

# *In Utero* and Lactational Exposure to Nicotine Alters the Intra-Ovarian IGF System in Adult Female Rats

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**Abstract:** In humans there is evidence that *in utero* exposure to cigarette smoke results in decreased fertility in female offspring. We have demonstrated in rats that fetal and neonatal exposure to nicotine alone results in impaired fertility and increased follicular atresia in the adult female offspring. In mammals, the insulin-like growth factor-I and -II (IGF-I and -II) and their binding proteins (IGFBPs) are considered stimulators and inhibitors of follicular growth and maturation. Therefore we hypothesized that dysregulation of the intra-ovarian IGF system could be implicated in the impaired fertility observed in nicotine-exposed offspring. Nulliparous female Wistar rats were exposed to nicotine (1 mg/kg/d) for 2 weeks prior to mating until weaning. Ovaries were collected on the morning of estrus from sexually mature saline- and nicotine-exposed offspring. Protein expression of IGF ligands and receptors (IGFR-I and IGFR-II) were quantified by western blot and immunohistochemistry. The expression of IGF-I, IGF-II; IGFR-I, IGFR-II; and IGFBP1–6 mRNA in the ovary was determined by semi-quantitative reverse transcriptase-PCR. Results showed that nicotine exposure significantly reduced IGF-I, IGF-II and IGFR-I protein expression ( $p < 0.01$ ) relative to saline controls. Furthermore, nicotine-exposed offspring had significantly reduced IGFR-II mRNA expression ( $p < 0.01$ ) in the ovary. Data from this study suggest that the decreased fertility and increased follicular atresia in nicotine-exposed animals may be due, in part, to disruption of IGF regulation in the ovary.

**Keywords:** nicotine, ovary, insulin-like growth factors, *in utero* exposure

## Introduction

It has been well documented that cigarette smoking is one of the most important and modifiable risk factors associated with impaired fertility in adult females.<sup>1</sup> Cigarette smoking is thought to affect female fertility via a number of alterations in ovarian function, including abnormal steroidogenesis, depleted ovarian reserves and increased frequency of oocyte chromosomal abnormalities.<sup>2–6</sup> However what is less well known is that fetal and neonatal exposure to cigarette smoke can lead to impaired fertility in the offspring. Indeed, population based studies have also revealed an association between fetal exposure to cigarette smoke and compromised fertility in adulthood.<sup>7,8</sup> However, the precise mechanism by which this phenomenon occurs remains unknown.

Of the over 4000 chemicals contained in cigarette smoke,<sup>9</sup> the compound(s) responsible for these adverse effects remains elusive, although animal studies suggest that nicotine, the major addictive component of cigarette smoke, may play an important role. Our laboratory has previously shown in a rat model that maternal exposure to nicotine during pregnancy and lactation results in reduced fertility, dysregulation of ovarian steroidogenesis, and altered follicle dynamics in the female offspring at six months of age compared to saline-exposed controls.<sup>10</sup> Although the mechanism by which prenatal nicotine exposure can adversely impact postnatal ovarian function is unknown, the change in follicle dynamics and decline in follicle health suggests that growth factors in the ovary may be affected.

Within the ovary, follicle growth, follicle differentiation, follicle maturation, release of viable oocyte and the formation and regression of the corpus luteum are precisely controlled by the coordinated regulation and action of the gonadal steroid hormones as well as factors that stimulate cellular proliferation and differentiation and those that inhibit these processes. Among potential intra-ovarian regulators, insulin-like growth factor has been widely studied and appears to serve as a central signal of a complete intra-ovarian growth factor system. The IGF family includes the IGF ligands (IGF-I and -II),

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type 1 and 2 IGF receptors (IGFR-I, -II), at least 6 high affinity binding proteins (IGFBP1-6) and IGFBP-directed endopeptidases.<sup>11</sup> These components are critical for normal ovarian function and indeed studies have demonstrated that the loss of IGF-I *in vivo* in animal models is associated with impaired ovulation and sterility.<sup>12</sup>

Cigarette smoking in adults has been associated with decreased serum IGF-I levels<sup>13,14</sup> and with decreased IGF-I levels in cord plasma of babies born to mothers who smoked.<sup>15</sup> Furthermore, the results from animal studies have demonstrated that the IGF axis is sensitive to perturbations during fetal life as chemical insults and metabolic stressors, including nicotine, have been shown to alter expression of key components of the IGF system in numerous organ systems postnatally.<sup>16–20</sup> Therefore the dysregulation of the intra-ovarian IGF system as a result of fetal and neonatal exposure to nicotine could have significant implications on ovarian function postnatally. The goal of this study was 1) to determine whether the observed reduction in fertility in nicotine-exposed offspring can be explained by dysregulation of the intra-ovarian IGF system and 2) determine whether the ability of nicotine to alter the intra-ovarian IGF system is via the nicotinic acetylcholine (nAChR) receptors in the fetal and neonatal ovary.

