon

CX37 (Figure 3A-C) was localized to the oocyte cell surface. At 8 days, CX37 protein staining was increased ($P < 0.05$) in small primary follicles by 12.5 nM ompared to 75 nM and vehicle-treated ovaries and in large primary and secondary follicles, CX37 was decreased ($P < 0.05$) by 12.5 nM and by 75 nM DMBA exposures, relative to vehicle-treated ovaries, respectively (Figure 3H). CX43 (Figure 3D-F) was localized to the granulosa cells of all follicular stages. CX43 total protein staining was decreased ($P < 0.05$) by 12.5 nM and 75 nM DMBA exposures compared to vehicle-treated ovaries at 8 days (Figure 3G). In follicles that were devoid of oocytes, CX43 protein localization was absent or negligible.

DMBA effect on *p53* and *Bax* mRNA level

Relative to vehicle-treated ovaries, DMBA exposure decreased ($P < 0.05$) *p53* mRNA (12.5 nM: 0.7-fold ± 0.04; 75 nM: 0.6-fold ± 0.1) after 4 days of exposure (Figure 4A). In contrast, DMBA increased ($P < 0.05$) *p53* mRNA (12.5nM: 0.6-fold ± 0.1; 75 nM: 0.4-fold ± 0.09), compared to vehicle-treated ovaries after 8 days of exposure (Figure 4B)

DMBA exposure did not affect *Bax* mRNA after 4 days of exposure (Figure 4C). In contrast, DMBA increased ($P < 0.05$) *Bax* mRNA (12.5nM: 0.9-fold ± 0.08; 75 nM: 0.8-fold ± 0.1), compared to vehicle-treated ovaries after 8 days of exposure (Figure 4D).

Discussion

DMBA is an ovotoxicant that depletes follicles at various stages of development (Rajapaksa *et al.*, 2007; Igawa *et al.*, 2009). The initiating event in DMBA-induced follicle destruction remains unclear; however, since cell to cell communication is vital for follicular viability, it is possible that perturbation to communication within the follicle could have detrimental consequences for the oocyte. CX37 and CX43 are the major ovarian gap junction proteins, shown to be essential for folliculogenesis and production of fertilizable oocytes (Granot *et al.*, 2002; Kidder and Mhawi, 2002b; Teilmann, 2005). There is little information on whether ovotoxic chemicals target CX proteins and communication within the follicle, thus,

we investigated whether DMBA had any impact on *Cx37* and *Cx43*. Prior to DMBA-induced follicle loss, *Cx37* mRNA and protein was increased and although *Cx43* protein was elevated, mRNA encoding the *Cx43* gene was decreased. These data indicate that DMBA may target *Cx43* at the transcriptional level initially, and that increased *Cx37* mRNA and protein and CX43 protein may be part of the ovarian protective response to DMBA exposure. Both *Cx37* and *Cx43* mRNA and protein were decreased by DMBA exposures at 8 days. This time coincides with DMBA-induced follicle depletion (unpublished data) and therefore may simply be a consequence of reduced follicle number.

Cx37-null mice display retarded oocyte growth (Carabatsos *et al.*, 2000) due to lack of nutrient intake from extracellular environment (Eppig, 1991). We found that CX37 protein was localized to the oocyte cell surface, in agreement with a previous report in the mouse ovary (Teilmann, 2005; Simon *et al.*, 2006). Lack of organized CX37 localization around the oocyte perimeter has been reported as an early sign of follicular atresia (Teilmann, 2005), thus, the perturbations to *Cx37* mRNA and protein could impact follicular viability during DMBA-induced ovotoxicity. We did not observe obvious localization changes in CX37, however, interestingly, increased CX37 protein was evident in the small primary follicles at 12.5 nM DMBA. Since small primary follicle numbers are not impacted by DMBA at this concentration (unpublished data), it is possible that increased CX37 is protective against DMBA-induced follicle loss.

Cx43 mRNA and protein level are correlated with follicular development in immature rats (Granot and Dekel, 1997). In extra-ovarian tissues, CX43 is inversely correlated with apoptosis, acting as a survival factor (Lin *et al.*, 2003). The increase in *Cx43* mRNA level after 4 days of DMBA exposure potentially indicates an anti-apoptotic role for ovarian CX43. Consistent with this posit, at the time point when follicle loss was observed (unpublished data), *Cx43* mRNA and protein were decreased by DMBA exposure which is consistent with lower expression of *Cx43* mRNA and protein expression in cryopreserved apoptotic mouse ovarian tissue (Lee *et al.*, 2008). This decrease in *Cx43* mRNA and protein is also associated with increased caspase 3 protein in ovaries treated with the same DMBA concentrations (Ganesan *et al.*, 2013). An elevated level of apoptosis associated with low expression of CX43 has also been reported in avian granulosa cells (Krysko *et al.*, 2004). CX43 protein was localized between the granulosa cells in all stage follicles, consistent with a previous study that reported CX43 localization in the gap junctions connecting rat ovarian granulosa cells, with highest expression in pre-mature and pre-ovulatory antral follicles (Okuma et al., 1996; Gittens and Kidder, 2005). Interestingly, in large primary and secondary follicles that were devoid of oocytes, CX43 protein was either absent or negligible, implying that some oocyte-derived factor may be involved in CX43 protein maintenance. Despite our observed reduction of CX43 protein from total protein homogenates, the number of CX43 positive foci was increased. This may be representative of an attempt to increase gap junction communication and promote survival.

