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Title: Androgen Excess Down-regulates Connexin43 in a Human Granulosa Cells Line

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Abstract: Objective: To investigate the effects of androgen excess on connexin43 (Cx43) expression in human granulosa cells in vitro.
Design and Setting: Controlled experimental study in a Medical Center
Intervention(s): Human granulosa cell line (HO-23) was treated by various dosages of dihydrotestosterone (DHT). The effect of DHT treatment on granulosa cell Cx43 was assessed by using Western blot. Gap junctional intercellular communication (GJIC) between granulosa cells was investigated by using the scrape-loading and dye transfer. Androgen receptor antagonist, flutamide, was used to test the specificity of the observed androgen responses.
Main Outcome Measure(s): Cx43 protein expression following DHT treatment.
Result(s): Treatment of the HO-23 cells with DHT showed a dose-dependent decrease in Cx43 protein expression. Flutamide significantly blocked the inhibitory effects of DHT on Cx43 expression. DHT-treated cells demonstrated a decreased enhancement of GJIC as assessed by dye transfer experiments.
Conclusion(s): High level androgen reduces Cx43 expression and impairs GJIC between human granulosa cells through androgen receptors. It may impair folliculogenesis, induce follicular atresia and lead to ovulatory dysfunction.

2010/6/16

Alan H. DeCherney, M.D.
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Department of Obstetrics and Gynecology
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Dear Prof. DeCherney:

We would like to resubmit our manuscript entitled "**Androgen Excess Down-regulates Connexin43 in a Human Granulosa Cells Line**" for publication in your cerebrated journal, **Fertility and Sterility**.

We have answered the reviewers' and editorial's comments in the revision as below:

Editorial's comment:

1) We had corrected Reference 4.
2) Our abstract had been shortened to two sentences.

Reviewer's comment:

1) We had corrected into "induce" and "lead". (Abstract, lines 30, 31)
2) We change mammalians into mammalian. (Page 10, line 39)
3) Figure 1-B, bottom: change B+D+ into B+D+F

In addition, we declare that the material contained in this manuscript has not been published, or is being submitted elsewhere. There is no interest conflict in this study.

With best wishes,

Looking forward to hearing from you soon, preferably by E-mail or by Fax.

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Running title: Androgen excess down-regulates Cx43 in granulosa cells

Androgen Excess Down-regulates Connexin43 in a Human Granulosa Cells Line

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Capsule:

Androgen excess reduces Cx43 expression and impairs GJIC between human granulosa cells.

By using a cell model, we found high level androgen reduces connexin 43 expression and impairs gap junction intercellular communication between human granulosa cells through androgen receptors. This finding suggests that high level androgen may impair folliculogenesis which in turn leads to ovulatory dysfunction in polycystic ovarian syndrome patients.

Androgen excess is the central defect in polycystic ovarian syndrome (PCOS) patients and androgen excess is believed to be involved in the pathogenesis of follicular development arrest (1,2). Jakimiuk *et al.* (3) have shown that women with PCOS express elevated levels of 5α reductase mRNA in the granulosa cells. Thus, it is conceivable that the deleterious effects of androgens in the ovary may be mediated through the conversion to 5α reduced metabolites.

Numerous literatures have shown that normal folliculogenesis relies on the bidirectional talk between granulosa cells and oocytes (4, 5). By facilitating the transfer of ions and small molecules from cell to cell, gap junction intercellular communication (GJIC) between granulosa cells and oocytes plays a critical role in folliculogenesis and oogenesis (6, 7). Connexins (Cx) are membrane-spanning proteins that assemble to form the intercellular channels of gap junctions. At least 20 rodent and 21 human connexins have been identified and named according to their molecular weights (8). Gap junctions between ovarian granulosa cells contain predominantly Cx43 which is present at all stages of follicle development (6, 7); while Cx37 is expressed at the oocyte surface in oocyte-somatic cell gap junctions, with little if any contribution from Cx43 (9; 10). Using chimeric ovaries, Gitten and Kidder (11) demonstrated that Cx37 is required for oocytes but not granulosa cells development; while Cx43 is required for granulosa cells but not oocytes development. The expression level of Cx43 protein is increased as the follicle grows and matures and decreased during follicular atresia (22). Taken together, these data suggested that Cx43 gap junction communication is critical for ovarian folliculogenesis.