## Materials and Methods

### Maintenance and treatment of animals

All animal experiments were approved by the Animal Research Ethics Board at McMaster University, in accordance with the guidelines of the Canadian Council for Animal Care. Nulliparous 200–250 g female Wistar rats (Harlan, Indianapolis, IN, U.S.A.) were maintained under controlled lighting (12:12 L:D) and temperature (22 °C) with *ad libitum* access to food and water. Two weeks prior to mating the dams were randomly assigned to receive either saline (vehicle) or nicotine (n = 7 per group). Dams were injected subcutaneously with 1 mg/kg/day nicotine bitartrate (Sigma Aldrich, St. Louis, MO, U.S.A.) or saline for 14 d prior to mating, during pregnancy and post-partum until weaning. The dose of nicotine used in this study (1 mg/kg per day nicotine bitartrate) results in maternal serum cotinine concentrations of 136 ng/ml,<sup>10</sup> which is within the range of cotinine

levels (80–163 ng/ml) reported in women who are considered as ‘moderate smokers.’<sup>21</sup> In addition, this dose of nicotine resulted in serum cotinine concentrations of 26 ng/ml in the nicotine-exposed offspring at birth,<sup>10</sup> which is also within the range (5–30 ng/ml) observed in infants nursed by smoking mothers.<sup>22</sup> Litter size was culled to eight at birth (postnatal day 1; PND1) to assure uniformity of litter size between treated (nicotine) and control (saline) litters. After weaning (postnatal day 21; PND21), female offspring were selected randomly and caged as sibling pairs. To examine whether nicotine could act directly at the fetal and neonatal ovary, ovaries were collected at PND1 and PND21, frozen in liquid nitrogen and analyzed by RT PCR to determine if nAChR subunits are present.

Beginning at 6 months of age, vaginal swabs were performed daily to determine the time of estrous. In the morning of estrus, animals were sacrificed by CO<sub>2</sub> asphyxiation. Blood samples were collected, allowed to clot at 4 °C, centrifuged, and stored at –80 °C. Ovaries were excised; one frozen in liquid nitrogen and stored at –80 °C, the other fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned for immunohistochemistry.

### RT PCR

Expression of nAChR subunits ( $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\beta 2$ ,  $\beta 3$ ,  $\beta 4$ ) and components of the IGF system (IGFI, IGFI, IGFR-I, IGFR-II, IGFBP1-6) were determined by semi-quantitative RT-PCR using previously published primer sequences.<sup>23–25</sup> Total RNA was extracted from the frozen ovarian tissue using RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada) according to manufacturer’s instructions. RNA samples were demonstrated to have intact 18s and 28s RNA bands on ethidium bromide-stained agarose gels. RNA samples (n = 5 per group) were reverse-transcribed to cDNA in a 20  $\mu$ L reaction mixture containing 1  $\mu$ g of extracted RNA, 1  $\mu$ L of random primers, 1  $\mu$ L of dNTPs (10 mM), 4  $\mu$ L of 5X First-Strand Buffer, 1  $\mu$ L of 0.1M DTT, 1  $\mu$ L of RNase OUT, 1  $\mu$ L of Superscript III RT, and DNase/RNase free water (Invitrogen, Carlsbad, CA, U.S.A.), according to the standard protocol supplied with each product. The reaction was carried out in an iCycler thermal cycler (Bio-Rad, Hercules, CA, U.S.A.) using the following program: primer annealing for 5 min at 25 °C, RT for 60 min at 50 °C, and inactivation reaction for 15 min at 70 °C. The cDNA

was stored at  $-20^{\circ}\text{C}$  until use. cDNA was subjected to PCR with a total reaction volume of  $50\ \mu\text{L}$  using primers listed in Table 1. All amplifications were performed in the iCycler thermal cycler (Bio-Rad, Hercules, CA, U.S.A.) using  $48\ \mu\text{L}$  Platinum Blue PCR Supermix (Invitrogen, Carlsbad, CA, U.S.A.),  $1\ \mu\text{L}$  of template cDNA, and  $1\ \mu\text{L}$  of primer mix ( $10\ \mu\text{M}$ ; MOBIX, McMaster University, Hamilton, ON, Canada). After initial denaturation/enzyme activation step at  $94^{\circ}\text{C}$  for 2 minutes, 35 cycles of PCR were performed under the following