We also found increased levels of pro-apoptotic *p53* and *Bax* at the time of follicle loss (8 days) but not before the time of follicle loss (4 days). Thus, DMBA-induced changes in the *Cx37* and *Cx43* genes occurred prior to changes in pro-apoptotic components. Decreased p53 prior to follicle loss could be due to interaction of p53 with mdm2, resulting in p53 inhibition (Lakin and Jackson, 1999). Additionally, there was no induction of *Bax* until the time-point at which follicle loss was observed (day 8). These results are in agreement with another study that demonstrated increased pro-apoptotic BAX and caspase-3 in granulosa cells of DMBA-exposed pre-ovulatory follicles (Tsai-Turton et al., 2007). Future work would include examining whether post-translational changes to BAX and p53 proteins occur during DMBA exposure.

In summary, DMBA alters ovarian *Cx37* and *Cx43* at the transcriptional and post-transcriptional levels. The initial increase in CX37 and CX43 proteins in response to DMBA exposure demonstrates the capacity of ovarian tissue to mount a compensatory response to an ovotoxic insult.

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Highlights

DMBA increases *Cx37* and *Cx43* expression prior to follicle loss

During follicle loss both *Cx37* and *Cx43* expression are reduced

CX43 protein is absent in follicle remnants lacking an oocyte

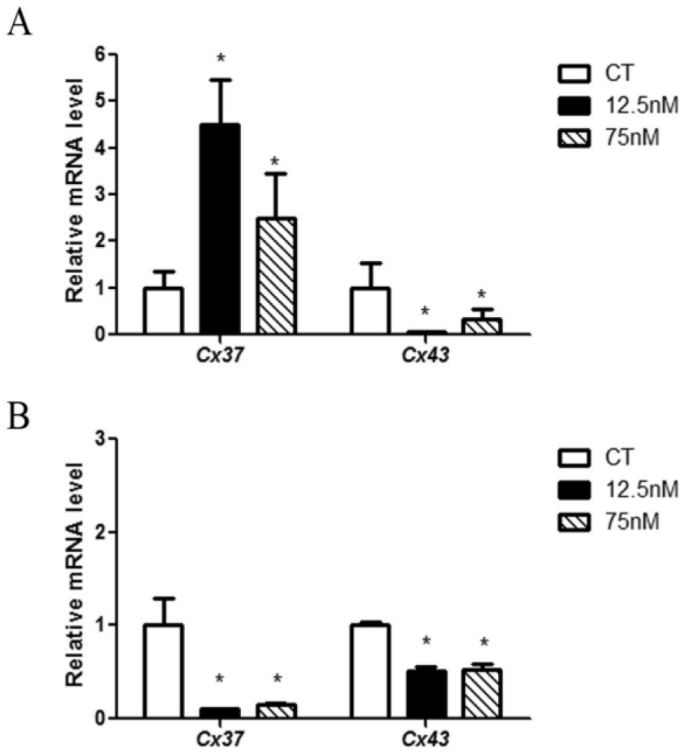

Figure 1. Effect of DMBA exposure on *Cx37* and *Cx43* mRNA expression

Total RNA was isolated from PND4 rat ovaries that were cultured in vehicle control medium for 4 days followed by a single exposure to control (CT), 12.5 or 75 nM DMBA for (A) 4 or (B) 8 days. qRT-PCR was performed to amplify *Cx37* or *Cx43* mRNA. Values are expressed as mean fold change ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = $P < 0.05$.