Hormones including estrogen, androgen, progesterone, gonadotropins, and thyroid

hormone, regulate expression of connexins. However, to the best of our knowledge there have been no reports regarding the effect of androgen on Cx43 expression in human granulosa cells. We hypothesize that decreased Cx43 expression and GJIC activity are induced by high levels of androgens in granulosa cells in PCOS ovaries, resulting in impaired folliculogenesis and anovulation - a hallmark of PCOS. We treated the human granulosa cell line (HO-23) cultured in vitro with different concentrations of dihydrotestosterone (DHT), a 5α reduced metabolite of testosterone that has the highest affinity for the androgen receptor (AR) , to investigate the Cx43 protein levels and to evaluate the effect of androgen on connexins expression in human granulosa cells.

The HO23 human immortalized luteinized granulosa cell line was provided by Dr. Abraham Amsterdam, Weizmann Institute of Science, Rehovot 76100, Israel (13). Granulosa cells were maintained at 37°C in 5% CO2 /air in Dulbecco's minimal essential medium (DMEM)/Ham's F12 (1:1), supplemented with 5% fetal calf serum and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). To test the effect of DHT on Cx43 expression, cells (2 x 10^6) were seeded on 100 mm culture dishes and incubated for 24 hours. The media were removed, and the cells were reincubated in fresh media with different concentrations of DHT. The cells were harvested for protein assays at 24 hours.

Protein content in cell lysate was determined by the bicinchoninic acid (BCA) protein assay method (Pierce, USA). After electrophoresis, the proteins were transferred onto

polyvinylidene difluoride membrane (Bio-Rad Laboratories, Ca, USA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 1 hours and incubated with mouse anti-human Cx43 antibody (Sigma-Aldrich Co. , USA) (1:3000) in 0.1% nonfat dry milk overnight at 4°C. After three washes in TBS containing 0.1% Tween-20, the membranes were incubated with goat anti-mouse IgG (Chemicon, USA) (1:5000) conjugated with alkaline phosphatase (Sigma-Aldrich) for 1 hour. The membranes were washed again as above, applied to the enhanced chemilunimescence (ECL) detection kit (Amersham, UK), and exposed to X-ray film (BioMAx films, Eastman Kodak Company, Rochester, NY, USA. To assess the amount of Cx43 protein loaded, filters were treated with 2% SDS and 100 mM β-mercaptoethanol in 62.5 mM Tris-HCl (pH 6.8) for 30 min at 60°C and reprobed for β-actin. Films were scanned, and the optical density of the bands was measured with Scion Image. Data shown are representative of at least three independent experiments with similar results.

Assessment of GJIC was determined using the scrape-loading and dye transfer (SLDT) technique with a fluorescent dye, Lucifer Yellow (LY, Sigma, USA) (14). Briefly, human granulosa cells, cultured as described above, were washed thoroughly with PBS. SL was performed applying three cuts on cell monolayer with a surgical scalpel, and then a mixture of 1% LY and 1% of rhodamine dextran in PBS was added to the cells. The cells were incubated for 5 min and washed three times with PBS to remove background

fluorescence. The cells were then fixed with 4% paraformaldehyde and photographed with a fluorescent microscope equipped with a camera. Cells that received the LY from the scrape-loaded cells were considered as communicating. The dye-coupled cell layers on either sides of the scrape were counted to evaluate the GJIC.

Data are expressed as mean ± SEM of at least three independent experiments performed at different time points. All data were analyzed using one-way analysis of variance (ANOVA). If the differences were significant, a Dunnett *post hoc* test was used for post-ANOVA multiple comparisons. Statistical significance was determined as $P < 0.05$.

The effect of DHT on Cx43 protein expression in response to 8-Br-cAMP was examined by Western blot analysis. 8-Br-cAMP, an analogue of cyclic AMP, is known to activate adenylate cyclase and increase the adenosine cAMP pool in the granulosa cells and up-regulates the Cx43 expression (15). A previous study showed the range of DHT in follicular fluid of normal population is 0.77 ± 0.11 ng/ml (16). To demonstrate the effect of androgen excess on Cx43 expression, cultured human granulosa cells were treated with increasing doses of DHT at 0, 1, 10, or 100 ng/ml. Western blot analysis of DHT-treated granulosa cell protein samples demonstrated decreasing amount of Cx43 protein in a dose-dependent manner. In addition, a significant decrease in the expression could be observed up to 24 h after treatment (data not shown).