conditions: denaturing at  $94^{\circ}\text{C}$  for 30 sec, annealing at  $55^{\circ}\text{C}$  for 30 sec, extension at  $72^{\circ}\text{C}$  for 1 min/kb. Electrophoresis analysis was conducted on a 3% agarose gel stained with  $1.875\ \mu\text{l}$  ethidium bromide (EMD, Gibbstown, NJ, U.S.A.). PCR products were visualized with the Epi Chemi II Darkroom and Labworks software (UVP Inc., Upland, CA, U.S.A.), and with the use of a 100 bp DNA ladder specific gene products were confirmed. The optical density of the samples was measured and after being normalized to a housekeeping gene (lamin or

**Table 1.** Primer sequences for reverse transcriptase PCR.

Gene	Forward 5'–3'	Reverse 5'–3'	Running weight (bp)
<i>nAChR-<math>\alpha</math>2</i>	CTC CTG CAG CAT CGA TGT GAC CTT CTT	ATG CAC AGC GTG ATC TTC TCT CCA C	362
<i>nAChR-<math>\alpha</math>3</i>	GAA GTG ACT TGG ATC C	GTG GGC ATG GTG TGT G	589
<i>nAChR-<math>\alpha</math>4</i>	GCC ATC TAT AAG AGC TCC TGC AGC ATC	CTT CTC GCC AAA CTC TGA AGG CAG ATA G	359
<i>nAChR-<math>\alpha</math>5</i>	CGA ACG TCT GGT TGA AGC	CAC CAT AAT GGA ATA GGG	706
<i>nAChR-<math>\alpha</math>6</i>	TCT TAA GTA CGA TGG GGT GAT AAC	AAC ATG GTC TTC ACC CAC TTG	614
<i>nAChR-<math>\alpha</math>7</i>	TTG CCA GTA TCT CCC TCC AG	CTT CTC ATT CCT TTT GCC AG	213
<i>nAChR-<math>\beta</math>2</i>	GCT GAC GGC ATG TAC GAA G	GGA GGT GGG AGG CAC AAT C	506
<i>nAChR-<math>\beta</math>3</i>	CTC ATT ATC CAC CTC CGT TT	CTG TAT CAC TCT CCT TTC CAT CC	304
<i>nAChR-<math>\beta</math>4</i>	GGT TGC CTG ACA TCG TGT TG	GCC AAT GAG CGG TAT GTC	556
<i>IGF I</i>	TAT GGC TCC AGC ATT CGG A	AGG CGA CGT TCG GAT GTT T	230
<i>IGF II</i>	GGC ATT TGC TGA CCA TCC TCT	ACA TAT GGC ATT CGA CGC G	207
<i>IGFBP1</i>	GGT TCT CAG CAT GAA GCG GCA	TGA GGT GGA AGC TAT CCA GCA	103
<i>IGFBP2</i>	AAC TGT GAC AAT CAT GGC CTG	TGG ATT GGC TCC CGG TAT T	110
<i>IGFBP3</i>	CCA TGG TGG TCT GGC AAT TAC A	AAG CTG ACA GGC AGG CAG ATT	126
<i>IGFBP4</i>	GCA TCC CAA CAA CAG CTT CAA	CCC CAC ACC CTT CAT CTT GCT	103
<i>IGFBP5</i>	CAA GAG AAA GCA GTG CAA GCC	TGA AAG TCC CCA TCG ACG TAC	111
<i>IGFBP6</i>	AGG CCC ACT CCT GTT CAA GAT	TGC TTG CGG TAG AAA CCT CTG	149
<i>IGFR-I</i>	GCA TCT GAT CAT TGC TCT GC	GCC CAA TCT GCT GTT ATT CC	103
<i>IGFR-II</i>	GCA AGG GCA TAA AGG TGA A	TGT AAG TCA CCC TGT GCA A	497
<i>Lamin</i>	GCA GTA CAA GAA GGA GCT A	CAG CAA TTC CTG GTC CTC A	300
<i><math>\beta</math>-actin</i>	TTG TAA CCA ACT GGG ACG ATA TGG	GAT CTT GAT CTT CAT GGT GCT AG	800

$\beta$ -actin), the mRNA expression of the genes of interest was compared between treatment groups.

