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Regulation of hormonal receptors of the hypothalamic-pituitary-gonadal axis by ghrelin in ovary of grass carp (*Ctenopharyngodon idella*)

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A R T I C L E I N F O

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A B S T R A C T

Ghrelin has been well known as an orexigenic hormone that regulates appetite and energy metabolism in animals. Recent studies imply the regulatory function of ghrelin on ovary development, however, the mechanism about the effects of ghrelin on key hormonal receptors in ovary is far away from systematically understood. The present study aimed to analyze the tissue distribution of ghrelin and related genes and elucidate the regulation of ghrelin on key hormonal receptors in ovary of grass carp (*Ctenopharyngodon idella*). High expression of ghrelin and ghrelin O-acyltransferase (GOAT) was found in the ovary compared to heart, kidney, spleen, muscle, brain, liver, gill and intestine. To understand the regulatory function of ghrelin on ovarian development, the effects of ghrelin on the key hormone receptors participated in ovarian maturation were determined by ghrelin, acyl-ghrelin and octanoic acid (substrate in the ghrelin acylation system) treatments with 1, 10 and 100 nM concentration. The in vivo study showed that all the 1, 10 and 100 nM ghrelin treatment stimulated follicle-stimulating hormone receptor (FSHR) expression compared to the control. Estrogen Receptor-α (ERα) was depressed by 10 nM acyl-ghrelin compared to the control, while ERβ1 and ERβ2 could be inhibited by 1 nM and 10 nM octanoic acid treatment compared to the control, respectively. The present results for the first time provided novel information about the regulatory role of ghrelin on key hormonal receptors in ovarian of grass carp providing potential new ideas for cultivating of grass carp broodstock.

1. Introduction

Grass carp (*Ctenopharyngodon idella*) is one of the most productive fish which provides the massive fish protein in the world (Xie et al., 2018). The species takes more than 4 years to achieve sexual maturity which is greatly restricted for development of breeding work (Zhao et al., 2020). Gynogenetic breeding and hybrid breeding can speed up the acquisition of individuals with dominant traits (Zhang et al., 2011), but it still takes a long time. In the 1950 s, China successfully completed artificial reproduction of grass carp by stimulating the gonad development using hormones such as luteinizing hormone-releasing hormone analogue (LHRH-a) (Zhang et al., 2018; Zhong et al., 2021a). During the reproductive seasons (from May to June) of each year, 15–20 μg LHRH-A injection of female grass carp and 5 μg LHRH-A injection of male grass carp could induce spawning in 10–16 h after injection at 23 ± 2 °C and the eggs and sperm could be obtained for artificial fertilization (Liu et al., 1993). Hormonal regulation is a key method for artificial operation to control reproductive development and behavior of fishes.

Ghrelin is a peptide hormone which has been reported as a promoter to stimulate growth hormone (GH) releasing and induce appetite firstly (Nakazato et al., 2001). It has been regarded that after being acylated by ghrelin O-acyltransferase (GOAT) (Gahete et al., 2010), ghrelin could interact with growth hormone secretagogue receptor (GHS-R) to exert various physiological effects (Liu et al., 2011). Ghrelin, GOAT and GHS-R form the Ghrelin/GOAT/GHS-R system involving in regulation of growth performance, food intake, energy balance, and reproductive process (Delporte, 2013; Lim et al., 2011). Recent evidences suggested that des-acyl to acyl ghrelin ratio regulate energy metabolism which is related to ovarian development and gamete maturation (Hatef and Unniappan, 2019; Sirini et al., 2017). In addition, it has been found that Ghrelin/GOAT/GHS-R system could interact with the hypothalamic-pituitary-gonadal (HPG) axis (Kluge et al., 2013). It is well known that HPG axis contains several hormones and plays crucial role in regulation of gonadal development (Maruska and Fernald, 2011), Ghrelin/GOAT/GHS-R system may affect ovarian development via regulating hormonal secretion in HPG axis. Unfortunately, we still lack of exact evidences about the Ghrelin/GOAT/GHS-R system function in regulation of hormone in HPG axis in grass carp.

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The HPG axis consists hormone released by hypothalamus, pituitary and gonad (Peper et al., 2010). Fish hypothalamus secrete neurohormones including gonadotropin hormone-releasing hormone (GnRH) (Barabutis and Schally, 2010) and GnRH could stimulate pituitary to produce gonadotropin (GTH) including follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Muñoz-Cueto et al., 2020; Yaron et al., 2003). FSH and LH could release to circulatory system and interact with FSH receptor (FSHR) and LH receptor (LHR), respectively (Rahman et al., 2003). The interaction promotes follicular maturation and ovulation in female via stimulation of estrogen (E2) secretion in ovary (Guiguen et al., 2010). By binding to estrogen receptor (ERα and ERβ), E2 participates in regulating oocyte maturation (Amenyogbe et al., 2020). Recent studies have shown that ghrelin regulated serum concentration of E2 and expression of ERβ suggesting the role of ghrelin in gonadal development (Fang et al., 2012; Rak-Mardyła et al., 2014). Meanwhile, E2 could directly regulate GTH expression in the female rat stomach (Matsubara et al., 2004). These clues suggest that a crosstalk exists between Ghrelin and hormones in HPG axis (Zhong et al., 2021b). In aquaculture, fish reproduction could be controlled by artificial manipulation of hormones (Mylonas et al., 2010). Such hormonal manipulation requires solid understanding of the regulatory mechanism of HPG axis.

