



Effects of GnIH treatment on ovarian steroid receptor proteins in cyclic mice: In vivo and in vitro studies

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Manuscript details:

Received: 21.05.2025

Revised: 28.05.2025

Accepted: 06.06.2025

Published: 10.06.2025

Cite this article as:

Padmasana Singh (2025) Effects of GnIH treatment on ovarian steroid receptor proteins in cyclic mice: *In vivo* and *in vitro* studies, *Int. J. of Life Sciences*, 13 (2): 165-172.

Available online on <http://www.ijlsci.in>

ISSN: 2320-964X (Online)

ISSN: 2320-7817 (Print)



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ABSTRACT

The aim of the present study was to evaluate the effects of treatment with different doses of GnIH *in vivo/in vitro* on ovarian follicular development, luteinization and steroidogenesis in the cyclic mice. Different doses of GnIH (low = 100 ng/day, high = 500 ng/day and pharmacological = 2 µg/day) were administered daily starting on proestrous for 8 days. One side of the ovaries of GnIH treated and control mice were fixed, serial sectioned and subjected to morphological analysis. Contralateral ovaries of the mice were processed for western blot analysis of progesterone receptor, estrogen receptor- α and GnRH I-receptor. Immunoblots were quantified by densitometry. The ovarian histology revealed dose dependent changes on follicular development and luteinization. The mice treated with pharmacological dose (2 µg/day) showed majority of antral follicles with abnormal morphological features. The antral follicles showed heterogeneous population of abnormal granulosa cells, hypertrophied thecal cells and abnormal corpus luteum. *In vivo* treatment with GnIH stimulated the expression of GnRH-I receptor, PR, and ER- α , whereas *in vitro* treatment with GnIH exerted an inhibitory effect on the expression of these proteins in mouse ovaries. These findings suggest that GnIH exerts a direct inhibitory effect on ovarian follicular development and steroidogenesis.

Key words: GnIH, folliculogenesis, steroid receptors, GnRH-I R, ovary, cyclic mice.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a decapeptide hormone produced by the hypothalamus that regulates reproductive functions in mammals by controlling the synthesis and secretion of gonadotropins—luteinizing hormone (LH) and follicle-stimulating hormone (FSH)—from the anterior pituitary gland (Conn & Crowley 1994; Stojilkovic *et al.* 1994). In addition to its well-established role at the pituitary level, gonadotropin-releasing hormone (GnRH) has also been implicated as a regulatory factor in several extrapituitary

tissues, including the gonads (Bauer-Dantoin & Jameson, 1995; Peng et al., 1994). Over the past several years, accumulating evidence has shown the presence of GnRH and its receptors in the ovaries of various vertebrate species where they exert autocrine and paracrine effects in the regulation of ovarian functions, such as steroidogenesis, folliculogenesis, and apoptosis (Dong et al., 1996; Singh et al., 2007). Billig et al., 1994; Srivastava et al., 1995; Tsafiriri & Adashi, 1994). Furthermore, the expression levels of GnRH and its receptor in the ovary appear to fluctuate across the estrous cycle in rats (Schirman-Hildesheim et al., 2005; Schirman et al., 2006). However, the specific factors that modulate GnRH action within the ovary remain poorly understood.

In 2000, Tsutsui and coworkers identified a novel hypothalamic neuropeptide containing RF amide at its C-terminal end, that directly inhibits gonadotropin release from cultured anterior pituitary cells of quail (Tsutsui 2000). They termed it gonadotropin inhibitory hormone (GnIH). Subsequently, Yin et al. found and replicated a new G protein-coupled receptor (GPR147) cDNA that encodes a potential GnIH-receptor (GnIH-R) in quail (Yin et al. 2005). Precise localization of GnIH in the avian brain was confirmed using *in situ* hybridization and immunocytochemistry. The results demonstrated that both GnIH protein and mRNA are localized in the paraventricular nucleus (PVN) of species such as quail, sparrow, and starling (Osugi et al., 2004; Ubuka et al., 2008; Ukena et al., 2003;). GnIH-expressing neurons were found in close proximity to GnRH neurons in the preoptic area of these avian species (Bentley et al., 2003; Ubuka et al., 2008).