### Immunohistochemistry

Protein expression of IGF-I and IGF-II, in saline- and nicotine-exposed ovarian sections was determined using immunohistochemistry. Histological tissue sections (5  $\mu$ m) were cut from paraffin embedded sections using a rotary microtome. Briefly, tissues were deparaffinized and rehydrated and antigen retrieval was performed by immersion in 10 mM Citrate buffer (90 °C) for 12 minutes. After inhibition of endogenous peroxidase activity with 2% (vol/vol) hydrogen peroxide, tissues were blocked in 5% (wt/vol) bovine serum albumin in PBS for 10 minutes. Tissues were then incubated overnight at 4 °C in a humidified chamber with anti-IGF-I antibody (1:500 R&D Systems, Minneapolis, MN, U.S.A.) and anti-IGF-II antibody (1:600, R and D Systems, Minneapolis, MN, U.S.A.). Anti-sera was diluted in 0.01 M PBS (pH 7.5) containing 2% (wt/vol) BSA and 0.01% (wt/vol) sodium azide (100  $\mu$ l/slide). All subsequent incubations were at room temperature. Biotinylated anti-rabbit, anti-mouse, or anti-goat IgGs (all 1:100 dilution; Sigma Aldrich, St. Louis, MO, U.S.A.) were diluted in the same buffer and incubated 1 hour. The slides were then washed in PBS and incubated with avidin and biotinylated horseradish peroxidase (1:30 dilution) (Extravidin, Sigma Chemical Co, St. Louis, MO, U.S.A.). Peptide immunoreactivity was localized by incubation in fresh diaminobenzidine tetrahydrochloride (DAB tablets, 10 mg, Sigma Aldrich, St. Louis, MO, U.S.A.) with 0.03% (vol/vol) hydrogen peroxide for 2 min. To determine antibody specificity, primary antibody omission controls were included. For these controls were treated the same as experimental tissue, with the exception of incubation with antibody diluent in the place of primary antibody. Tissue sections were counterstained with Carazzi's Hematoxylin for 1 min. Tissues were dehydrated and placed under a coverslip with Permount (Fisher Scientific, Pittsburgh, PA, U.S.A.). Stained slides were imaged using a brightfield microscope and immunostaining was quantified using image analysis software (Image Scope, Aperio, CA, U.S.A.). This software allows for the detection of DAB-positive staining, indicative of specific antibody localization. Staining was quantified as the

percentage of immunopositive ovarian tissue in 6 fields of view per section, with a minimum of 4 animals per group.

### Western blotting

IGFR-I, and IGFR-II protein expression was measured in whole ovary homogenates (n = 5 per group) of nicotine and saline-exposed offspring. Protein was extracted from the frozen tissue using RIPA lysis buffer (15 mM Tris-HCL, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 167 mM NaCl, 0.5% (w/v) sodium deoxycholate acid), with Complete Mini EDTA-free protease inhibitors (Roche Applied Science, Laval, QC, Canada). For Western Blots, 20  $\mu$ g of protein plus 4X SDS-PAGE sample loading buffer ( $\beta$ -mercaptoethanol, (2%), Glycerol (29%), SDS (8%), Bromophenol Blue (0.25%), 0.5M Tris, pH 6.8 (42.75%)) was loaded into precast gels (Pierce, Rockford, IL, U.S.A.) and subjected to SDS-PAGE and then electro-transferred to PVDF blotting membrane (BioRad Laboratories, Hercules, CA, U.S.A.). Membranes were blocked for 1 hour at room temperature and then overnight at 4 °C with 5% (wt/vol) skim milk in TTBS on a rocking platform. Membranes were then incubated for 1 h at room temperature in primary antibody on a rocking platform IGFR-I: 1:1000, R&D Systems, Inc., Minneapolis, MN, U.S.A.; IGFR-II: 1:1000, BD Transduction Laboratories, San Jose, CA, U.S.A. After washing with TTBS (TBS, 0.5% (v/v) Tween 20), blots were incubated with peroxidase-conjugated secondary, anti-goat IGFR-I – 1:2000; Sigma Aldrich, St. Louis, MO, U.S.A. or anti-mouse IGFR-II – 1:2000; Amersham Biosciences, Piscataway, NJ, U.S.A. antibodies for 1 h at room temperature on a rocking platform. Blots were washed thoroughly in TTBS, followed by TBS after immunoblotting. Reactive protein was detected with ECL Plus chemiluminescence (Amersham Biosciences, Piscataway, NJ, U.S.A.) and Bioflex X-ray film (Clonex Corporation, Markham, ON, Canada). Densitometric analysis of immunoblots was performed using ImageJ 1.27v software; all proteins were quantified relative to the loading control,  $\alpha$ -tubulin (Abcam Inc. Cambridge, MA, U.S.A.).