Grass carp needs artificial inducing of ovulation in aquaculture. Considering the long term for maturation, stimulation of HPG axis by other hormone may promote final maturation of the eggs. Recent studies have shown that ghrelin may be an important regulator for regulation of HPG axis (Tena-Sempere, 2007). In the present study, we focus on the ghrelin and acyl-ghrelin effects on FSHR, LHR and ER in ovary grass carp. By evaluating the effects, we provide new evidences of ghrelin function in regulation of key receptors in HPG axis and extend knowledge about ovarian development controlled by peptide hormone secreted by the gastrointestinal tract. The present results would aid to provide potential method to cultivate grass carp for breeding.

2. Materials and methods

2.1. Fish sampling

The experimental grass carp were provided by the State Key Laboratory of Developmental Biology of Freshwater Fish, Hunan Normal University (Changsha, China). The grass carp were reared in a 667 m^2 pond and were fed twice a day at 7:00 and 17:00. Tissues from three 5-years old individuals were collected for the study. Before tissue collection, the grass carp were anesthetized with MS-222 (200 mg/L). The heart, kidney, spleen, muscle, brain, ovary, liver, gill and intestine were collected and frozen immediately in liquid nitrogen and stored at −80 °C until RNA isolation. A part of intestine tissues was collected for immunofluorescence. Meanwhile, ovarian tissues were collected for primary cell culture. All the experiments were approved by the Animal Care Committee of Hunan Normal University (Changsha, China).

2.2. Immunofluorescence

Ghrelin and GHS-R were detected in intestinal tissues of grass carp by immunofluorescence. After the fixation with 4% paraformaldehyde, the tissues were prepared to embedded in OCT (Sakura Fine-Tek, Japan) and slices into 10 μm thick section. After blocked by 4% dry milk powder, the sections were incubated with primary antibody (Anti-ghrelin antibody and Anti-GHS-R antibody, Sigma-Aldrich, USA). Subsequently, the sections were detected by secondary antibody Goat Anti-Rabbit IgG H&L (FITC) (Sigma-Aldrich, USA). After three times washes by TBST, DAPI (Sigma-Aldrich, USA) was used for staining the nuclear. The sections were imaged on a Leica inverted DMIRE2 microscope image system (Leica, Wetzlar, Germany).

2.3. Real-time quantitative PCR (qPCR)

For analysis of tissue distribution of ghrelin, GHS-R and GOAT, the total RNAs from heart, kidney, spleen, muscle, brain, ovary, liver, gill and intestine were extracted using TRIzol (Invitrogen, USA). The RNA was detected by 1% agarose gel and spectrophotometer (Sigma-Aldrich, USA). The first-strand cDNA was prepared by RT reagent Kit with genomic DNA Eraser (Takara, Japan) using 1 μg total RNA. The specific primers were synthesized from previous report (Cai et al., 2015) (Table 1). The qPCR was performed using PowerUp™ SYBR™ Green qPCR Master Mix (Applied Biosystems, USA) with the following condition: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 45 s at 60 °C by a CFX96 Real-time PCR Detection System (Bio-Rad, USA). The reaction system contained 2 μL cDNA, 0.8 μL forward and reverse primer, 10 μL PowerUp™ SYBR ™ Green qPCR Master Mix and 6.4 μL ddH_2O. β-actin was used as internal reference. The relative expression was calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Melt curve analysis was performed to ensure the specific amplification.

2.4. Ovarian cell culture and treatment

Ovarian tissues were collected from the grass carp and washed three times in sterile culture medium (DMEM/F-12 1:1 mixture supplemented with 10% bovine fetal serum and 1% antibiotic–antimycotic solution, all from Gibco, Carlsbad, USA). Then, the tissues were separated by repeated pipetting and digested with 4 mg/ml collagenase IV, 2.5 mg/ml hyaluronidase (Sigma), 2 mg/ml trypsin (Sigma) and 1 mg/ml DNase I for 15 min following three times washes by phosphate-buffered saline (PBS). The cells were cultured in 24-well plates (Corning, New York, USA) and incubated for 2–3 h at 38.5 °C under 5% CO_2 in humidified air. The cells were treated by ghrelin, acyl-ghrelin and octanoic acid for 6 h at 0, 1, 10, 100 nM, respectively. The mature peptides of ghrelin (GTSFLSPAQKPQGRRPPRV) and acyl-ghrelin (GTS (octanoic-acid) FLSPAQKPQGRRPPRV) were synthesized (GenScript Biotech Corp., Nanjing, China) (Sigma-Aldrich, USA). The octanoic acid was purchase from Sigma-Aldrich (USA). The expression of FSHR, LHR, ERα, ERβ1 and ERβ2 were assayed by qPCR. The primers were shown in Table 1 (She et al., 2015).

