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Research Article

Gonadotropins for ovarian stimulation mediate uterine fluid secretions through Cystic fibrosis transmembrane conductance regulator gene

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SUMMARY

Objective: Controlled ovarian hyperstimulation (COH) for ovulation stimulation is associated with formation and/or sudden increase in hydrosalpinx fluid (HF) as well as reflux into the uterine cavity leading to poor outcome of in vitro fertilization (IVF) treatments. However, the mechanisms underlying fluid formation during gonadotropin administration for ovarian hyperstimulation have not been thoroughly investigated. Cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-dependent ion channel is known to modulate fluid secretion in the female reproductive tract. The present study investigated whether ovarian stimulation increases CFTR expression in vivo after gonadotropin administration.

Methods: We examined CFTR expression after gonadotropin administration using reverse transcriptase-polymerase chain reaction (RT-PCR) and immunofluorescence staining.

Results: RT-PCR revealed that CFTR expression in gonadotropin-treated rats was significantly greater than control. Immunostaining showed significantly enhanced CFTR immunoreactivity in the uterine epithelium of rats with intact ovaries.

Conclusions: These results suggest that ovulation induction using gonadotropins up regulate CFTR expression thus transepithelial fluid transport and this regulation may be mediated via the ovary.

INTRODUCTION

Ovulation induction for assisted reproductive technology (ART) using gonadotropins is associated with the presence of fluid in the uterine cavity and formation or enlargement of hydrosalpinx.[1-9] Fluid accumulation in the Fallopian tubes and reflux into the uterine cavity may disturb embryo implantation leading to infertility.[3, 5, 7-8] Sudden re-accumulation of uterine fluid after aspiration prior to embryo transfer has also been reported especially in those patients with hydrosalpinges.[7-9] Transient fluid accumulation could be found during gonadotropin stimulation for ART but some reports show that massive fluid formation and uterine accumulation usually follow human chorionic gonadotropin (HCG) administration leading to poor treatment out come in these patients. [9, 10] It has also been reported that fluid accumulation was evident during gonadotropin stimulation and increased after HCG administration. [9, 10] Taken together, these studies suggest that gonadotropins including HCG may induce fluid formation in the female reproductive tract during ovarian stimulation. Although an association between hormones including those for ovulation induction and fluid secretion has been suggested,[11] the mechanisms underlying fluid formation and accumulation in the uterine cavity and enlargement of hydrosalpinx encountered during gonadotropin administration for ovarian stimulation have not been thoroughly investigated.

Leutenizing hormone (LH), known to trigger ovulation, activates adenylate cyclase and increase cellular levels of cAMP:[12] Human chorionic gonadotropin (HCG) and follicular stimulating hormone (FSH) activate the protein kinase A-dependent signalling pathway.[13] Therefore, gonadotropin may be modulating fluid secretion via the activation of adenylate cyclase – cAMP, protein kinase A-dependent signalling pathway. Cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP – protein kinase A-dependent chloride channel present on epithelial cells, has been shown to be involved in mediating a variety of neurohormonal secretory responses in the endometrium.[14-16] Previous study also showed that CFTR mediates transepithelial fluid transport in mouse female reproductive tract epithelia.[17] Therefore, CFTR may be involved in fluid secretion in the uterine cavity encountered during gonadotropin administration. The present study investigated whether ovarian stimulation increases CFTR expression in vivo in a rat model.

MATERIALS AND METHODS

Animals

Mature (60-90 days old) ovariectomized and immature (23 days old) female Sprague-Dawley (S-D) rats were used in this study. The rats were kept in the Animal Service Centre of University of Hong Kong prior to experiments and were fed laboratory chow and water *ad libertum*. They were maintained under controlled conditions of 12 hours light and 12 hours dark, at an average temperature of 21 - 23^{o}C.

Experiments were conducted in accordance with the University guidelines on animal experimentation.

Ovariectomy

The rats were anesthetized using an intraperitoneal (IP) injection of 0.4ml of a mixture of ketamine hydrochloride (100mg/ml) and xylazine (20mg/ml) in 20ml of distilled water. The dorsal surface of the rat was disinfected prior to surgery. A small midline incision of 1.5-2.5cm was made on the dorsal surface of the back halfway between the hump and the base of the tail. Entrance to the peritoneal cavity was gained on both sides through the incision. The muscles were incised and the ovaries dissected out by freeing them from the peri-ovarian fatty tissues. The ovaries were pulled out, clamped at the uterine horns and then carefully cut out with a pointed pair of scissors in order not to allow even a small piece to detach. This was done for each of the ovaries and the uterine horns put back into the peritoneal cavity. The skin incisions were closed using surgical clips and the rats were kept warm under droplight. The rats were allowed to recover and adjust for one month prior to further experiments.