### Statistical analysis

All statistical analyses were performed using Student's t test (SigmaStat, v.2.03, SPSS, Chicago,

IL, U.S.A.). Data were tested for normality as well as equal variance, and when normality or variance tests failed, data were analyzed using the Mann-Whitney rank sum test. Values are presented as mean  $\pm$  SEM.

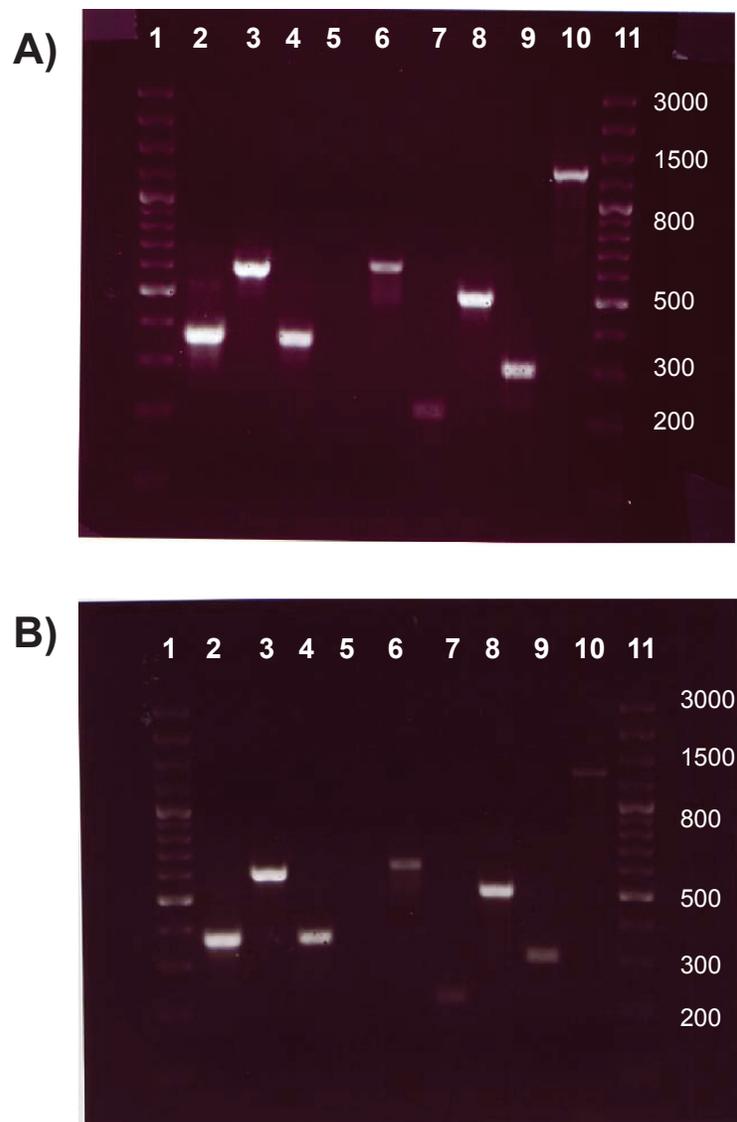
## Results

### Expression of nAChR subunits in neonatal ovarian tissue

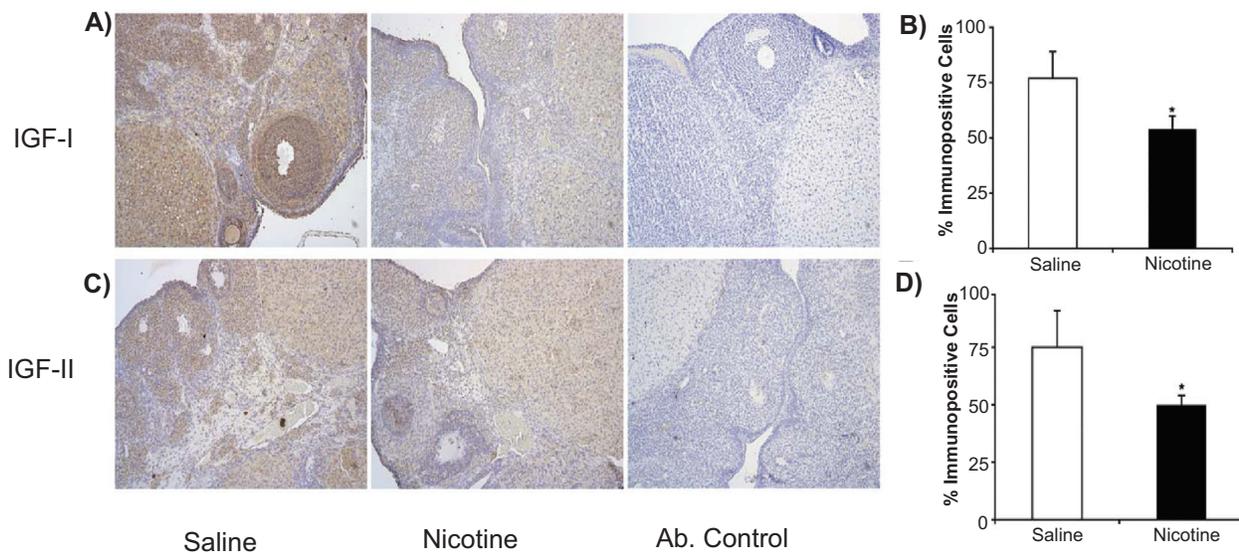
At PND1 and PND21 all of the nAChR subunits except  $\alpha 5$  were present in the ovary of both saline- and nicotine-exposed offspring (Fig. 1).