2.5. Statistical analyses

All the gene expression data were shown as average ± standard deviation. The significant differences among the tested tissues and treated groups were identified by One-way ANOVA analysis followed by Tukey's multiple comparison test. When $P < 0.05$, the significances were confirmed. All the statistical analysis was performed by SPSS 23.0.

3. Results

3.1. Tissue distribution of ghrelin, GHS-R and GOAT

To determine the tissue distribution of ghrelin and GHS-R in intestine, immunofluorescence was performed. The signals of ghrelin were

Table 1
The primers used in the present study.

Gene name	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
Ghrelin	CGCTCTTTACTTATGTCTCG	AGCACAGGACCGTATTTCT
GHS-R	GAGAAAGAGGGAGACGAT	GCACGAAGGCAAACA
GOAT	GCTCGCCAGTGGAACAGAAC	ACGCTGAGAACCCAAATGTCA
FSHR	CGGCTTCTTCACGGTCTTCT	ACCAGCGCTCCAGGGTAAT
LHR	TCTCCGCGGCCTTCAAA	AGCACCAGGAGGATCTTCGA
ERα	AGAGAAGCATTCAAGGTCAC	TGTCACGAGCCTCATTACTG
ERβ1	AAGGCATTGAGCATCTGTC	TCTGTCTCCATGTCCTCTC
ERβ2	CGTCCAGATTCAGAGAACT	GGTCCATGCTGAGAAGTG
β-actin	GGCTGTGCTGTCCCTGTATG	GGTAGTCAGTCAGGTCACGGC

observed in mucosal layer of intestine at the base of folds in the mucosa. The GHS-R signals were found in the mucosal epithelium and the submucosal layer in intestine of grass carp (Fig. 1).

Furthermore, the mRNA expression levels in different tissues were assayed by qPCR. The ghrelin mRNA expression levels were most abundant in the ovary but lower in the heart, kidney, spleen, intestine, and the brain (Fig. 2A). For GHS-R, the highest expression was found in brain while lower expression was observed in other tested tissues (Fig. 2B). Similar to ghrelin, the present result showed that ovary had the highest GOAT expression, while other tissues showed lower expression of GOAT mRNA (Fig. 2C).

3.2. Ghrelin and octanoic acid promote expression of FSHR in ovarian cells

The effects of ghrelin, acyl-ghrelin and octanoic acid on FSHR in ovary from grass carp were determined. The result showed that 1, 10 and 100 nM ghrelin treatment stimulated FSHR mRNA expression compared to the control significantly ($P < 0.05$) (Fig. 3A). In contrast, acyl-ghrelin did not change FSHR mRNA expression in ovary of grass carp (Fig. 3B). For octanoic acid treatment, only 10 nM octanoic acid group showed the increase of FSHR expression significantly ($P < 0.05$) while 1 and 100 nM octanoic acid did not change FSHR mRNA expression (Fig. 3C).

3.3. Ghrelin, acyl-ghrelin and octanoic acid did not affect LHR in ovarian cells

The expression of LHR mRNA levels in ovarian cells of grass carp affected by ghrelin, acyl-ghrelin and octanoic acid was examined by qPCR. The results showed that there was no significant difference in the expression level of LHR in the ovarian cells by ghrelin and acyl-ghrelin treatment ($P > 0.05$) (Fig. 4A, B). Similar result was shown with 1, 10, and 100 nM octanoic acid treatment. No significant differences could be found in LHR expression with the octanoic acid treatment ($P > 0.05$) (Fig. 4C).

3.4. Effects of ghrelin, acyl-ghrelin and octanoic acid on ER in ovarian cells

No significant difference of ERα expression was found among the ghrelin treated groups ($P > 0.05$) while 10 nM acyl-ghrelin group significantly decrease ERα expression compared to the control ($P < 0.05$). The octanoic acid treatment did not change the ERα expression in ovarian cells (Fig. 5). For ERβ1, ghrelin and acyl-ghrelin showed no significant effects on ERβ1 mRNA expression, while 1 nM octanoic acid treatment decreased ERβ1 mRNA expression significantly ($P < 0.05$) (Fig. 6). Similar to ERβ1, ghrelin and acyl-ghrelin treatment did not change ERβ2 mRNA expression, while 10 nM octanoic acid treatment group showed significant decrease of ERβ2 mRNA expression ($P < 0.05$) (Fig. 7).