To assess whether the suppressive effect of DHT on Cx43 is mediated through the AR, cells were incubated with DHT (10 ng/ml) and flutamide (100 ng/ml, a 10-fold excess above that of DHT). Flutamide, an AR antagonist, significantly blocked the inhibitory effects of DHT on Cx43 expression as shown in Figure 1-A.

To understand the association between reduced Cx43 expression and GJIC activity, we assessed the ability of HO-23 granulosa cells to transfer Lucifer yellow through gap junctions using the scrape-loading dye transfer (SLDT) technique in corresponding cultures of cells with reduced Cx43 expression. As shown in Figure 1-B, the number of dye-coupled cell layers was significantly reduced on either side of the scrape in HO-23 granulosa cells grown in 8-Br-cAMP with DHT medium compared with cells grown in 8-Br-cAMP medium. The phenomenon was prevented upon replenishing the medium with flutamide. This gap junctional defect could be induced with DHT and the effect was recovered with addition of flutamide to the culture medium.

Although anovulation is frequently observed in PCOS patients with androgen excess, the underlying mechanism has not been fully elucidated. Several possibilities have been raised to explain how androgens might mediate anovulation. In this study, we propose a new pathway through which high androgen level down-regulates Cx43 expression, reduce GJIC in human granulosa cells, and finally lead to impaired folliculogenesis.

Androgens and their 5α reduced metabolites like DHT have been shown to perturb the

ovarian physiology in animals (17, 18). One of the targets of this disruptive action is the granulosa cell within the ovarian follicle. While both androgen and gap junctions are reported to have important roles in follicular development, our study provides a novel mechanism which links androgen excess and Cx43 expression in the granulosa cells. We showed reduced GJIC activity in the milieu of high androgen and reduced Cx43 expression which occurs at the translation level mediated through androgen receptor.

A link between reduced GJIC activity and reduced connexin gene expression has been previously reported in rat ovarian cells exposed to luteinizing hormone (LH). LH inhibited GJIC activity by down-regulating Cx43 synthesis (19). Recently, Wang et al. (20) provided the evidence that the strength of gap junctional conductance was positively correlated with Cx43 level in human cumulus cells. Although it is possible that the effect of high androgen on GJIC activity may be mediated via other junction protein components, our findings indicate that reduced GJIC activity in human granulosa cells exposed to high androgen is at least in part due to inhibition of Cx43 gene expression.

The expression and function of Cx43 protein in the ovary of mammalian species have been discussed previously (21). In the mouse ovary, Cx43 is an important gap junction protein required for granulosa cell to granulosa cell communication, which in turn is required for follicle and oocyte maturation (7). Decreased Cx43 protein level is associated with follicular atresia, supporting the hypothesis that a loss of gap junctional communication plays

a coordinating role in the process of atresia (12). Disruption of granulosa cell coupling not only impairs follicle growth, but also impacts negatively on the quality of the enclosed oocytes. The knock-out mice of Cx43 showed that GJIC activity was reduced and oocyte growth in mutant follicles was retarded (22). However, we know little about Cx43 in human ovarian follicles and their roles in human folliculogenesis and fertility. A recent study shows that Cx43 was primarily localized in the membrane where it forms gap junction-like plaques between human cumulus cells, and its expression level may influence pregnancy outcome from in vitro fertilization (20). Furthermore, high Cx43 levels in granulosa cells are linked to good prognosis of human oocytes (9).

The roles of androgen and androgen receptor (AR) are well-established in the development of male reproductive organs and spermatogenesis. However, the functions of this steroid and its receptor in the ovary remain elusive. AR expression in the ovary has been described at various stages of folliculogenesis in several species. In human ovaries, AR protein expression is observed at different stages in granulosa and theca cells (23). Using a Cre–loxP system to generate AR-knockout (ARKO) mice, investigators showed that activated ARs are indispensable for normal folliculogenesis (24, 25). In this study, we demonstrate that androgen regulates Cx43 expression in cultured human granulosa cells by an AR-dependent process considering suppressive effect of DHT on Cx43 was blocked by an AR antagonist, flutamide. The findings provide a theoretical rationale for the clinical use of flutamide in the

treatment of patients with PCOS and anovulation. In fact, flutamide is able to restore ovulation in some women with PCOS (26, 27). However, there is still concern about the use of flutamide in women with infertility considering its possible teratogenic effect (28).