Beyond the hypothalamus, both GnRH and GnIH have also been identified in reproductive tissues, suggesting that these neuropeptides may have additional roles outside of traditional neuroendocrine regulation (Singh et al., 2011).

Since last 15 years, GnIH has been found in a variety of vertebrate species, including fish to mammals. By inhibiting gonadotrophin synthesis and release via its effects on the hypothalamus and pituitary glands, it contributes to the regulation of both metabolism and reproduction (Ubuka et al., 2008). The study also showed that GnIH administration *in vivo* decreases the seminiferous tubule diameter and the number of germ cells in the quail (Ubuka et al., 2008). Whether these

effects of GnIH on testis are via suppression of pituitary gonadotropin synthesis or due to direct action of GnIH on testis are not clear. Mice administered with GnIH showed marked disruptions in steroidogenesis, attributed to its inhibitory effects on the ovarian functions (Singh et al., 2011). However, the detail physiological roles and biological significance of GnIH on steroid receptors in the ovary remained to be elucidated. Therefore, in the present study, the effect of *in vivo* and *in vitro* treatment of GnIH on ovarian follicular development and steroid receptor expression was investigated in the cyclic mice. In order to understand the relationship of GnIH with GnRH, effect of GnIH treatment on ovarian expression of GnRH I-receptor protein was also investigated.

MATERIALS AND METHODS

Animals

All experiments were conducted in accordance with the principles and procedures approved by the Departmental Research Committee of Banaras Hindu University. Parkes strain mice (10–12 weeks old) were used in this study. The animals were housed under controlled conditions of temperature and humidity in the institutional animal facility, with a 13:11 h light-dark cycle (lights on from 0700 to 2000 h), and food and water provided *ad libitum*. Estrous cycles were monitored by daily examination of vaginal cytology, and only animals exhibiting at least two consecutive regular cycles of 4–5 days were included in the study

Treatment

To examine the effects of GnIH treatment on ovarian follicular development and steroid receptor expression, cycling mice were daily injected, subcutaneously, with different doses of GnIH (low = 100 ng/day, high = 500 ng/day and pharmacological = 2 µg/day) dissolved in normal saline for 8 days (n = 8-10). GnIH was a generous gift from Dr. K. Tsutsui, Tokyo, Japan that was isolated from quail brain. The treatment with GnIH was started on proestrus morning for each mouse. The mice in the control group (n = 10) were injected with vehicle only. Vaginal cytology was evaluated throughout the course of experiments as an indirect indication of ovarian cyclicity. The animals were sacrificed by decapitation under mild anesthesia (anesthetic ether) within 30 min after the last injection. Ovaries were excised out, cleaned from any adhered fat tissue and oviduct, and weighed. The ovary

of one side from each animal was snap frozen and kept at -40 °C until protein extraction for immunoblots and the contralateral ovary was fixed in Bouin's fixative at room temperature for histological evaluation of follicular development.

Histology

Ovaries collected from the mice injected with different doses of GnIH were dehydrated, embedded in paraffin wax, sectioned serially (5 µm) and stained with hematoxylin and eosin. The slides were then observed under a Nikon Eclipse Microscope E200 (Nikon, Tokyo, Japan). Estimation of different types of follicles (both preantral and antral excluding primordial) and number of corpus luteum in the ovary were made by examining every fifth serial section of each ovary and then counting the follicles whose plane of section passed through the nucleolus of the oocytes.

Ovaries collected from mice treated with varying doses of GnIH were dehydrated, embedded in paraffin wax, and serially sectioned at 5 µm thickness. Sections were stained with hematoxylin and eosin and examined under a Nikon Eclipse E200 microscope (Nikon, Tokyo, Japan). Follicle classification (preantral and antral, excluding primordial follicles) and corpus luteum counts were analyzed from every fifth serial section of each ovary. Only follicles with a visible nucleolus in the oocyte were analysed. Preantral and antral follicles were classified based on standard morphological criteria. Follicular atresia was identified by the presence of pyknotic nuclei in granulosa cells or morphological changes in oocytes, such as loss of the nuclear membrane and/or nuclear fragmentation (Ingram, 1962). Follicles exhibiting features such as hypertrophied granulosa cells with condensed nuclei, enlarged theca cells, and abnormal oocyte morphology were categorized as abnormal follicles.