4. Discussion

Ghrelin, GHS-R and GOAT have been considered as key genes in brain-gut axis playing crucial role in appetite and energy metabolism regulation (Nakazato et al., 2001). Recent clues suggested that they also participated in gonadal development (Tena-Sempere, 2008; Zhong et al., 2021b). In the present study, we demonstrated the regulation of ghrelin system on key sexual hormone receptors in the ovary of grass carp. The present results would promise possible usage for regulating HPG axis. In addition, the evidences may provide valuable information for the development of breeding technology of grass carp by elucidating the new regulatory factor of receptors in HPG axis.

Ghrelin is a hormone that releases in gastrointestinal tract (Kojima and Kangawa, 2002). The immunofluorescence results showed the

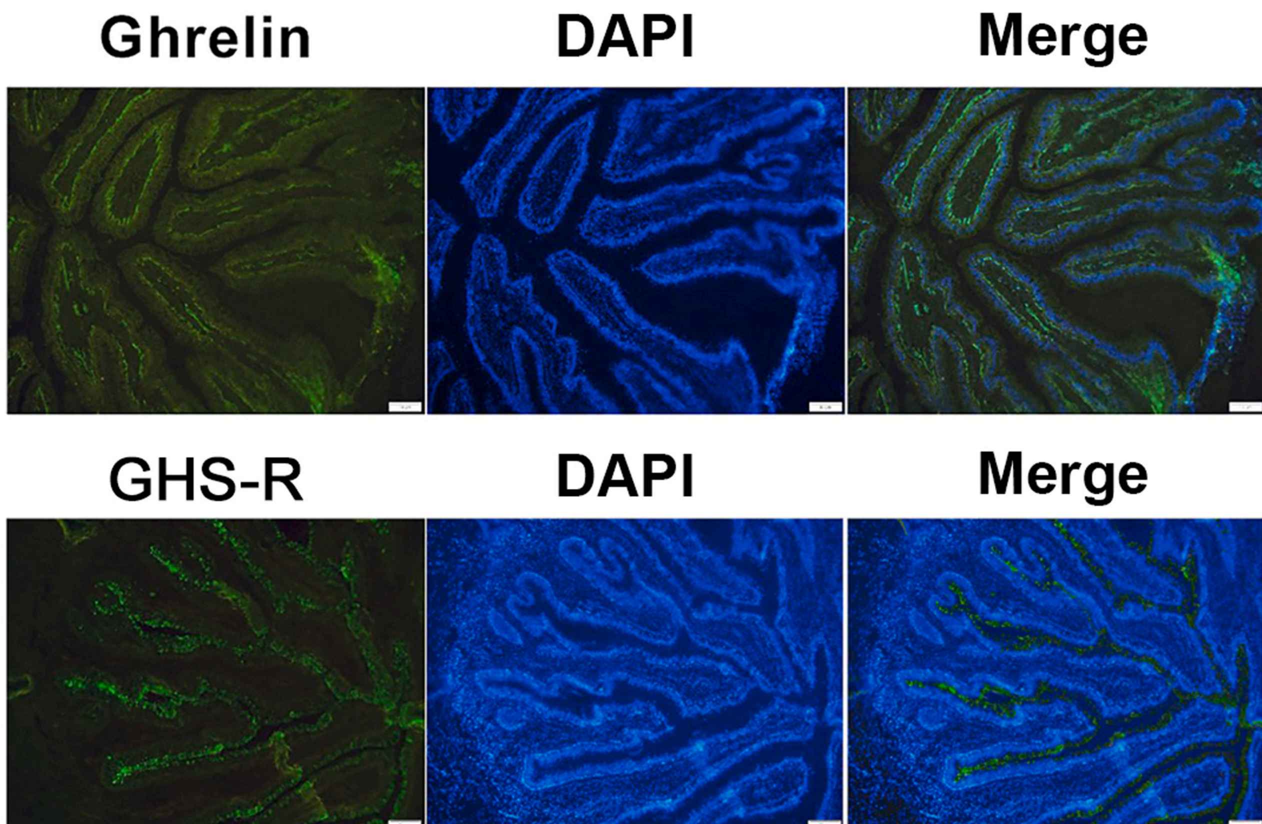

Fig. 1. Immunofluorescence analysis of ghrelin and GHS-R of intestine from grass carp. The bar = 100 μm.