### Protein expression of intra-ovarian IGF axis components

Fetal and neonatal exposure to nicotine caused significant changes in components of the intra-ovarian IGF system. Both IGF-I and II were expressed in ovarian stroma, theca and granulosa cell compartments (Fig. 2). The IGF ligands (IGF-I and IGF-II) were significantly ( $p < 0.05$ ) decreased in nicotine-exposed offspring (Fig. 2). Loss of expression of IGF-I and -II occurred in all ovarian cell compartments and the loss did not appear elevated or reduced in any compartment compared to the others. IGFR-I protein expression was significantly decreased in the nicotine exposed



**Figure 1.** mRNA expression for nAChR subunits in a single representative saline control ovary at: **A)** PND1 and **B)** PND21. Lane: (1) 100 bp DNA ladder; (2)  $\alpha 2$ ; (3)  $\alpha 3$ ; (4)  $\alpha 4$ ; (5)  $\alpha 5$ ; (6)  $\alpha 6$ ; (7)  $\alpha 7$ ; (8)  $\beta 2$ ; (9)  $\beta 3$ ; (10)  $\beta 4$ ; (11) 100 bp DNA ladder. Ovaries from nicotine-exposed animals had the same pattern of nAChR subunit expression.



**Figure 2.** Immunohistochemical staining and semi-quantification of IGF Ligands: **B)** IGF-I, **D)** IGF-II in saline—(white bars) and nicotine—exposed (black bars) ovary at 26 weeks of age. A representative image for each ligand is presented in panel **A)** and **C)**. Primary antibody omission controls demonstrate a lack of non-specific antibody localization. Data are presented as mean  $\pm$  SEM. Values with an asterisk are significantly different from the saline control ( $p < 0.05$ ).

ovaries (Fig. 3A,  $p < 0.05$ ), whereas there was no effect of the nicotine exposure on the expression of IGFR-II (Fig. 3B).

#### mRNA expression of the intra-ovarian IGF axis components

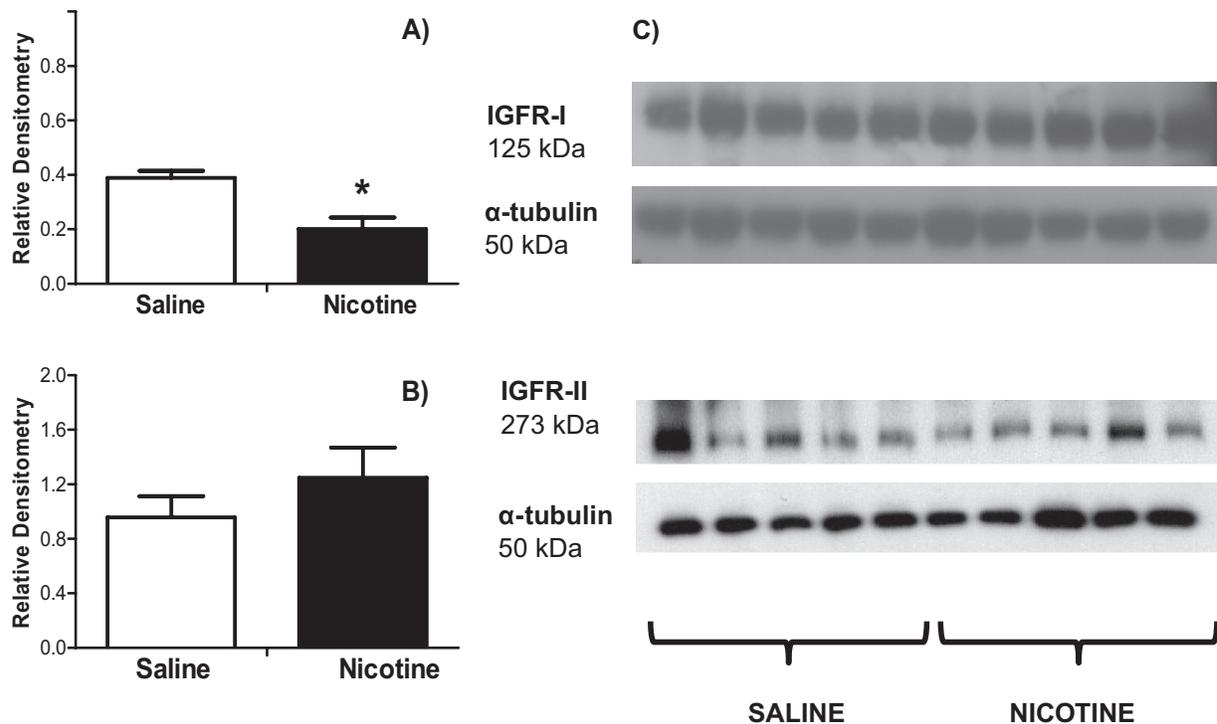
Fetal and neonatal exposure to nicotine did not affect the gene expression of the two IGF ligands, nor did it affect the expression of any of the six IGF binding proteins. Fetal and neonatal exposure to nicotine did significantly ( $p < 0.01$ ) decrease IGFR-II mRNA but did not alter IGFR-I mRNA expression (Table 2).