In vitro study

The *in vitro* study was performed on the ovaries collected during proestrus to determine the direct effects of different doses of GnIH on ovarian activity. Animals were sacrificed on proestrus having multiple large antral follicles. Their ovaries were quickly dissected out and cleaned for any adhered fat tissue and oviduct in medium Dulbecco Modified Eagle's Medium (DMEM; Himedia, Mumbai, India) containing 250 U/ml penicillin and 250 µg/ml streptomycin sulfate. Culture media was a mixture of DMEM (with sodium pyruvate and L-glutamine) and Ham's F-12

(1:1; v:v) (Himedia, Mumbai, India) containing 100 U/ml penicillin, 100 µg/ml streptomycin and 0.1% BSA (Sigma Chemicals Co., St Louis, USA). Following an initial 2-hour incubation at 37 °C, the culture medium was discarded, and ovaries (one per tube) were cultured in 1 ml of fresh medium for 24 hours in a humidified atmosphere of 95% air and 5% CO₂ (pH 7.4) at 37 °C. GnIH was administered at doses of 1 ng, 10 ng, and 100 ng in a total volume of 10 µl per ml of medium per tube, based on results from preliminary studies (Singh et al., 2011). Control tubes received 10 µl of saline per ml of medium and did not receive GnIH. Each treatment group was run in triplicate. Ovaries cultured under these conditions remained healthy and showed no signs of necrosis. At the end of the culture period, ovaries were collected, washed several times with PBS, and stored at -40 °C for subsequent immunoblot analysis.

Immunoblotting

Ovaries were homogenized to produce 10% homogenate. Further, protein extraction and immunoblot was performed (Singh et al. 2011). Equal amount of proteins (15-50 µg) as determined by Lowry's method (Lowry et al. 1951) was loaded on to SDS-PAGE (10% or 12%) for electrophoresis. Thereafter, proteins were transferred electrophoretically to PVDF membranes (0.45µm, Millipore, USA) overnight at 4 °C. Membranes were blocked for 60 min in blocking solution (100 mM phosphate buffered saline pH 7.4, 150 mM NaCl, 5% fat free dry milk) and then incubated with primary antibody for 1 h at room temperature (PR and ER-α at a dilution of 1:1000). Membranes were then washed with three changes of phosphate buffered saline (0.1 M PBS pH 7.4) over 10 min. Immunodetection was performed with anti-rabbit or anti-mouse IgG-horse radish peroxidase antibody (at a dilution of 1:500). Finally, the blots were washed three times with PBS and detected with enhanced chemiluminescence detection system (Bio Rad, Hercules, USA). Immunoreactive bands were later scanned from X-ray film and then quantified by densitometry (Image J vs 1.36, NIH, USA). Experiments were repeated three times with the same result. Equal loading was confirmed with Ponceau S staining and β-actin. All immunoblots were normalized to β-actin.

Statistical analysis

The data were analyzed by one-way analysis of variance followed by Duncan's multiple range post hoc test. The difference was considered significant if P < 0.05.

RESULTS

Effect of *in vivo* treatment of GnIH on ovarian histology and follicular development

The mice treated with different doses of GnIH showed a dose dependent change in the follicular morphology and development (**Fig. 1**). The ovary of control mice showed numerous small and medium sized healthy antral follicles (**Fig. 1 A**). The mice treated with the low dose of GnIH showed no change in the morphology of follicle and they appeared healthy antral follicles (**Fig. 1 B**). The antral follicles and corpus luteum showed nearly normal histological features in the mice treated with the low dose of GnIH. But the mice treated with high (500 ng/day) and pharmacological dose (2

µg/day) of GnIH showed many apparently abnormal follicles together with healthy preantral and antral follicles. The abnormal follicles showed the marked changes in the morphology of granulosa cells (GCs), which either undergoes hypertrophied or condensed nuclei. (**Fig. 1 C, E**). Both the types of GCs can be seen in the same follicles. Theca cells consist of only 2-3 layers of hypertrophied and vacuolated cells (**Fig. 1 E**). The corpus luteum in the ovary in the ovaries of mice treated with high and pharmacological doses of GnIH exhibited subnormal morphology, characterized by loosely arranged and highly vacuolated luteal cells that suggests impaired luteal differentiation and function (**Fig. 1 D, F**).