Administration of gonadotropins for ovarian stimulation

The rats received IP injections of 10 IU pregnant mare's gonadotropin (PMSG, Sigma USA), human menopausal gonadotropin (HMG), HCG (Merck Serono, USA), and similar volume of saline as control. They were sacrificed 48 hours later by cervical dislocation. Uteri and oviducts received from the rats were dissected free of fatty tissues and immediately snap-frozen in liquid nitrogen, and later stored in –70°C for RNA isolation or fixed in 4% paraformaldehyde for immunofluorescence staining.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Semi-quantitative RT-PCR was performed using RNA obtained from the rats' uteri. The specific oligo nucleotide primers for rat GAPDH were: GAC CAC AGT CCA TGA CAT CAC TGC (sense) and GCT GTT GAA GTC GCA GGA GAC AAC (antisense), corresponding to nucleotides 565-904 with expected cDNA of 340 base pairs (bp). The specific oligo nucleotide primers for CFTR were: CAT CTT TGG TGT TTC CTA TGA TG (sense) and GTA AGG TCT CAG TTA GAA TTG AA (antisense), corresponding to nucleotides 1655-2135 with expected cDNA of 481 bp. The conditions were: denaturation at 94°C for 45 sec; annealing at 53 °C and 58°C for 60 sec; extension at 72°C for 60 sec; 25 and 30 cycles for GAPDH and CFTR respectively. Optimal amplification cycles were determined based on the linear relationship between the amount of PCR product detected and the number of amplification cycles. The intensities of the bands of CFTR subunits were normalized to that of GAPDH, which was amplified simultaneously. Experiments in the absence of reverse transcriptase were conducted as negative controls and repeated five times.

Immunofluorescence staining

Tissue sections dried onto Superfrost microscope slides (Fisher brand, Fisher scientific) were deparafinized in xylene, rehydrated in graded ethanol and rinsed in tap water. The slides were then incubated in a solution of 3% hydrogen peroxide in water for 30 minutes to quench endogenous peroxidase activity. Thereafter, the slides were washed in water and incubated in normal blocking serum (Vectastain Elite ABC kit, Vector Laboratories, Inc., CA, USA) for another 30 minutes to block non-specific sites. Excess serum was removed from the slides by blotting using clean tissue paper. The slides were then incubated overnight in primary antibody (CFTR1: 500 V/V in buffer, Neo Markers, Lab Vision Corp., Fremont, CA, USA) at 4°C. Tissue sections were then washed for 5 minutes for three times with Phosphate buffered solution (PBS) and incubated for 30 minutes in a diluted biotinylated secondary antibody solution (Vectastain Elite ABC kit, Vector Laboratories, Inc., CA, USA). Then tissue sections were again washed three times for 5 minutes in PBS and incubated for 20-30 minutes in FITC-conjugated anti-mouse antibody (Vector Laboratories, Inc., CA, USA), washed again in PBS, mounted with glycerol and observed under an Olympus epifluorescence microscope (Olympus IX-70, Tokyo, Japan).

Statistical analysis

The ratio of CFTR/GAPDH expression intensities was analyzed using 2% agarose gel electrophoresis of RT-PCR products. Data presented are mean and SEM for immunofluorescence staining intensities. Differences between groups were compared using Newman Keuls multiple comparison test. P value of 0.05 or less was considered significant. Statistical analysis was carried out using Graph Pad Prism (Graph Pad Software Inc., San Diego, CA, USA).

RESULTS

RT-PCR

Due to the influence of different estrus cycle stages on reproductive tissues, we conducted this study using sexually mature ovariectomized and 23-day-old immature non-cycling S-D rats with intact ovaries. There was no difference in CFTR mRNA expression found between the treatment groups and control group of the ovariectomized rats (Figure 1A). However, CFTR was highly upregulated when compared to the controls relative to the intensities of CFTR bands and those of GAPDH that were simultaneously amplified and used as internal marker, in the immature rats with intact ovaries (Figure 1B).

Immunofluorescence staining

Immunoreactive CFTR obtained using CFTR antibody was detected strongly in the epithelium of gonadotropins treated immature rats (Figure2B-D) and in the controls (Figure 2A). Densitometric measurements showed that the immunoreactive intensity in gonadotropins treated immature rats was significantly different from that of controls ($p<0.0001$, Figure 2E).

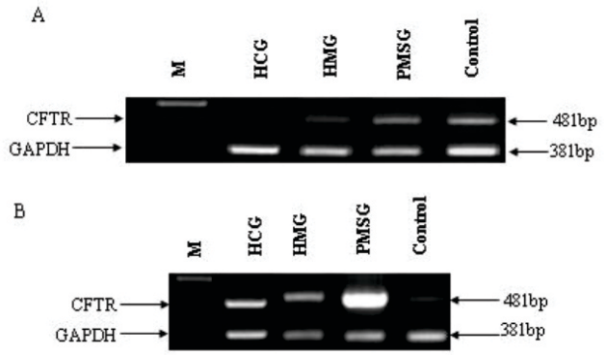

Figure 1: Cystic fibrosis transmembrane conductance regulator (CFTR) mRNA expression in the ovairectomized (Fig.1A) and 23-day old immature non-cycling with intact ovary rats (Fig.1B). Representative gels of 2% agarose gel electrophoresis of RT–PCR products for CFTR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Band for GAPDH corresponds to 381base pairs (bp) whereas CFTR band corresponds to 481 bp. HCG – human chorionic gonadotropin, PMSG - pregnant mare's gonadotropin, HMG- human menopausal gonadotropin, M – DNA marker.