### Discussion

In the past few decades there has been an increased concern globally that fetal exposure to environmental chemicals is resulting in adverse health consequences in offspring. These concerns have largely focused on reproductive disorders and infertility as chemical insults *in utero* have been shown to cause impaired postnatal fertility.<sup>26–30</sup> The majority of previous research has focused on compounds with estrogenic activity including polychlorinated biphenyls, herbicides, chlorinated hydrocarbon pesticides and plasticizers. More recently there has been an increased interest in the reproductive health consequences to the offspring as a result of maternal lifestyle choices, such as smoking.<sup>8</sup>

Epidemiological studies have shown an association between maternal smoking and reduced fertility in the female offspring in adulthood although the mechanisms are unknown.<sup>7,31</sup> Results from *in vivo* and *in vitro* studies have clearly shown that nicotine alone can have adverse effects on adult ovarian function, including an increased number of atretic follicles, reduced ovarian and uterine weights, and irregularities in the estrous cycle<sup>32,33</sup> Nicotine has been shown to cross the placenta and accumulates in breast milk during lactation resulting in both fetal and neonatal exposure to nicotine<sup>34</sup> We have previously demonstrated in our rat model that prenatal and lactational exposure to nicotine results in reduced fertility in the female offspring at 6 months of age as well as altered follicle dynamics resulting in increased atresia of growing follicles.<sup>10</sup> Because a loss of IGF-I *in vivo* has been associated with impaired ovulation and sterility and cigarette smoke has been associated with reduced IGF-I serum levels<sup>12–15</sup> we hypothesized that the reduced fertility seen in the nicotine-exposed female offspring is due to alteration in the intra-ovarian IGF system.

In this study, we have shown that nicotine-exposed animals have decreased expression of IGF-I and IGF-II, and the IGFR-I at the same age where we have previously observed decreased fertility and increased follicle atresia.<sup>10</sup> In the ovary, IGF-I and to a lesser degree IGF-II bind



**Figure 3.** Quantification of protein expression of IGF receptors: **A)** IGFR-I, **B)** IGFR-II in saline—(white bars) and nicotine-exposed (black bars) ovary at 26 week of age ( $n = 5$  per group). A representative Western blot for each receptor is presented in panel **C)**. All protein expression was quantified relative to  $\alpha$ -tubulin loading controls. Data are presented as the mean  $\pm$  SEM. Values with an asterisk are significantly different from the saline control ( $P < 0.05$ ).

to the IGFR-I to stimulate follicle growth and maturation. IGF-I activity has previously been detected in the granulosa cells of healthy follicles but, in contrast, expression of IGF-I mRNA is not observed in atretic follicles.<sup>35</sup> In response to nicotine

exposure, a decrease in IGFR-II was detected, while IGFR-I was unchanged. Conversely, at the protein level, IGFR-I was decreased while IGFR-II was not different. The reason for this discrepancy is not clear, but likely involves changes in transcriptional

**Table 2.** mRNA expression of components of the IGF system in the ovary at 26 weeks of age, as determined by semi-quantitative RT PCR.