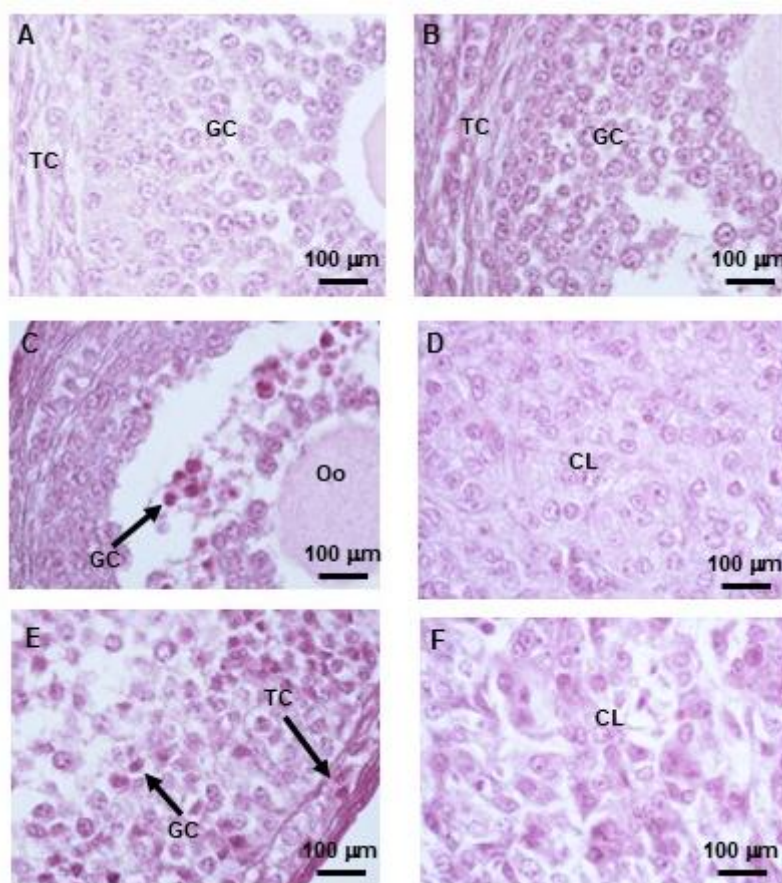


Figure 1 : Transverse sections of the ovaries (stained with haematoxylin-eosin) of cyclic mice showing histological changes following administration of GnIH:

(A) ovary of control mice - ovary of vehicle treated normal control showing antral follicle containing healthy granulosa cells (GC) and theca cells (TC); (B) ovary of mice treated with 100 ng/day GnIH - normal healthy antral follicle was observed; (C) & (D) ovary of mice treated with 500 ng/day GnIH; (C) a portion of ovary showing abnormal follicle having condensed (black arrow) nuclei of granulosa cells; (D) regressed corpus luteum (CL); (E) & (F) ovary of mice treated with 2 µg/day GnIH - (E) a portion of ovary showing abnormal follicle having condensed and hypertrophied granulosa cells (GC), hypertrophied theca cells (TC), (F) regressed corpus luteum (CL).