The root cause of anovulation in PCOS is now thought to be associated with the significant abnormalities in the very earliest stages of folliculogenesis (29). The normal 'dialogue' between oocyte and granulosa cells in these early growing follicles is altered. Recently, Li et al. (30) raised a hypothesis that if dysfunctional communications exist between granulosa cells or granulosa cells and the oocyte, unnecessary androgen will enter the oocyte and bind to the AR, provoking some follicles to undergo atresia. We therefore propose a working mode for the effect of androgen excess on folliculogenesis and oogenesis. High androgen level down-regulates Cx43 translation and impairs the communication between granulosa cells and possibly communications between granulosa cells and oocyte. Impaired communications negatively impacts folliculogenesis. The adverse effect of androgen excess is further strengthened by "shunting" of excessive androgens to the oocytes secondary to impaired communications between granulosa cells.

In conclusion, the present study provides the first evidence that increased levels of androgen down-regulate Cx43 expression and impair communications between granulosa cells. The effect may compromise folliculogenesis and oogenesis in PCOS patients. Caution should be taken to extrapolating in vitro results using immortalized granulosa cell lines to the

clinical setting considering few clinical data in this study. In addition, how DHT affects Cx43 translation still awaits further investigation.

Acknowledgment:

This study was supported by grants from the Changhua Christian Hospital (94-CCH-IRP-07)

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Figure legends

Figure 1-A. Antiandrogen treatment blocked the inhibitory effects of DHT on Cx 43 expression. HO-23 cells were cultured for 24 h under serum-free conditions in the presence of 8-Br-cAMP (0.1 mM), DHT (10ng/ml), and the antiandrogen flutamide (100 ng/ml)**.** Data are expressed as the mean ± SD of three different experiments. (* : $p < 0.05$ vs. 8-Br-cAMP)

B: 8-Br-cAMP D: DHT F: flutamide

Figure 1-B: Analysis of GJIC activity in HO-23 cells using SLDT technique. RD remains in the cells immediately bordering the scrape, while the LY diffuses to neighboring cells in gap junction-enabled cells. GJIC was evaluated as the counts from dye-coupled cell layers and is represented as a histogram. GJIC is blocked in HO-23 cells treated with 8-Br-cAMP (0.1 mM) and DHT (10ng/ml) (* : $p < 0.05$ vs 8-Br-cAMP). Flutamide (100 ng/ml) reversed the

blockage of GJIC induced by DHT to a comparable level to 8-Br-cAMP (ns, not significant vs 8-Br-cAMP). Data are expressed as the mean ± SD of three different experiments.

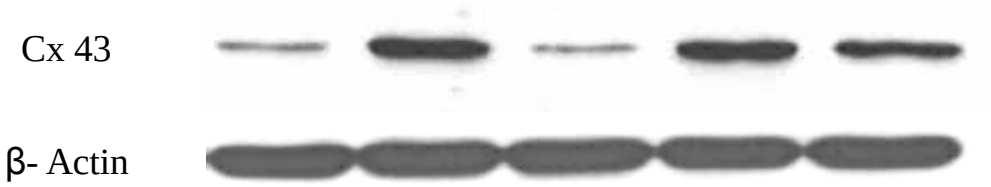
B: 8-Br-cAMP D: DHT F: flutamide

GJIC: gap junction intercellular communication

SLDT: scrape-loading and dye transfer

RD: rhodamine dextran LY: Lucifer Yellow

Figure 1-A.



Effect of DHT on the connexin 43 level of human granulosa cell line (HO23)