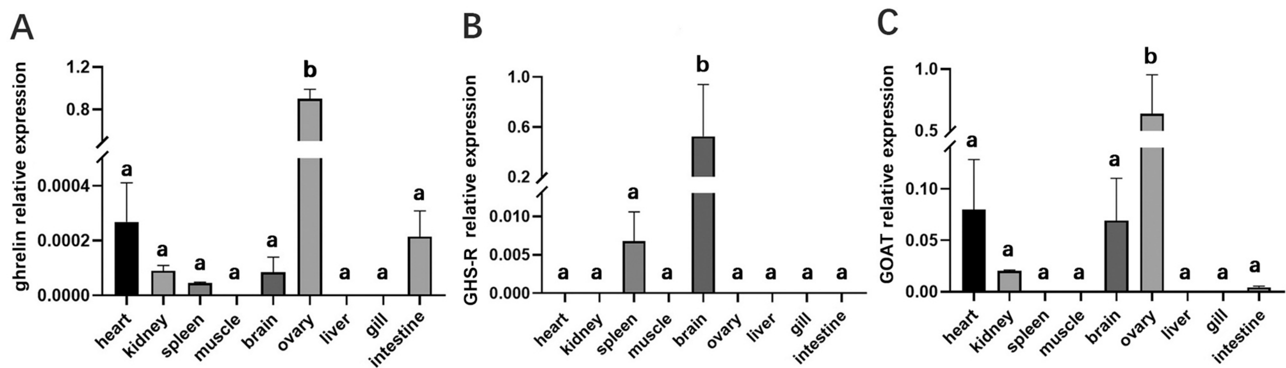

Fig. 2. Tissue distribution of ghrelin (A), GHS-R (B) and GOAT (C) in grass carp. Different letters indicate statistically significant differences ($P \leq 0.05$) ($n = 3$).

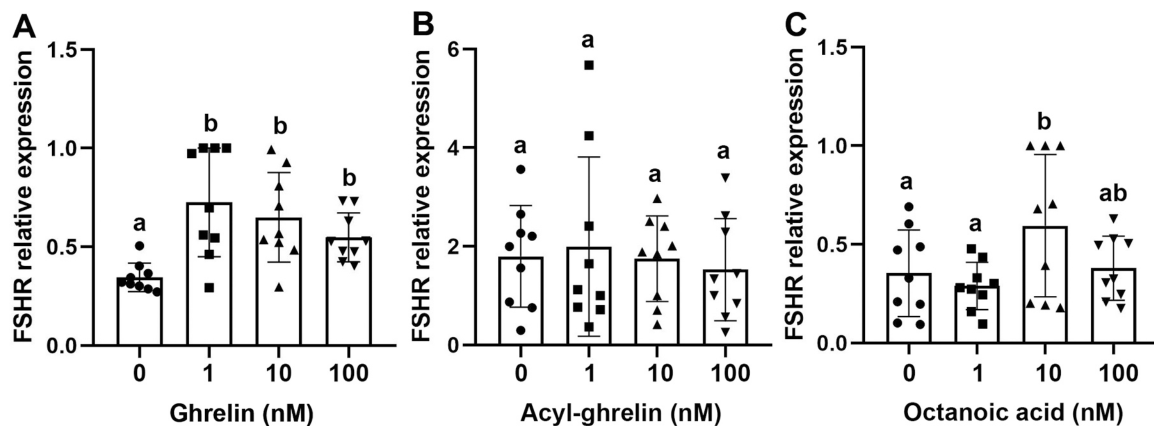

Fig. 3. Effects of ghrelin (A), acyl-ghrelin (B) and octanoic acids (C) on expression of FSHR in ovary of grass carp. Different letters indicate statistically significant differences ($P \leq 0.05$) ($n = 4$).

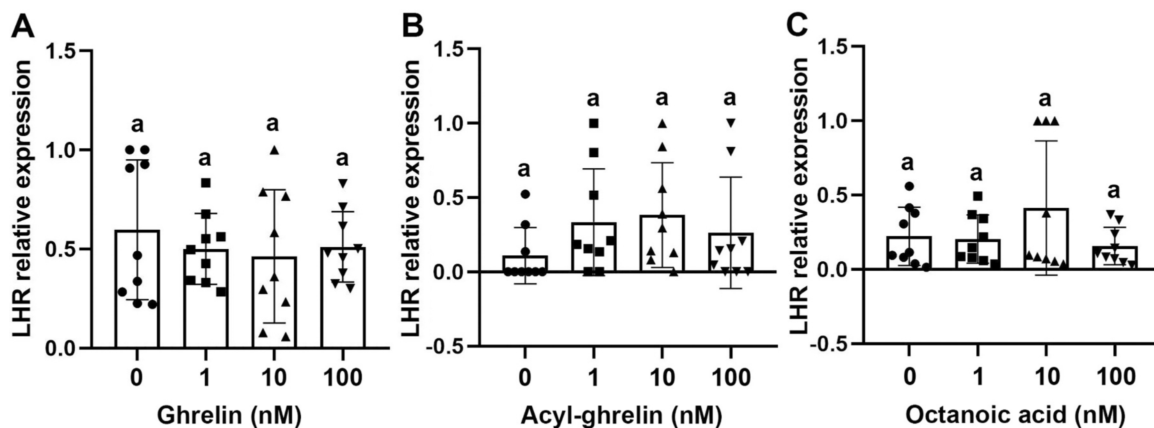

Fig. 4. Effects of ghrelin (A), acyl-ghrelin (B) and octanoic acids (C) on expression of LHR in ovary of grass carp. Different letters indicate statistically significant differences ($P \leq 0.05$) ($n = 4$).