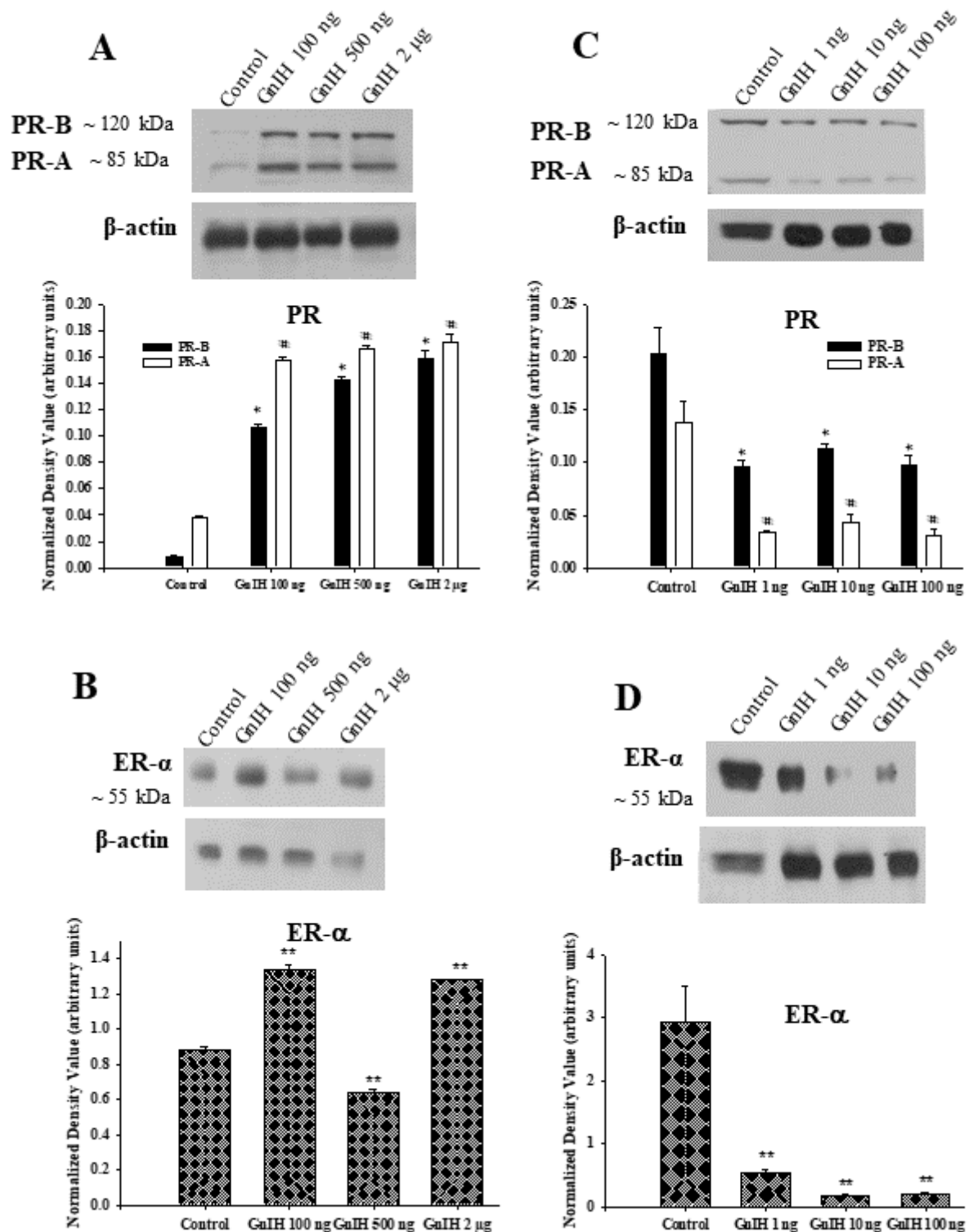


Figure 2 : Western blot analyses of PR (Fig. A) and ER-α (Fig. C) proteins in the ovaries of cyclic mice after *in vivo* treatment of different doses of GnIH. Western blot analyses of PR (Fig. B) and ER-α (Fig. D) proteins in the ovaries after *in vitro* treatment of different doses of GnIH. Mice treated with normal saline served as control for *in vivo* and ovaries treated with medium alone served as control for *in vitro* experiments. Values are represented as mean ± S.E.M. (n = 6)

* Values for PR-B are significantly different ($P < 0.05$) versus control.

Values for PR-A are significantly different ($P < 0.05$) versus control.