Gene	Nicotine	Saline	P value
<i>IGF-I</i>	0.64 $\pm$ 0.13	0.53 $\pm$ 0.07	$p = 0.505$
<i>IGF-II</i>	2.16 $\pm$ 0.32	2.02 $\pm$ 0.38	$p = 0.785$
<i>IGFR-I</i>	1.37 $\pm$ 0.31	1.37 $\pm$ 0.32	$p = 0.715$
<i>IGFR-II</i>	0.75 $\pm$ 0.17	1.43 $\pm$ 0.14	$p = 0.008^*$
<i>IGFBP1</i>	0.64 $\pm$ 0.13	0.53 $\pm$ 0.07	$p = 0.505$
<i>IGFBP2</i>	1.58 $\pm$ 0.47	1.04 $\pm$ 0.19	$p = 0.306$
<i>IGFBP3</i>	1.07 $\pm$ 0.2	1.01 $\pm$ 0.07	$p = 0.768$
<i>IGFBP4</i>	1.59 $\pm$ 0.23	1.18 $\pm$ 0.10	$p = 0.136$
<i>IGFBP5</i>	1.61 $\pm$ 0.23	1.19 $\pm$ 0.1	$p = 0.134$
<i>IGFBP6</i>	1.17 $\pm$ 0.23	1.01 $\pm$ 0.1	$p = 0.542$

All gene expression was quantified relative to a  $\beta$ -actin or lamin loading control. Data are presented as the mean  $\pm$  SEM. Values with an asterisk are significantly different from the saline control ( $p < 0.01$ ).

regulation of the IGF receptors, or changes in receptor mRNA stability. Nicotine has been shown to increase gene transcription of pro-enkephalin in bovine chromaffin cells<sup>36</sup> and alters tyrosine hydroxylase mRNA stability in rodent vascular cells.<sup>37</sup> Although the effects of nicotine on IGF gene transcription or mRNA stability have not been studied to date, it is likely that nicotine does alter gene and protein expression through these mechanisms. Studies have also shown that IGFs not only stimulate ovarian cell growth, but are also responsible for the attenuation of apoptotic cell death that occurs at various stages of follicle development.<sup>12,38</sup> In bovine ovaries, IGF availability and the expression of IGFs have been suggested to play a role in determining whether a follicle will progress to dominance and ovulate or succumb to atresia.<sup>38</sup> A reduction in IGF signaling in the ovary of the nicotine-exposed females may therefore be sufficient to explain the reduced number of growing follicles and impaired fertility we have previously reported.<sup>10</sup> The IGFs have been shown to be involved in follicle selection, dominance, and ovulation and reduced IGF expression is associated with decreased ovulatory events.<sup>35,39</sup> It is possible that nicotine, acting via the nAChR in the ovary directly initiated a change in key components of the intra-ovarian IGF axis. However, the mechanism by which fetal and neonatal exposure to nicotine can permanently alter the ovarian IGF axis is currently unknown.

The consequence of a dysregulated intra-ovarian system may extend beyond altered follicle growth and dynamics, as the ovarian steroidogenic pathway is modified by a number of growth factors including IGF-I and IGF-II. IGF-I has been shown to stimulate basal and FSH-supported synthesis of progesterone, estrone and androgens by granulosa and theca cells.<sup>40–43</sup> The stimulatory effects of IGFs on steroidogenic cells have been attributed to increased cAMP generation and enhanced uptake of lipoproteins.<sup>44</sup> Moreover, it has been shown that IGF-I can also affect ovarian steroidogenesis by increasing the activity and expression of key steroidogenic enzymes including steroid acute regulatory protein (StAR),<sup>42</sup> cholesterol side-chain cleavage cytochrome P450 (CYP11A1),<sup>41,45</sup> 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD)<sup>41,46</sup> and aromatase (CYP19A1).<sup>47</sup> Whether these key steroidogenic enzymes are altered in our animal model as a result of changes in the expression of the IGF system is currently under investigation.

One-fifth of pregnant women smoke.<sup>48</sup> Results from this study have shown that maternal nicotine use resulting in prenatal and lactational exposure to nicotine may cause alterations in the intra-ovarian IGF system, providing a potential mechanism behind the decreased fertility seen in the adult offspring. Increasingly, pregnant women who are unable to quit smoking are encouraged to use nicotine replacement therapy (NRT), even though the safety of this practice has not been well studied. Although there are no reports of decreased fertility in NRT exposed children, evidence from this study raises concerns about the long-term health effects of NRT and nicotine product use during pregnancy.

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## Disclosure

The authors report no conflicts of interest.

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