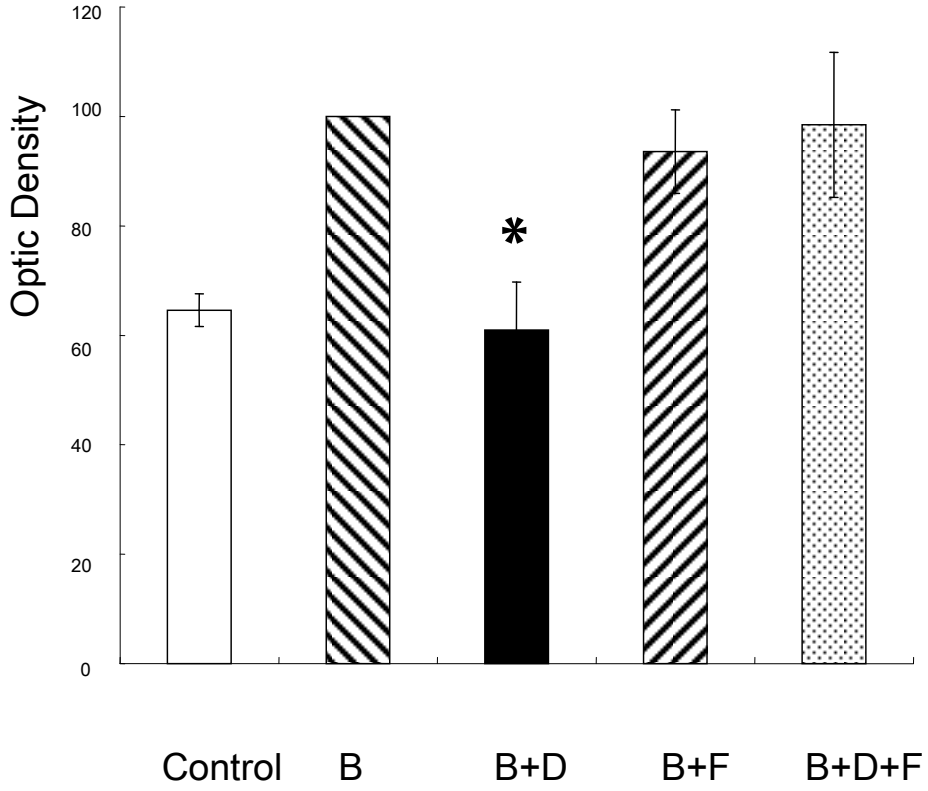
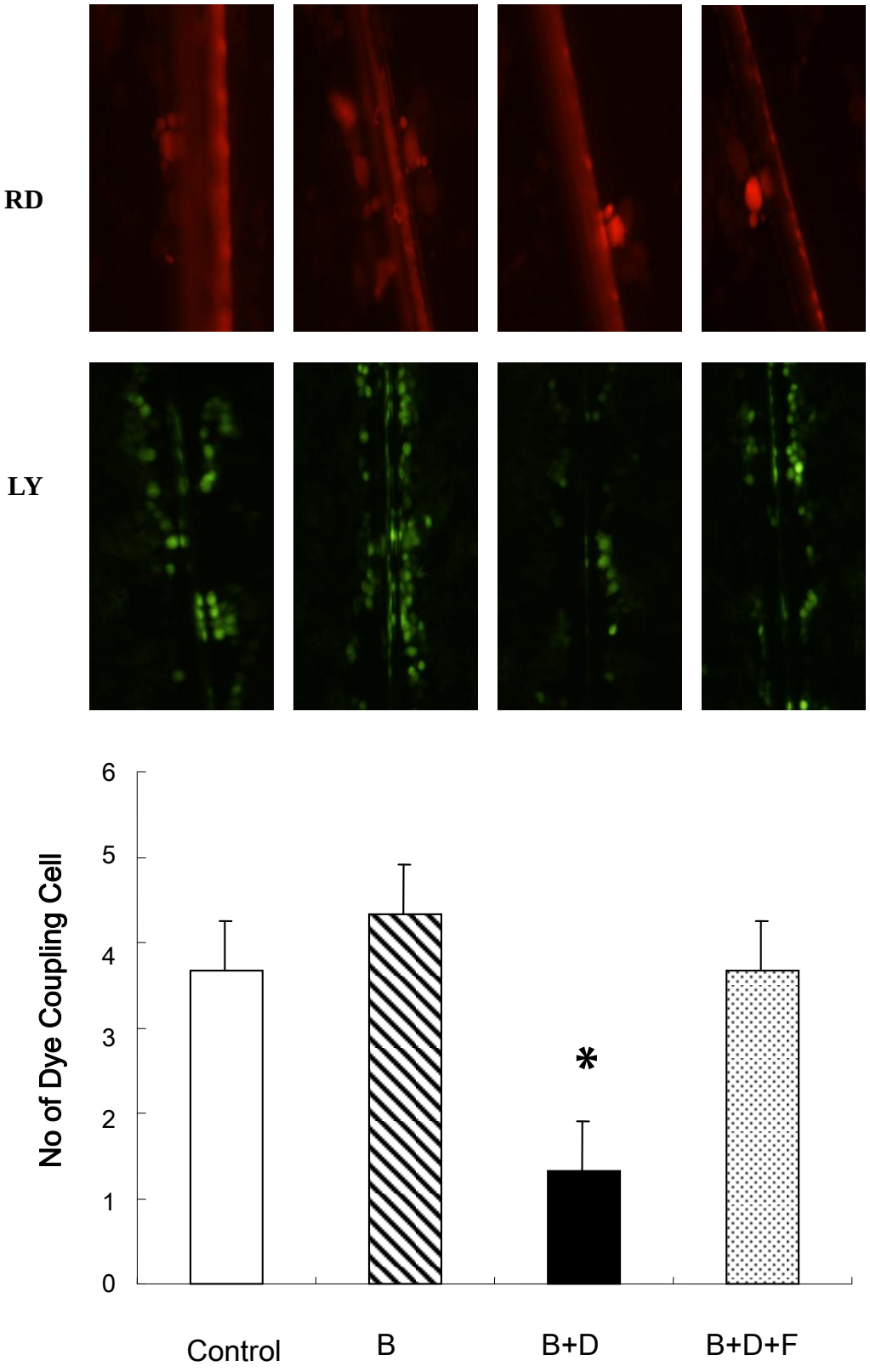