strong positive signals of ghrelin in mucosal layer of intestine. Previously, ghrelin has been reported expressed in mucosal layer as well in other vertebrates, such as rat, mouse and chicken (Kojima and Kangawa, 2002). Grass carp is the species without stomach which is similar to several fishes such as goldfish (*Carassius auratus*). Similar results of ghrelin expression in intestine were found in grass carp and goldfish (Sánchez-Bretaño et al., 2015). Thus, the expression of ghrelin has high conservation among different species. Previous study indicated that brain was the main tissue to express GHS-R (Sánchez-Bretaño et al., 2015). The present results also supported that GHS-R was mainly expressed in brain. GOAT participates in ghrelin acylation which is thought co-expression in the same cells with ghrelin (Romero et al., 2010). The present results also in accordance with this proposal. Similar tissue distribution was found in ghrelin and GOAT in grass carp. Interestingly, high expression of ghrelin and GOAT was found in ovary. In zebrafish (*Danio rerio*), high expression of ghrelin was found in ovary (Amole and Unniappan, 2009; Shepperd et al., 2012). These results suggested that ovary may be another key tissue to secret ghrelin and for ghrelin acylation implying the function of ghrelin in regulation of ovarian development.

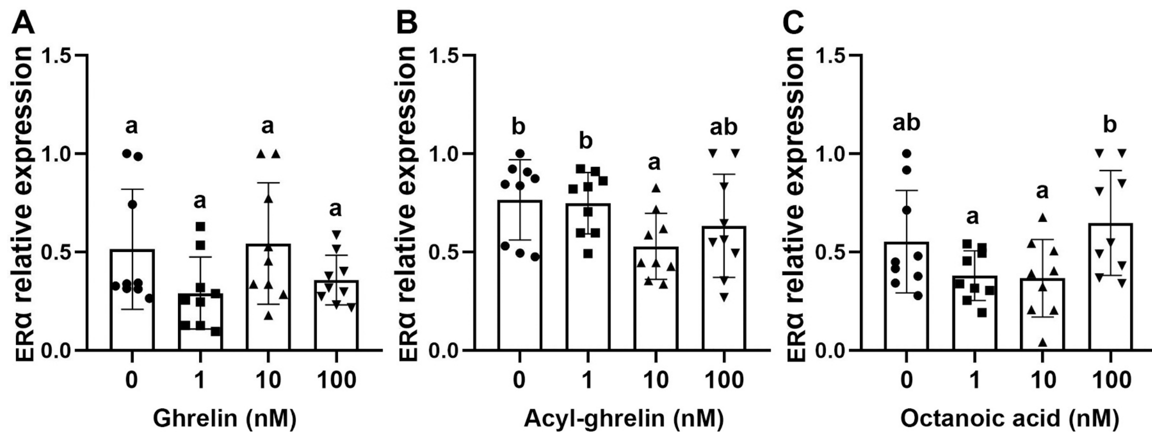

Fig. 5. Effects of ghrelin (A), acyl-ghrelin (B) and octanoic acids (C) on expression of ERα in ovary of grass carp. Different letters indicate statistically significant differences ($P \leq 0.05$) ($n = 4$).