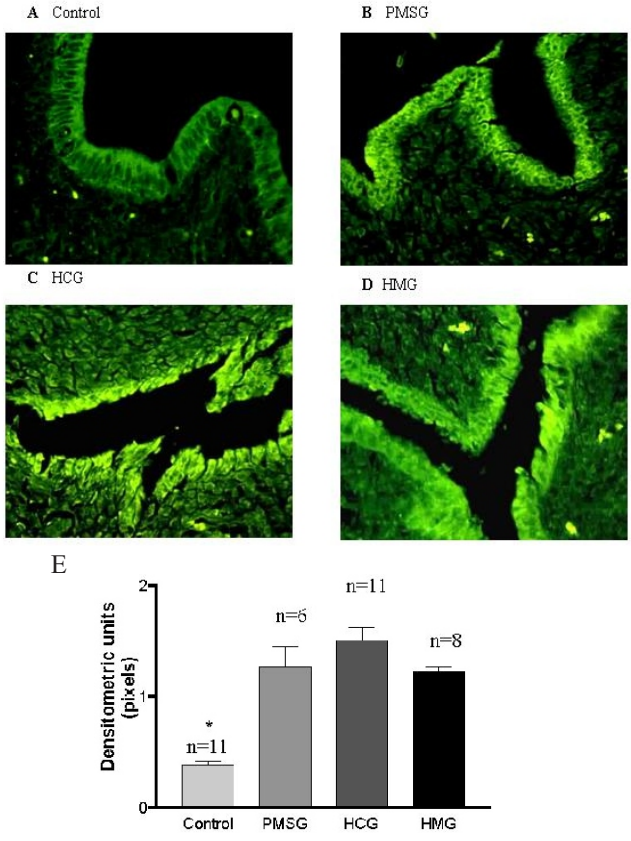

Figure 2: Immunofluorescence staining of the uterus of 23-day old immature non-cycling SD rats treated with gonadotropins, paraffin sections showing cystic fibrosis transmembrane conductance regulator (CFTR) protein localization on the epithelium (B–D) and control (A). Note the apical localization of CFTR on control uterine tubes epithelium (A) and enhanced expression on the epithelium of B–D. Magnification × 40, Densitometric measurements using Meta Morph image analysis software showed that CFTR immuoreactive intensity of B–D was significantly different from that of A ($P<0.0001$, Figure 2E). HCG – human chorionic gonadotropin, PMSG - pregnant mare's gonadotropin, HMG- human menopausal gonadotropin, n- number of rats.

DISCUSSION

This study is the first to specifically examine gonadotropins for ovarian stimulation as secretory hormones involved in fluid excretion via the expression of CFTR, a cAMP-dependent chloride channel known to mediate neurohormonal mechanisms, involved in mediating the secretory activities in the female reproductive tract [18] and increase in osmotic water permeability [19] after ovulation induction. Although it has been suggested that hormones for ovarian stimulation may modulate CFTR expression,[11, 20, 21] our present data confirm that ovulation induction upregulate CFTR expression in the female reproductive tract and possibly acts as secretory hormones to increased fluid secretion as previously suggested. [22]

The increased expression of CFTR in the present study that was not seen in ovariectomized S-D rats suggests that ovarian hormones may be involved in the upregulation of CFTR during ovarian stimulation. Although we did not measure the plasma levels of ovarian hormones in this study, it is well established that estradiol but not progesterone [23] upregulates CFTR expression and hyperestrogenemia is seen during ovarian hyperstimulation.[21]Therefore, upregulation of CFTR possibly mediated via increased estrogen levels during ovulation induction may be the reason for increased fluid secretion and or reflux into the uterine cavity encountered during gonadotropin administration.

Movement of ions across the tubal epithelium is essential for the movement of fluid, which is not actively transported but moves in response to osmotic gradients largely established by the transport of ions across epithelium particularly Cl- ions through ion channels such as CFTR mainly responsible for Cl- secretion in many epithelia. [24, 25] The demonstration of cAMP-activated oviductal Cl- secretion in normal but not CFTR mutant mice [26] indicates a functional role of CFTR in oviductal secretory function. Some studies have provided evidence of CFTR involvement in transepithelial fluid transport in the female reproductive tract of mice [16,17] and our previous study also demonstrated increased CFTR expression in human hydrosalpinx.[27] Pregnant mare's serum gonadotropin (PMSG) has been shown to up regulate CFTR both in vivo and in vitro.[28] This is consistent with our present result that gonadotropin administration for ovarian stimulation significantly increased the expression of CFTR in vivo leading to fluid formation and uterine accumulation.

CONCLUSION

It is likely that elevated CFTR expression, by gonadotropins during ovulation induction may lead to abnormal fluid secretion and absorption balance, mediating increased transepithelial fluid secretion. More experiments are needed to determine the signaling mechanisms involved and using electrophysiological methods to elucidate transepithelial fluid transport after gonadotropins administration during ovarian stimulation.

Conflict of Interest

The authors declare no conflict of interest.

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