Figure 1-B.



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Running title: Androgen excess down-regulates Cx43 in granulosa cells

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The HO23 human immortalized luteinized granulosa cell line was provided by Dr. Abraham Amsterdam, Weizmann Institute of Science, Rehovot 76100, Israel (13). Granulosa cells were maintained at 37°C in 5% CO2 /air in Dulbecco's minimal essential medium (DMEM)/Ham's F12 (1:1), supplemented with 5% fetal calf serum and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). To test the effect of DHT on Cx43 expression, cells (2×10^6) were seeded on 100 mm culture dishes and incubated for 24 hours. The media were removed, and the cells were reincubated in fresh media with different concentrations of DHT. The cells were harvested for protein assays at 24 hours.

Protein content in cell lysate was determined by the bicinchoninic acid (BCA) protein assay method (Pierce, USA). After electrophoresis, the proteins were transferred onto

polyvinylidene difluoride membrane (Bio-Rad Laboratories, Ca, USA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 1 hours and incubated with mouse anti-human Cx43 antibody (Sigma-Aldrich Co. , USA) (1:3000) in 0.1% nonfat dry milk overnight at 4°C. After three washes in TBS containing 0.1% Tween-20, the membranes were incubated with goat anti-mouse IgG (Chemicon, USA) (1:5000) conjugated with alkaline phosphatase (Sigma-Aldrich) for 1 hour. The membranes were washed again as above, applied to the enhanced chemilunimescence (ECL) detection kit (Amersham, UK), and exposed to X-ray film (BioMAx films, Eastman Kodak Company, Rochester, NY, USA. To assess the amount of Cx43 protein loaded, filters were treated with 2% SDS and 100 mM β-mercaptoethanol in 62.5 mM Tris-HCl (pH 6.8) for 30 min at 60°C and reprobed for β-actin. Films were scanned, and the optical density of the bands was measured with Scion Image. Data shown are representative of at least three independent experiments with similar results.

Assessment of GJIC was determined using the scrape-loading and dye transfer (SLDT) technique with a fluorescent dye, Lucifer Yellow (LY, Sigma, USA) (14). Briefly, human granulosa cells, cultured as described above, were washed thoroughly with PBS. SL was performed applying three cuts on cell monolayer with a surgical scalpel, and then a mixture of 1% LY and 1% of rhodamine dextran in PBS was added to the cells. The cells were incubated for 5 min and washed three times with PBS to remove background

fluorescence. The cells were then fixed with 4% paraformaldehyde and photographed with a fluorescent microscope equipped with a camera. Cells that received the LY from the scrape-loaded cells were considered as communicating. The dye-coupled cell layers on either sides of the scrape were counted to evaluate the GJIC.

Data are expressed as mean ± SEM of at least three independent experiments performed at different time points. All data were analyzed using one-way analysis of variance (ANOVA). If the differences were significant, a Dunnett *post hoc* test was used for post-ANOVA multiple comparisons. Statistical significance was determined as $P < 0.05$.

