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Novel hGHRH homodimer promotes fertility of female infertile hamster by up-regulating ovarian GHRH receptor without triggering GH secretion

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ABSTRACT

Extra-hypothalamic growth hormone-releasing hormone (GHRH) plays an important role in infertility. The female infertility models were formed by intraperitoneally injecting cyclophosphamide in 5-week-old Chinese hamster once in a week for 5 weeks. All the models mated with healthy male hamster in the ratio of 1:1 in the experimental 6–8th week and the couples were separated to breed in the 9–10th week. 20 mg/kg of cyclophosphamide induced temporary interference of reproduction and did not cause significant difference in the weight of body, bilateral ovaries, or liver. By intramuscularly injecting twice in a week during the experimental 4–10th week, 2, 4, 8 mg/kg of *Grin* induced 30, 42.9, 60% of total pregnancy rates in a dose-dependent manner whereas 200 U/kg of hMG induced 50% of total pregnancy rates. The single cyclophosphamide dose caused strongly eosinophilic ovarian cells, scattered early follicles, many atretic follicles, and no corpora luteum was observed. The hMG group individually presents many follicles at all levels, especially secondary ones in the ovarian cortex and medulla. Much of loose connective tissue, vacuoles, and sparse interstitial cells distribute in the medulla. *Grin* induced many follicles at all dose levels and corpora lutea in the cortex, and the compactly aligned interstitial cells occurred in the whole ovarian tissue. The less TUNEL staining and higher expression of ki67 showed the proliferation and protection effect of *Grin* on ovarian cells. *Grin* obviously promotes fertility by up-regulating ovarian GHRH receptor and strengthening the development and maturation of follicles without triggering central and ovarian GH secretion.

1. Introduction

The maturation of oocyte is involved in the co-regulations of the gonadotropin–releasing hormone (GnRH)-gonadotropin-sex hormone pathway and the growth hormone-releasing hormone (GHRH)-growth hormone (GH)-insulin-like growth factor 1 (IGF1) pathway.

The central or autocrine GHRH and/or GH plays important roles in fertility. Kotani et al. (1998) reported that GHRH significantly stimulated progesterone production and cAMP accumulation in cultured rat ovarian granulosa cells in a dose-dependent manner. In the presence of luteinizing hormone (LH), GHRH significantly inhibited LH-stimulated progesterone production in a dose-dependent manner. GHRH has effect on rat ovarian steroidogenesis, which may be involved in modulation of key steroidogenic steps concerned with progesterone degradation rather than formation in LH-stimulated rat granulosa cells. Hugues et al. (1996) thought that the significant effect of GHRH on ovarian function is probably to be exerted *via* activation of the somatotrophic axis and the subsequent amplification of ovarian follicle-stimulating hormone (FSH) responsiveness by IGF 1. Pals et al. (2008) reported that the majority of GHRH target cells in the 14-day-old rat pituitary are cells expressing LH beta mRNA alone or in combination with GH and/or proopiomelanocortin (POMC) mRNA, showing that glucocorticoids and GH co-regulates the gonadotroph and somatotroph population sizes, the former preserving bihormonal gonadotrophs and the latter repressing LH beta-only cell abundance. GHRH may not expand the somatotroph population unless glucocorticoid hormone is present to maintain terminal differentiation. GHRH has effect on *in vitro* bovine oocyte maturation, which is not on nuclear maturation or cumulus expansion

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of bovine cumulus oocyte complexes (COCs), but retard cytoplasmic maturation by delaying cortical granule migration (Beker et al., 2005). hGHRH(1-29) [^{1}Tyr-hGHRH(1-29), molecular weight 3358 Da] was administered to improve the ovarian responsiveness to gonadotropins in poor responder women (Hugues et al., 1991). Many women with polycystic ovary syndrome (PCOS) have an impaired GH response to L-dopa and GHRH. Hyperandrogenism in PCOS may contribute to the reduced GH secretion because testosterone directly stimulates somatostatin release. Reduction of the excessive androgens facilitates the dopaminergic control of GH. GHRH shows a prospective therapy to the female infertility with hypofunction of ovary.

Spiliotis (2003) reported that GH and IGF 1 play significant roles in pubertal development, menarche, the menstrual cycle, and reproduction. GH directly provides an important modulatory effect on gonadotropin-dependent and independent functions. GH acts on the ovary affecting gametogenesis and steroidogenesis, *i.e.* the maturation of the follicle and gamete. GH deficiency or insufficiency causes a delay in the onset of puberty and in its normal course. The majority of women with GH-deficiency require assisted reproductive technologies to induce ovulation. Yoshimura et al. (1994) discovered that GH affects the ovary indirectly through amplification of gonadotropic action. Childs (2000) reported that GH stimulates ovarian follicles and Leydig cells by working alone or synergistically with LH and FSH. GH cells acts as co-gonadotrope in the regulation of the reproductive system. Childs (2002) suggested that the anterior pituitary contains a subset of GH cells that have the capacity to respond to multiple releasing hormones and support more than one system. Adashi (1993) thought that a population of multihormonal/multipotential cells exists in the anterior pituitary to support the extra needs of the gonadotrope and somatotrope population at mid-cycle. In the anterior pituitary, the cells begin as GH cells with GHRH receptors. Then, estrogens stimulate the expression of GnRH receptors. The cells are potentially capable of responding as gonadotropic cells. Most GH cells have GHRH receptor expression, indicating that the subset of cells is multifunctional. Izadyar et al. (1999) reported that the ovarian GH synthesis is not controlled by GHRH, which suggests that the multifunctional cells are both correlated and independent to some extent. Bartke et al. (1994) over-expressed human GH in the mouse. The transgenic mice lead to additional endocrine and reproductive abnormalities including stimulation of LH secretion, loss of responsiveness to testosterone feedback, overstimulation of mammary glands, enhanced mammary tumorigenesis, and hypertrophy of accessory reproductive glands in males. So hGH had a contradictory result on human infertility. Hartmann et al. (1997) reported the effect of hormone replacement therapy (HRT) on growth hormone stimulation in women with premature ovarian failure (POF). The results were the following: Women with POF have similar GH secretion patterns as healthy age-matched women; Physiologic HRT has no impact on GHRH-induced GH stimulation; and HRT has no impact on body weight in women with POF, which suggests the extra-genetic GHRH does not induce GH effect.

