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Regulation of AMH and SCF expression in human granulosa cells by GnRH agonist and antagonist

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With the progress of cancer treatment, fertility preservation has become an urgent requisition. Gonadotropin-releasing hormone agonist (GnRH-a) and antagonist (GnRH-ant) have been used to protect fertility for these patients. However, studies showed that although GnRH-a and GnRH-ant had a comparable down-regulating effect on the pituitary, GnRH-ant could not preserve ovarian function. Moreover, GnRH-ant alone could deplete primordial follicles. It might be speculated that an additional intraovarian system existed except the pituitary system. Anti-Müllerian hormone (AMH) and Stem cell factor (SCF) proved to be the key factors in follicle recruitment and development. The balance between AMH and SCF was tightly related to ovarian reserve. To investigate the intraovarian effect of GnRH-a or GnRH-ant on ovarian reserve, we examined AMH and SCF expression in human granulosa cells (hGCs). GCs were isolated from follicular aspirates after oocyte removal from the patients undergoing assisted reproduction techniques. After pretreated with GnRH-a (triptorelin) or GnRH-ant (cetrorelix) for 48 h, mRNA and protein of AMH and SCF were analyzed by Real-time PCR and Immunoblot assay respectively. The results indicated that AMH mRNA and protein expressions were down-regulated in the GnRH-ant groups, SCF mRNA and protein expressions were up-regulated in the high-dose GnRH-ant group. There was no difference of AMH and SCF expression in the GnRH-a group or GnRH-a + GnRH-ant group compared with control. These results suggested the effects of GnRH-a and GnRH-ant on the regulation of AMH and SCF were different, which may provide insight into the mechanism of GnRH-a and GnRH-ant interventions on ovarian reserve.

1. Introduction

The use of aggressive chemotherapy causes the damage of ovarian function resulting in infertility (Chemaitilly et al. 2006; Sklar et al. 2006). Therefore, it is urgent to develop viable way to preserve fertility in these patients.

Several protocols for preserving fertility have been reported in past decade, including *in vitro* fertilization (IVF), embryo cryopreservation, ovarian tissue cryopreservation, and administration of a gonadotropin-releasing hormone (GnRH) agonist (GnRH-a) (Schwartz 1999; Blumenfeld et al. 2002, 2003; Lutchman Singh et al. 2005; Lobo 2005). Among them GnRH-a is a non-invasive and promising technique that minimize the gonadotoxic effect. Several possibilities provided to explain the beneficial effect of GnRH analogues, such as inhibiting the pituitary-ovary axis, furnishing a hypogonadotropic milieu, decreasing the number of primordial follicles entering the differentiation stage, which is more vulnerable to chemotherapy (Blumenfeld et al. 2002; Lobo 2005; Flaws et al. 1997; Knight et al. 2006; Ataya et al. 1995; Yacobi et al. 2004). Although GnRH-a has been used widely to adjunctive therapy, there are still some limitations. For example, GnRH-a has a "flare-up" effect, which may accelerate follicle recruitment. The GnRH antagonist (GnRH-ant) is much more advantageous over the agonist by competitive blockade of the receptors and to achieving a faster pituitary-ovarian desensitization (Kovacs et al. 2001; Murase et al. 2005), so it was believed to have a similar fertility protective effect and less limitations comparing with GnRH-a. However, previous studies on the protective role of GnRH-ant in rodents led to contradictory results (Sonmezer et al. 2006; Danforth et al. 2005). Moreover, it was suggested that GnRH-ant alone could deplete the primordial follicles (Danforth et al. 2005). The results of our previous study also support this opinion (Peng et al. 2007). It might be speculated that an additional intraovarian regulating system existed except the pituitary system. The mechanisms implied need further investigation.

Follicles are the basic functional units of the ovary (Richardson et al. 1987). Follicle depletion caused infertility. The number of primordial follicles was thought to represent the ovarian reserve. Follicle development is a process not only regulated by the hypothalamic-pituitary-ovarian (H–P–O) axis but also influenced by autocrine/paracrine factors of ovaries. Anti-Müllerian hormone (AMH) and Stem cell factor (SCF) are two important intraovarian factors (Kang et al. 2003; Hoyer et al. 2005; Carlsson et al. 2006; Durlinger et al. 1999). Previous studies (Durlinger et al. 1999) found that AMH null mice contained almost threefold more small nonatretic growing follicles than their wild-type littermates. Also *in vitro* culture of neonatal ovaries in the presence of AMH confirmed the inhibitory effect of AMH on primordial follicle recruitment (Durlinger et al.