The effect of DHT on Cx43 protein expression in response to 8-Br-cAMP was examined by Western blot analysis. 8-Br-cAMP, an analogue of cyclic AMP, is known to activate adenylate cyclase and increase the adenosine cAMP pool in the granulosa cells and up-regulates the Cx43 expression (15). A previous study showed the range of DHT in follicular fluid of normal population is 0.77 ± 0.11 ng/ml (16). To demonstrate the effect of androgen excess on Cx43 expression, cultured human granulosa cells were treated with increasing doses of DHT at 0, 1, 10, or 100 ng/ml. Western blot analysis of DHT-treated granulosa cell protein samples demonstrated decreasing amount of Cx43 protein in a dose-dependent manner. In addition, a significant decrease in the expression could be observed up to 24 h after treatment (data not shown).

To assess whether the suppressive effect of DHT on Cx43 is mediated through the AR, cells were incubated with DHT (10 ng/ml) and flutamide (100 ng/ml, a 10-fold excess above that of DHT). Flutamide, an AR antagonist, significantly blocked the inhibitory effects of DHT on Cx43 expression as shown in Figure 1-A.

To understand the association between reduced Cx43 expression and GJIC activity, we assessed the ability of HO-23 granulosa cells to transfer Lucifer yellow through gap junctions using the scrape-loading dye transfer (SLDT) technique in corresponding cultures of cells with reduced Cx43 expression. As shown in Figure 1-B, the number of dye-coupled cell layers was significantly reduced on either side of the scrape in HO-23 granulosa cells grown in 8-Br-cAMP with DHT medium compared with cells grown in 8-Br-cAMP medium. The phenomenon was prevented upon replenishing the medium with flutamide. This gap junctional defect could be induced with DHT and the effect was recovered with addition of flutamide to the culture medium.

Although anovulation is frequently observed in PCOS patients with androgen excess, the underlying mechanism has not been fully elucidated. Several possibilities have been raised to explain how androgens might mediate anovulation. In this study, we propose a new pathway through which high androgen level down-regulates Cx43 expression, reduce GJIC in human granulosa cells, and finally lead to impaired folliculogenesis.

Androgens and their 5α reduced metabolites like DHT have been shown to perturb the

ovarian physiology in animals (17, 18). One of the targets of this disruptive action is the granulosa cell within the ovarian follicle. While both androgen and gap junctions are reported to have important roles in follicular development, our study provides a novel mechanism which links androgen excess and Cx43 expression in the granulosa cells. We showed reduced GJIC activity in the milieu of high androgen and reduced Cx43 expression which occurs at the translation level mediated through androgen receptor.

A link between reduced GJIC activity and reduced connexin gene expression has been previously reported in rat ovarian cells exposed to luteinizing hormone (LH). LH inhibited GJIC activity by down-regulating Cx43 synthesis (19). Recently, Wang et al. (20) provided the evidence that the strength of gap junctional conductance was positively correlated with Cx43 level in human cumulus cells. Although it is possible that the effect of high androgen on GJIC activity may be mediated via other junction protein components, our findings indicate that reduced GJIC activity in human granulosa cells exposed to high androgen is at least in part due to inhibition of Cx43 gene expression.

The expression and function of Cx43 protein in the ovary of mammalian species have been discussed previously (21). In the mouse ovary, Cx43 is an important gap junction protein required for granulosa cell to granulosa cell communication, which in turn is required for follicle and oocyte maturation (7). Decreased Cx43 protein level is associated with follicular atresia, supporting the hypothesis that a loss of gap junctional communication plays

a coordinating role in the process of atresia (12). Disruption of granulosa cell coupling not only impairs follicle growth, but also impacts negatively on the quality of the enclosed oocytes. The knock-out mice of Cx43 showed that GJIC activity was reduced and oocyte growth in mutant follicles was retarded (22). However, we know little about Cx43 in human ovarian follicles and their roles in human folliculogenesis and fertility. A recent study shows that Cx43 was primarily localized in the membrane where it forms gap junction-like plaques between human cumulus cells, and its expression level may influence pregnancy outcome from in vitro fertilization (20). Furthermore, high Cx43 levels in granulosa cells are linked to good prognosis of human oocytes (9).