** Values for ER-α are significantly different ($P < 0.05$) versus control.

Evidence that the GnIH modulates ovarian progesterone receptor (PR) and estrogen receptor- α (ER- α) proteins in mice

Effect of *in vivo* treatment of GnIH on the expression of PR and ER- α in the ovaries of mice are shown in **Fig 2 A, B**. Western blot analysis of PR in the ovaries of GnIH-treated mice revealed two immunoreactive bands corresponding to ~85 kDa (PR-A) and ~120 kDa (PR-B). *In vivo* treatment with all the doses of GnIH caused significantly increased expression of both the isoforms of PR. Western blot analysis of ER- α in the ovaries of GnIH-treated mice revealed a single

immunoreactive band at ~55 kDa. The expression of ER- α protein increased significantly in the mice treated with low and pharmacological doses of GnIH, whereas expression of ER- α protein decreased significantly in the mice treated with high dose of GnIH.

Effect of GnIH treatment on expression of PR and ER- α *in vitro* in the ovary of mice are shown in **Fig 2 C, D**. *In vitro* treatment of mouse ovaries with all three doses of GnIH significantly decreased the expression levels of PR and ER- α proteins.

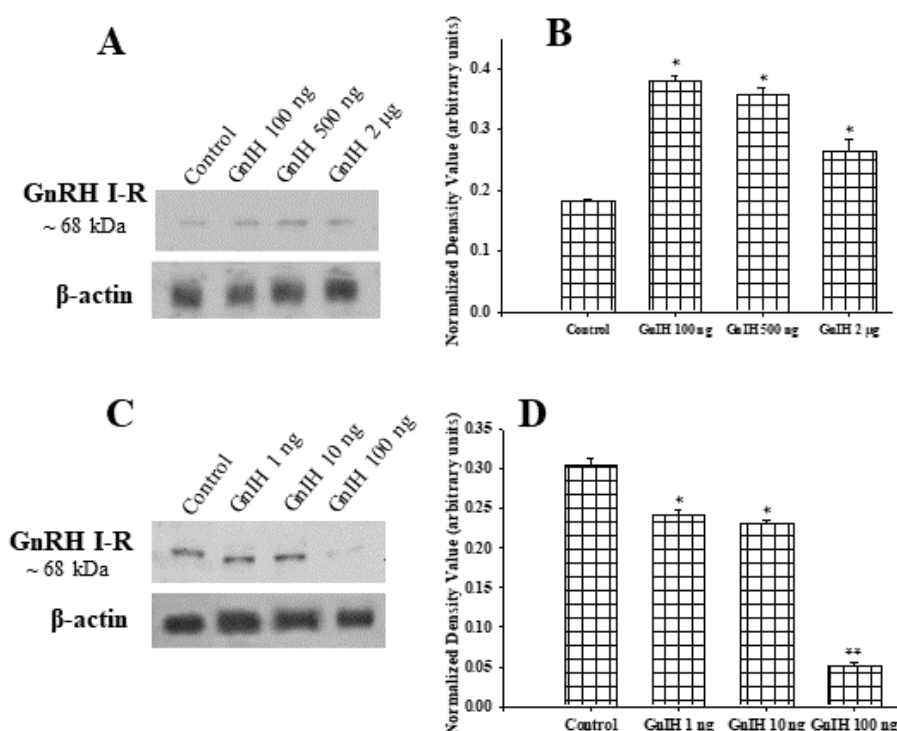


Figure 3: Western blot analyses of different doses of GnIH treatment on GnRH I-receptor protein *in vivo*, (Fig. A) and *in vitro* (Fig C) in the ovaries of mice. Mice treated with normal saline served as control for *in vivo* and ovaries treated with medium alone served as control for *in vitro* experiments. Densitometric analyses of the blots are shown in bar graphs. Values are represented as mean $\pm$ S.E.M. (n = 6) obtained from three immunoblots

* Values are significantly different ($P < 0.05$) versus control.

Evidence that the GnIH modulates ovarian GnRH I-receptor proteins in mice

The effects of *in vivo* and *in vitro* treatment of GnIH on the expression of GnRH I-receptor in the ovary of mice are shown in **Fig. 3**. Both *in vivo* and *in vitro* treatment with GnIH caused a marked variation in the expression of GnRH I-receptor protein in the ovaries of mice. Treatment with GnIH *in vivo* showed a significant

increase in the expression of GnRH I-receptor protein in the ovaries of mice (**Fig. 3 A, B**). All the three doses of GnIH treatment *in vitro* caused the significant decline in the expression of GnRH I-receptor protein (**Fig. 3 C, D**). The declined in GnRH I-receptor protein expression was more pronounced in the ovary of mice treated *in vitro* with pharmacological dose of GnIH.