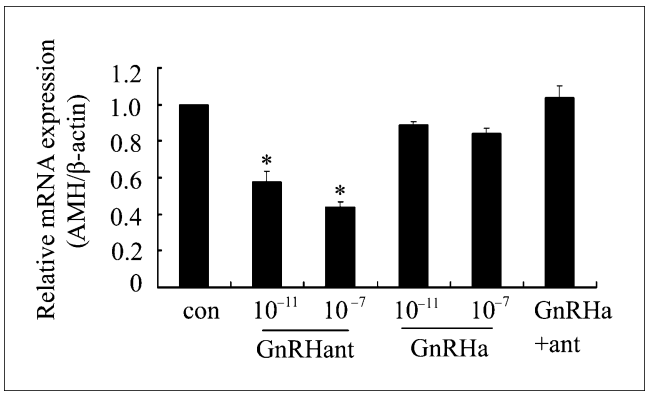

Fig. 1: AMH relative mRNA expression in hGCs (AMH/β-actin). The GnRH-ant groups (10^{-11}mol/L and 10^{-7}mol/L) had significantly decreased expression of mRNA ($P<0.05$ vs control). No significant difference was found between GnRHant (10^{-11}mol/L) and GnRHant (10^{-7}mol/L). There was no difference of AMH mRNA expression in GnRH-a group or the GnRHa + ant group compared with control. Data are presented as mean ± SD. *, means $P<0.05$

2002). Parrott and Skinner (Parrot et al. 1999) reported that SCF markedly promoted the follicle growth and induced primordial follicle activation *in vitro*. These results provided strong evidence that AMH could inhibit follicle development while SCF could promote follicle growth. It is more plausible that ovarian reserve was exquisitely regulated by the balance between AMH and SCF.

In the present study, the effects of GnRH agonist (triptorelin) and antagonist (cetrorelix) on AMH and SCF expression were investigated, which may shed light on the mechanism of GnRH-a and GnRH-ant interventions on ovarian reserve.

2. Investigations and results

2.1. mRNA expression of AMH and SCF in human granulosa cells

The GnRH-ant groups had significantly decreased mRNA expression of AMH. No significant difference was seen between GnRH-ant (10^{-11}mol/L) and GnRH-ant (10^{-7}mol/L). There was no difference of AMH mRNA expression in GnRH-a group or the GnRHa + ant group compared with control (Fig. 1).

SCF mRNA expression was significantly increased in the GnRH-ant group (10^{-7}mol/L). A significant difference was seen between GnRH-ant (10^{-11}mol/L) and GnRH-ant (10^{-7}mol/L). There was no difference of SCF mRNA expression in GnRH-a group or the GnRHa + ant group compared with control (Fig. 2).

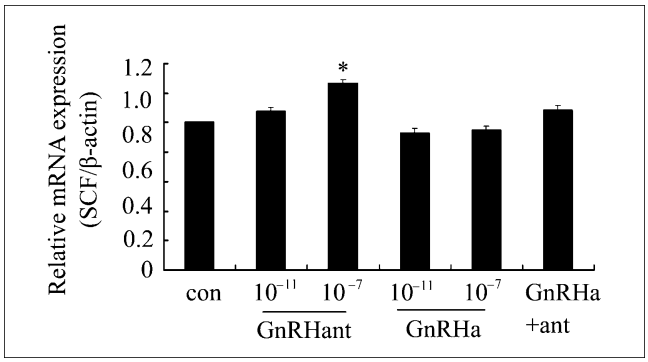

Fig. 2: SCF relative mRNA expression in hGCs (SCF/β-actin). The GnRH-ant group (10^{-7}mol/L) had significantly increased expression of mRNA ($P<0.05$ vs control). Significant difference was found between GnRH-ant (10^{-11}mol/L) and GnRH-ant (10^{-7}mol/L) ($P<0.05$). There was no difference of SCF mRNA expression in GnRH-a group or the GnRHa + ant group compared with control. Data are presented as mean ± SD. *, means $P<0.05$