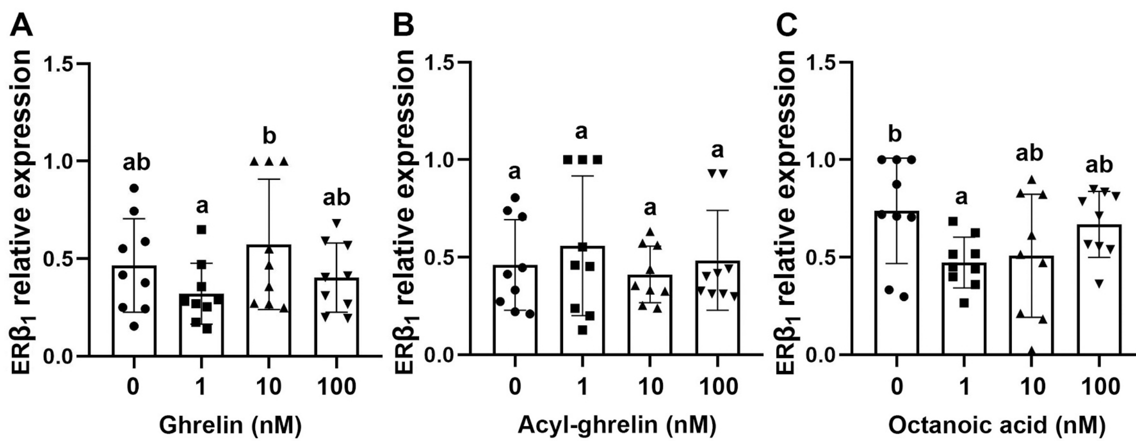

Fig. 6. Effects of ghrelin (A), acyl-ghrelin (B) and octanoic acids (C) on expression of ERβ1 in ovary of grass carp. Different letters indicate statistically significant differences ($P \leq 0.05$) ($n = 4$).

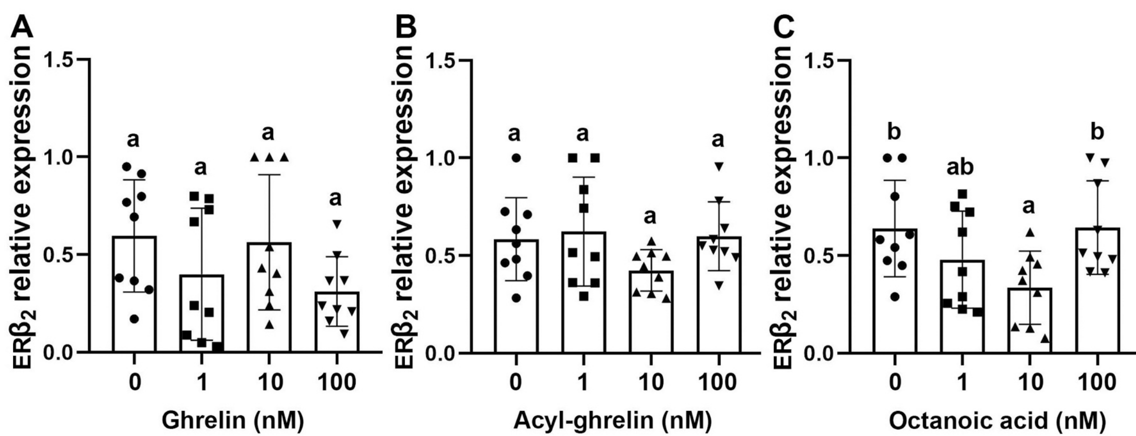

Fig. 7. Effects of ghrelin (A), acyl-ghrelin (B) and octanoic acids (C) on expression of ERβ2 in ovary of grass carp. Different letters indicate statistically significant differences ($P \leq 0.05$) ($n = 4$).

After determined the ovarian expression of ghrelin and GOAT, we further focused on the regulation of ghrelin on the receptors of GTH in ovary including FSHR and LHR. FSHR is specific receptor for FSH which stimulates follicle development (Shinoda et al., 2010). It has been reported that 50 ng/ml ghrelin inhibited FSHR expression in zebrafish oocyte maturation samples (Shepperd, 2012). The results are quite different with our present results in grass carp. We supposed the different species and/or treated dose may lead to the differences.

According to previous study, the expression pattern of FSH in grass carp and zebrafish is different (Zhou et al., 2010). FSH was dominantly expressed in early stage to stimulate follicular development in zebrafish, while in grass carp, FSH was increased until 5 years old fish to promote ovarian development lately (Zhou et al., 2010). Thus, there might be significant differences of ghrelin on the regulation of FSH and FSHR. Further study is still needed to discuss the effects in different fish species. For LHR, no changes could be found by ghrelin in ovary from grass carp. This is similar to the results in zebrafish (Shepperd, 2012). Thus, the present study showed the special FSHR regulation by ghrelin in ovary in grass carp which may due to the different expression during the developmental stages of FSHR expression in the species.

Generally, FSH and LH stimulate releasing of several steroid hormones in ovary such as E2 by binding their receptors (Nilsson and Gustafsson, 2002). It has been regarded the E2-ER binding is the final hormonal action for controlling the ovarian development and ovulation (Li et al., 2019). The E2 interacts with multiple isoforms of ER. In fishes, three major isoforms have been reported including ERα, ERβ1 and ERβ2 (Meng et al., 2010). The present study suggested that 10 nM acyl-ghrelin depressed ERα while 1 nM and 10 nM octanoic acid decreased ERβ1 and ERβ2, respectively. These clues hinted that ghrelin could inhibit expression of ER in ovary of grass carp in acyl-ghrelin form. Commonly, ghrelin needs to be acylated to play its regulatory role by GOAT (Al Massadi et al., 2011). All of these clues suggested that the effects of ghrelin on ER is mediated by acyl-ghrelin. The most known function of E2-ER pathway is to regulate ovarian development especially the final maturation of oocyte and ovulation (Reading et al., 2018). Based on these evidences, we suggested that acyl-ghrelin inhibited E2-ER pathway which may be a potential internal mechanism for control maturation of oocytes. In addition, the inhibitors of GOAT may be useful for controlling the final maturation or inhibit ovulation in grass carp providing a possible method to artificial regulating the ovarian maturation for grass carp.