The roles of androgen and androgen receptor (AR) are well-established in the development of male reproductive organs and spermatogenesis. However, the functions of this steroid and its receptor in the ovary remain elusive. AR expression in the ovary has been described at various stages of folliculogenesis in several species. In human ovaries, AR protein expression is observed at different stages in granulosa and theca cells (23). Using a Cre–loxP system to generate AR-knockout (ARKO) mice, investigators showed that activated ARs are indispensable for normal folliculogenesis (24, 25). In this study, we demonstrate that androgen regulates Cx43 expression in cultured human granulosa cells by an AR-dependent process considering suppressive effect of DHT on Cx43 was blocked by an AR antagonist, flutamide. The findings provide a theoretical rationale for the clinical use of flutamide in the

treatment of patients with PCOS and anovulation. In fact, flutamide is able to restore ovulation in some women with PCOS (26, 27). However, there is still concern about the use of flutamide in women with infertility considering its possible teratogenic effect (28).

The root cause of anovulation in PCOS is now thought to be associated with the significant abnormalities in the very earliest stages of folliculogenesis (29). The normal 'dialogue' between oocyte and granulosa cells in these early growing follicles is altered. Recently, Li et al. (30) raised a hypothesis that if dysfunctional communications exist between granulosa cells or granulosa cells and the oocyte, unnecessary androgen will enter the oocyte and bind to the AR, provoking some follicles to undergo atresia. We therefore propose a working mode for the effect of androgen excess on folliculogenesis and oogenesis. High androgen level down-regulates Cx43 translation and impairs the communication between granulosa cells and possibly communications between granulosa cells and oocyte. Impaired communications negatively impacts folliculogenesis. The adverse effect of androgen excess is further strengthened by "shunting" of excessive androgens to the oocytes secondary to impaired communications between granulosa cells.

In conclusion, the present study provides the first evidence that increased levels of androgen down-regulate Cx43 expression and impair communications between granulosa cells. The effect may compromise folliculogenesis and oogenesis in PCOS patients. Caution should be taken to extrapolating in vitro results using immortalized granulosa cell lines to the

clinical setting considering few clinical data in this study. In addition, how DHT affects Cx43 translation still awaits further investigation.

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Figure legends

Figure 1-A. Antiandrogen treatment blocked the inhibitory effects of DHT on Cx 43 expression. HO-23 cells were cultured for 24 h under serum-free conditions in the presence of 8-Br-cAMP (0.1 mM), DHT (10ng/ml), and the antiandrogen flutamide (100 ng/ml)**.** Data are expressed as the mean ± SD of three different experiments. (* : $p < 0.05$ vs. 8-Br-cAMP)

B: 8-Br-cAMP D: DHT F: flutamide

Figure 1-B: Analysis of GJIC activity in HO-23 cells using SLDT technique. RD remains in the cells immediately bordering the scrape, while the LY diffuses to neighboring cells in gap junction-enabled cells. GJIC was evaluated as the counts from dye-coupled cell layers and is represented as a histogram. GJIC is blocked in HO-23 cells treated with 8-Br-cAMP (0.1 mM) and DHT (10ng/ml) (* : $p < 0.05$ vs 8-Br-cAMP). Flutamide (100 ng/ml) reversed the

blockage of GJIC induced by DHT to a comparable level to 8-Br-cAMP (ns, not significant vs 8-Br-cAMP). Data are expressed as the mean ± SD of three different experiments.

B: 8-Br-cAMP D: DHT F: flutamide

GJIC: gap junction intercellular communication

SLDT: scrape-loading and dye transfer

RD: rhodamine dextran LY: Lucifer Yellow

***Revision Notes**

We have answered the editorial's and reviewers' comments in the revision as below:

Editorial's comment:

1) We had corrected Reference 4.
2) Our abstract had been shortened to two sentences.

Reviewer's comment:

1) We had corrected into "induce" and "lead". (Abstract, lines 30, 31)
2) We change mammalians into mammalian. (Page 10, line 39)
3) Figure 1-B, bottom: change B+D+ into B+D+F

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A copy of this form must be completed and signed by each author. All authors must disclose any commercial interest, financial interest, and/or other relationship with manufacturers of pharmaceuticals, laboratory supplies, and/or medical devices and with commercial providers of medically related services. All relationships must be disclosed. All non-FDA-approved uses of products must be clearly identified.

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