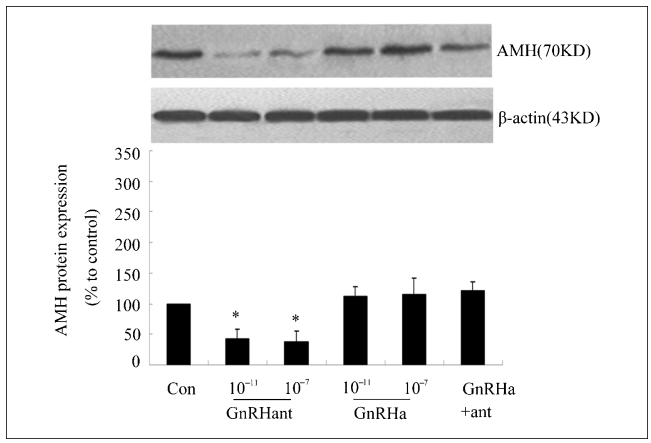

Fig. 3: AMH protein expression in hGCs (% to control). The GnRH-ant groups (10^{-11}mol/L and 10^{-7}mol/L) had significantly decreased expression of protein ($P<0.05$ vs control). No significant difference was found between GnRH-ant (10^{-11}mol/L) and GnRH-ant (10^{-7}mol/L). There was no difference of AMH protein expression in GnRH-a group or the GnRHa + ant group compared with control. Data are presented as mean ± SD. *, means $P<0.05$

2.2. Protein expression of AMH and SCF in human granulosa cells

AMH protein expression was significantly decreased in the GnRH-ant groups. No significant difference was showed between GnRH-ant (10^{-11}mol/L) and GnRH-ant (10^{-7}mol/L). There was no difference of AMH protein expression in GnRH-a group or the GnRHa + ant group compared with control (Fig. 3).

SCF protein expression was significantly increased in the GnRH-ant group (10^{-7}mol/L). A significant difference was seen between GnRH-ant (10^{-11}mol/L) and GnRH-ant (10^{-7}mol/L). There was no difference of SCF protein expression in GnRH-a group or the GnRHa + ant group compared with control (Fig. 4).

3. Discussion

Follicular development and differentiation are sequential events which are tightly regulated by endocrine hormones, intraovarian regulators and cell-cell interactions (Suh et al. 2002). AMH and SCF are important autocrine/paracrine factors involved in follicle development. AMH serves as an inhibitor to suppress the primordial follicles recruitment while SCF serves conversely. Ovarian reserve was exquisitely regulated by the

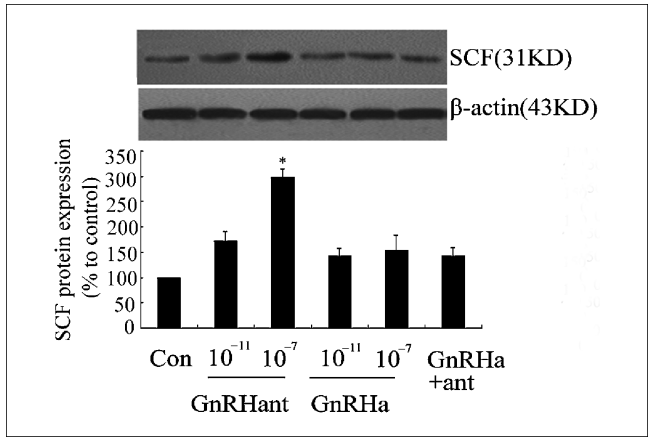

Fig. 4: SCF protein expression in hGCs (% to control). The GnRH-ant group (10^{-7}mol/L) had significantly increased expression of protein ($P<0.05$ vs control). Significant difference was found between GnRH-ant (10^{-11}mol/L) and GnRH-ant (10^{-7}mol/L) ($P<0.05$). There was no difference of SCF protein expression in GnRH-a group or the GnRHa + ant group compared with control. Data are presented as mean ± SD. *, means $P<0.05$

balance between AMH and SCF (Durlinger et al. 1999, 2002; Parrott et al. 1999; Tai et al. 2000; Suh et al. 2002; Jayaprakasan et al. 2008; Liu 2006).