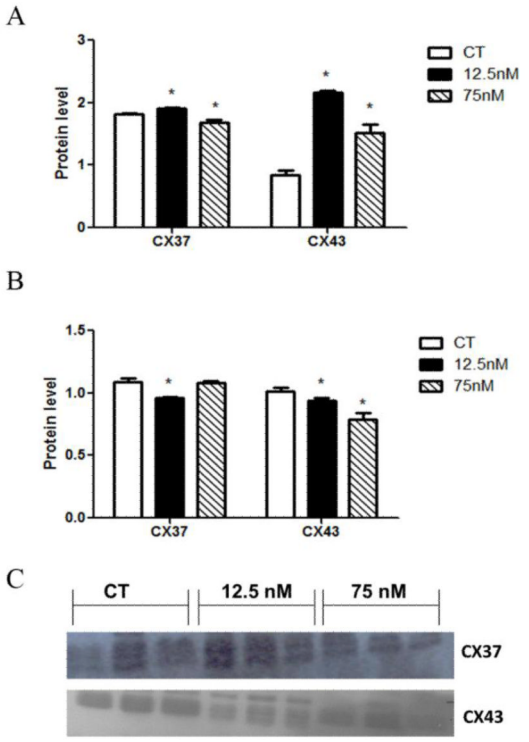

Figure 2. Effect of DMBA exposure on CX37 and CX43 protein level

Total protein was isolated from PND4 rat ovaries that were cultured in vehicle control medium for 4 days followed by a single exposure to control (CT), 12.5 or 75 nM DMBA for (A) 4 or (B) 8 days. (C) Western blotting was performed to quantify CX37 or CX43 protein level (day 8). Values are expressed as densitometric mean ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = $P < 0.05$.

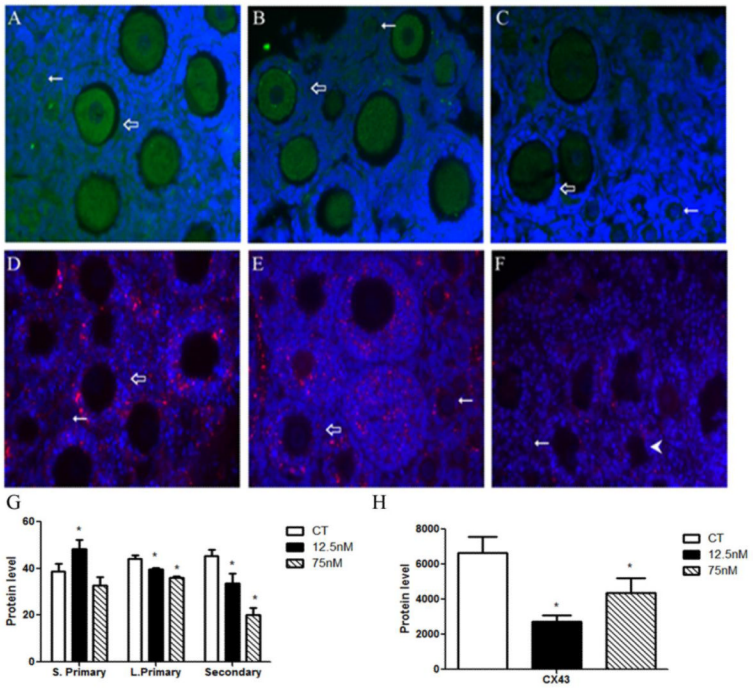

Figure 3. Localization of and impact of DMBA exposure on ovarian CX37 and CX43 protein level

PND4 rat ovaries were cultured in control medium for 4 days followed by a single exposure to vehicle control (CT), 12.5 or 75 nM DMBA for 8 days. Ovarian sections were immunostained using primary (A-C) CX37 (green stain) or (D-F) CX43 (red stain) antibodies to check the localization and intensity of staining. Blue staining represents nuclear DNA staining. Thin arrows indicate primordial/small primary follicles; thick arrows indicate large primary/secondary follicles; chevron indicates follicle lacking an oocyte. ImageJ software was used to analyze the intensity of staining. 10-15 follicles per ovary (n=3) were used for (G) CX37 quantification and 3 ovaries were used for (H) CX43 positive foci quantification. Values are expressed as densitometric mean ± SE. *= $P < 0.05$; different from control.

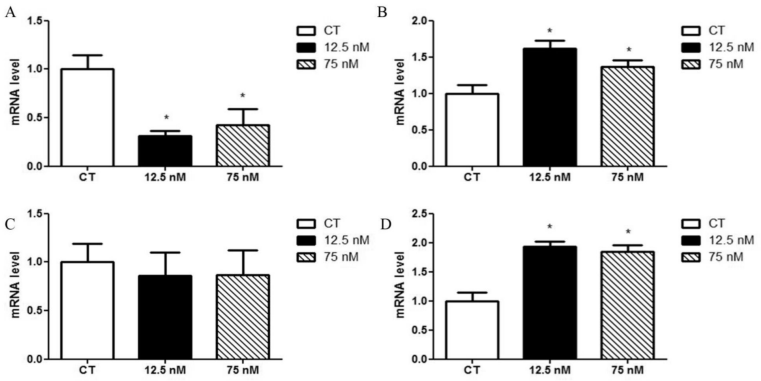

Figure 4. Effect of DMBA exposure on *p53* and *Bax* mRNA expression

Total RNA was isolated from PND4 rat ovaries that were cultured in vehicle control medium for 4 days followed by a single exposure to vehicle control (CT), 12.5 or 75 nM DMBA for (A, C) 4 or (B, D) 8 days. qRT-PCR was performed to amplify (A, B) *p53* or (C, D) *Bax* mRNA. Values are expressed as mean fold change ± SE; n=3 (10 ovaries per pool). Statistical significance was defined as * = $P < 0.05$.