From the above publications, GH secretion does not definitely follow GHRH. The effect of GHRH on ovarian function probably was not exerted *via* activation of the somatotrophic axis GHRH-GH-IGF1.

In our previous publication (Zhou et al., 2015), *Grin* peptide [^{1}P-hGHRH(2-44)-GGC-CGG-hGHRH(44-2)-P^{1}] (also known as 2F) showed the strongest and long-lasting *in vitro* effect on rat GH release and similar species-specificity compared to natural hGHRH(1-44)NH_2. Human GHRH-like peptide was not evaluated in the pharmacodynamics of female infertility. The treatment effect and mechanism of *Grin* on the infertility models of female hamsters were reported in the paper.

2. Materials and methods

2.1. Sequence and synthesis of Grin

Grin monomer [(H)PADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARLGGC(OH)] and hGHRH(1-44)NH_2 [(H)YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARL(NH_2)] were manufactured by solid phase synthesis (*China Peptides Co., Ltd.*, China). *Grin* [(H)PADAIFTNSYRKVLGQLSARKLLQDIMSRQQGESNQERGARARLGGC(OH)-C(OH) GGLRAR-AGREQNSEGQQRSMIDQLLKRASLQGLVKRYSNTFIADAP(H), MW 10381 Da] was synthesized in a special dimerization protocol. All the structure-activity research about *Grin* referred to our previous publication (Zhou et al., 2015).

2.2. Experimental animal models

Chinese hamsters (*Cricetulus griseus*) (5-week-old female and 10-week-old male) were purchased from Sichuan Dasuo Animal Center (China). They were bred in the Sichuan Dasuo Animal Center for female infertility models and mating test. Kunming mice (female, 18–20 g) were purchased from the Animal Center of Guangzhou University of Traditional Chinese Medicine (China) for the maximal tolerated dose evaluation. Animals were treated in accordance with the Institutional Guidelines for Care and Use of Experimental Animals. The test animals were kept under controlled lighting (14 h light /10 h dark) at a constant temperature (23 ± 2 °C) and chow supplied *ad libitum*. The studies were approved by the Animal Centers on animal care. After all hamsters used were raised for 2 days, they were randomly grouped.

2.3. Experimental design

Fifty female hamsters were randomly allocated into 5 groups (single cyclophosphamide, hMG, H-, M-, L-*Grin*) (n = 10). All the animals were intraperitoneally injected 20 mg/kg of CPA (Ratification No.12032925, *Jiangsu Henrui Pharmaceutical Co.,* China) once in a week for 5 weeks. After the fourth CPA injection, *Grin* (H-, M-, L-*Grin*: 8, 4, 2 mg/kg of dose) or hMG (200 U/kg or 46.2 mg/kg, FSH: LH = 1:1, Ratification No. 120506, *Livzon Pharmaceutical Group Co., Ltd*. China) as positive drug was intramuscularly injected twice in a week in the hind leg muscle until the end of the ten-week experiment. The single CPA group as model control received saline vehicle in the latter five-week experiment. In the experimental 6–8th week, all the hamster models mated with healthy male hamsters (10 weeks age) (1:1 mating ratio). The couples were separated to breed in the experimental 9–10th week.

2.4. Fertility of female model and sample collection

During the 6-8th week of the experiment, one female hamster model (10 weeks old) was bred with one healthy male hamster (10 weeks old), and provided with standard pellet chow. The pregnant female hamsters were recorded by observing the beaded feta in the ovarian duct. The hamster models were weighed per week and their daily activities were observed. The total pregnancy rate was calculated according to the pregnant hamster number and new baby. After the 10th week, the body weights of the female models were determined and they were sacrificed by cervical dislocation. Blood was collected by taking eyeball without anticoagulant. Serum was separated by centrifuging at 6000*g* for 30 min and stored at −70 °C for ELISA of GH or estrogen. Livers and ovaries were quickly dissected out and weighed. Ovaries were kept at −70 °C for ELISA and Western blot assays (n = 6–10) or fixed in 10% formaldehyde in PBS for histological evaluation (n = 3).

2.5. Histochemical analysis of ovarian tissue

2.5.1. H-E staining

Ovaries were fixed overnight in 10% formalin-PBS solution, dehydrated in ethanol, and embedded in paraffin. Tissue sections (6 μm) were mounted on glass slides and dried at 60 °C for 10 h. The sections were then deparaffinized with xylene and rehydrated with alcohol and water. The rehydrated sections were stained with haematoxylin and eosin, mounted with 50% glycerol in PBS and examined under a light