GnRH-a can inhibit the pituitary-ovary axis and preserve a relatively static follicle status and follicles in static status is insensitivity to chemotherapy drugs. In this regards GnRH-a may be benefit to relieve the gonad damage. Ideal GnRH-ant was believed to have similar effects, however, Danforth′ work (Danforth et al. 2005) and our previous studies showed the contradictory results (Peng et al. 2007). In our previous research, the functions of GnRH-a and GnRH-ant on the ovarian reserve on rats exposed to chemotherapy were observed, and the results suggested that although GnRH-ant could downregulate Gonadotropin-releasing hormone receptor (GnRHR) more completely compared with GnRH-a in the pituitary, they had different effects on ovaries. In the ovary, GnRH-a depressed follicle growth, recruitment and decreased chemotoxic sensitivity; conversely, GnRH-ant promoted follicle growth, sensitized follicles to chemotoxicity and caused a decline in ovarian reserve. These results indicated that the GnRH-ant had adverse effect on follicle recruitment and ovarian reserve. Hsueh et al. (1981) also found that the extra-pituitary action of GnRH-a was due to anti-differentiation on granulosa cells and depression of steroid synthesis in the ovary, while GnRH-ant probably relieved suppression of ovarian follicle growth, consequently led to sexual precocity. This result demonstrated that the different intraovarian effects existed between GnRH-a and GnRH-ant. The dichotomy between GnRH agonists and antagonists as defined in pituitary gonadotrophs might not be applied to the GnRH system in ovarian compartment (Gründker et al. 2004). Precise mechanism of GnRH-a and GnRH-ant different effect on ovarian compartments is still under exploration.

In the present study, we found that (1) AMH mRNA and protein expressions were down-regulated in GnRH-ant groups, while SCF mRNA and protein expressions were up-regulated. (2) These effects were dose-dependent and statistically difference was showed in the higher doses of the GnRH-ant group (10^{-7}mol/L). (3) When combined GnRH-a with GnRH-ant the converse effect induced by GnRH-a and GnRH-ant could be eliminated. Based on these results, we suggested that GnRH-a and GnRH-ant worked differently on autocrine/paracrine factors AMH and SCF. These results could partly explain the different effect of GnRH-a and GnRH-ant on ovarian reserve.

So far, two kinds of GnRH receptor (GnRHR) have been found in human central and peripheral tissue named GnRHR- I and GnRHR-II, which belong to members of the G protein-coupled receptor superfamily (GPCR) (Chegini et al. 2002; Chou et al. 2003). GnRHR- I is thought to couple to multiple G protein subunits (Gqα, Gsα, and Giα) and activate multiple signaling pathways (Conn 1994; Stojilkovic et al. 1995; Stanislaus et al. 1998). Increasing evidence that multiple G proteins can mediate the effects of GnRHR- I raised the possibility that the same kind of GPCR exerts regulation through differential signal transduction pathways in distinct tissues or cells (Hori et al. 1998; Sim et al. 1995). Documents also show that GnRH binding sites were both up and down regulated by GnRH in the pituitary and ovaries (Mason et al. 1994; Kakar et al. 1994). In general, low doses or pulsatile treatment of GnRH upregulate its receptor, whereas high doses or continuous treatment downregulate the receptor numbers. Based on these results, we speculated that GnRH-a and GnRH-ant worked differently on autocrine/paracrine factors AMH and SCF. The discrepancy of AMH and SCF expression may be mediated through GnRHR-I; high-dose treatment may down-regulate the receptor numbers in ovarian compartments. Another possible explanation is that this regulation of GnRHR may couple different G protein subunits and activate corresponding signaling pathway.

Taken together, our research provided the new evidence that GnRH-a and GnRH-ant showed different effect on ovarian reserve. We further suggest that this discrepancy might be caused by different regulation on intraovairan autocrine/paracrine factors AMH and SCF through GnRHR-I system.