DISCUSSION

GnIH recently identified as a novel hypothalamic neuropeptide inhibiting gonadotropin release in the bird (Tsutsui *et al.* 2000). However, intraovarian function of GnIH has not been so far demonstrated in mammals. The purpose of the present study was to evaluate the effects of *in vivo* and *in vitro* treatment of GnIH on morphological structure of the ovary and expression of steroid receptors in the ovary. The results of this study provide evidence for inhibitory effect of GnIH on ovarian activity including follicular development and steroidogenesis in mice.

The results presented in this study assessed by the determination of decreased number of healthy follicles demonstrate the dose dependent inhibitory changes in the effects of GnIH treatment *in vivo* on ovarian follicular development and luteinization in cyclic mice. The mice treated with 500 ng and 2 µg/day dose of GnIH showed the presence of a number of large antral follicles with abnormal morphological features in the ovary. The abnormal antral follicles showed dispersed and heterogenous population of GCs. Some of the GCs were hypertrophied contained large and vacuolated nuclei, whereas other GCs showed small, condensed and irregular shaped nuclei. Pyknotic cells are seen in the abnormal antral follicle. The morphological features of oocytes were also not normal. Decrease in the number and size of corpus luteum in the mice treated with GnIH suggest qualitatively and quantitatively sub-normal ovulation. This might be due to the suppressive effects of GnIH on synthesis and release of LH (Ciccione *et al.* 2004; Tsutsui *et al.* 2000; Tsutsui *et al.* 2006; Ubuka *et al.* 2006). Inhibitory effect of GnIH on plasma LH level were found to be main physiological actions of GnIH both in quail and Syrian hamster (Kriegsfeld *et al.* 2006). The reduction in thecal layer thickness observed in the abnormal antral follicles may also be due to deficiency of LH support for thecal development. However, the presence of many late antral follicles in the ovaries of mice treated with GnIH suggests that FSH secretion is not suppressed completely. Unfortunately, LH and FSH levels could not be measured in the present study. However, a degree of residual level of gonadotropin secretion would be expected to persist after GnIH treatment with FSH being less suppressed than LH (Ubuka *et al.* 2006). While this suppression is sufficient to reduce the ability of antral follicles to be selected as dominant follicles, the low levels of LH and

FSH available may be sufficient to maintain follicular development up to antral follicles.

The result of the present study showed that GnIH treatment also regulates expression of GnRH I-receptor, PR and ER-α proteins in the ovaries of mice. The treatment of GnIH *in vivo* showed a stimulatory effect on the expression of GnRH I-receptor, PR and ER-α, whereas the treatment of GnIH *in vitro* showed inhibitory effect on the expression of these proteins in the ovaries of mice. Osugi (2004) recently reported that treatment with GnIH *in vivo* rapidly inhibited GnRH mediated LH release in the sparrow Osugi *et al.* (2004). The reason for this differential expression pattern of these receptor proteins in response to GnIH treatment *in vivo* or *in vitro* is not known. However, it is possible that the GnIH induced regulation of multiple ovarian functions in mice may be the result of change in the concentration of steroids and GnRH I-receptors.

In brief, the present study provides evidence suggesting that GnIH acts directly, as well as indirectly on the ovary and inhibit the development of dominant follicles. To our knowledge, GnIH is the first identified neuropeptide that has a direct inhibitory action on gonadal functions. The present *in vitro* study showed inhibitory effect of GnIH on ovarian expression of steroid receptors and GnRH I-receptor. This suggests that GnIH might be acting by directly inhibiting ovarian steroid and GnRH I-receptor. GnIH may directly influence antral follicle development and apoptosis and luteinization. The present study provides evidence for interaction between GnIH, GnRH and steroid receptors at the ovarian level.

Conflict of Interest: The authors declare no conflict of interest in relation to this research.

Data Availability Statement: Not applicable.

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Peer review information

IJLSCI thanks the anonymous reviewers for their contribution to the peer review of this work. A peer review file is available.

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