4. Experimental

4.1. Reagents

GnRH analogues: GnRH-I agonist [D-Trp6] (triptorelin, 3.75 mg/vial) was purchased from Ipsen pharma biotech (France), The GnRH-I antagonist cetrorelix acetate (0.25 mg/vial) (D-20761) was supplied by Serono SA (Switzerland); Phenol-red-free M199 (Gibco, Invitrogen Corporation, Carlsbad, CA, USA), FBS (fetal bovine serum) (Hyclone, Invitrogen Corporation, Carlsbad, CA, USA), Ficoll-Hypaque (Sigma, Steinheim, Germany). Monoclonal anti-human AMH antibody (MAB1737, R&D Systems), Rabbit monoclonal to SCF (ab52603, abcam), beta-actin (Santa Cruz). Except where otherwise noted, all chemicals were purchased from Sigma (Steinheim, Germany).

4.2. Human granulosa cell culture and pharmacological treatments

Follicular aspirates were collected during oocyte retrieval from women undergoing *in vitro* fertilization in the IVF centre of the second affiliated hospital of Sun-Yat-San University. All protocols involving human subjects were approved by the Institutional Review Boards of Sun Yat-sen University. Granulosa cells (GCs) were prepared as previously described (Tai et al. 2000). Freshly harvested GCs were mechanically dissociated, after centrifugation on a density gradient (Ficoll-Hypaque; Sigma) at 2,500 rpm for 20 min, GCs were purified from red blood cells and washed with 10 mL PBS at 1,000 rpm for two times, The cell pellet was resuspended in M199 supplemented with 10% FBS, 100 U/ml penicillin G, and 100 mg/ml streptomycin at a density of 10^5 cells/ml. The cells were seeded in 35 mm culture dishes and allowed to adhere for 24 h at 37 °C in a humidified atmosphere of 5% CO_2–95% air. Cell viability was determined by trypan blue exclusion. The entire procedure was completed within 1 h after follicle aspiration to prevent cell death. Subsequently, the cells were rinsed with PBS, and on day 3 the cells were cultured in phenol red-free M199, then treated with GnRH agonist and antagonist respectively or together with the concentrations of 10^{-11}mol/L or 10^{-7}mol/L, on day 5 in culture (GnRH-a or GnRH-ant pretreated 48 h), media were removed, whereas cells from six to eight wells were pooled for RNA and protein extraction.

4.3. Real-time PCR

After treatment with triptorelin or cetrorelix 48 h, the total RNA was extracted using Trizol (Invitrogen) from GCs. RNA was reversely transcribed into cDNA according to the manufacturer's instructions (TaKaRa). The primers for AMH are: sense, 5′- AGG TCG CGG CAG AGG AGA TA - 3′; and antisense, 5′- TTA AGT GAG CCG AGT GGA AGG TG - 3′. The primers for SCF are: sense, 5′- GAG TCG CCA CAC CAC TGT TTG - 3′; and antisense, 5′- GTC ACA CGA TTC CTG CAG ATC C - 3′. The primers for beta-actin are: sense, 5′- TGG CAC CCA GCA CAA TGA A - 3′; and antisense, 5′- CTA AGT CAT AGT CCG CCT AGA AGC A - 3′. The reactions were set up with 12.5 µl SYBR Green PCR Master Mix (TaKaRa). All real-time experiments were run in triplicate, and a mean value was used for the determination of mRNA levels. The steady-state concentrations of mRNA for AMH and SCF in GCs were normalized to the amount of beta-actin mRNA.

4.4. Immunoblot assay

After culture for 5 days (GnRH-a or GnRH-ant pretreated 48 h), the cells were washed twice with ice-cold PBS and lysed in ice-cold RIPA buffer and PMSF (Sigma). The extracts were placed on ice for 30 min and centrifuged to remove cellular debris. Protein amount of supernatants was determined using the BCA Protein Assay Kit (Bio-world). Total protein (30 mg) was run on 10% SDS-PAGE gels and electrotransferred to a nitrocellulose membrane (Invitrogen). The membrane was immunoblotted using specific primary antibodies at 4 °C overnight. The signals were detected with horseradish peroxidase-conjugated secondary antibody for 1 h, and visualized using the ECL System (Invitrogen). Gray level semiquantitative analysis was performed using the Molecular Analyst System in Gel Doc 1000 (BIO-RAD). The OD ratios were presented as relative expression using beta-actin (43KD) as a control (gray relative levels).

4.5. *Statistical analysis*

Results were subjected to statistical analysis using SPSS 12.0 software. Data were represented as means ± standard deviation (SD). Statistical analysis was performed by one-way ANOVA. Differences were considered significant as $P < 0.05$.

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