The interaction of acyl-ghrelin and GHS-R is regarded as essential action for the function of ghrelin. However, recent study suggested that ghrelin may play its regulator role directly without acylation and its receptor. Although the evidences about the function unacylated ghrelin have not been unveiled in grass carp yet, the present result suggested that ghrelin may directly mediated the stimulation of FSHR expression in ovary of grass carp. In contrast, the depression of ghrelin on ER was mediated by acylation via GOAT and interaction with GHS-R. Thus, based on our findings, both unacylated ghrelin and acyl-ghrelin have their function in regulation of hormone receptors in ovary in grass carp (Fig. 8).

In summary, the present study first showed the high expression of ghrelin and its receptor in grass carp. Ghrelin could induce FSHR mRNA expression while acyl-ghrelin or octanoic acid inhibited ER expression in ovarian cells. These clues suggested the ghrelin system plays multiple regulatory function in different ways. Further study is needed to clarify the ghrelin regulation on follicular development and develop method to artificial control of ovarian development of grass carp.

CRediT authorship contribution statement

Jiamin Pi: Investigation, Resources, Visualization, Writing – original draft, Writing – review & editing. **Huan Zhong:** Methodology, Conceptualization Supervision, Project administration, Funding acquisition. **Yi Zhou:** Writing – review & editing, Supervision, Validation. **Tao Dai:** Resources, Investigation, Resources, Data curation, Writing – review & editing. **Weiling Qin:** Resources, Investigation, Resources, Data Curation, Writing – review & editing. **Hui Liu:** Validation, Resources, Data curation, Writing – review & editing. **Yi Zhang:** Validation, Resources, Data curation, Writing – review & editing. **Yuling Zhou:** Validation, Resources, Data curation, Writing – review & editing.

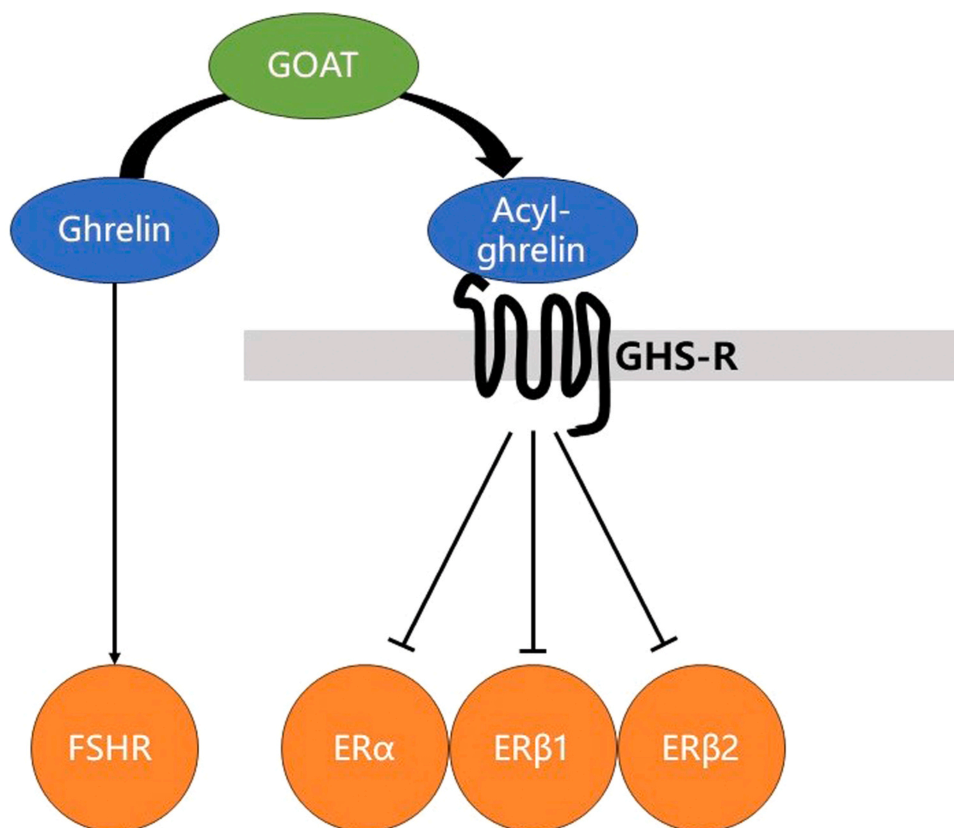

Fig. 8. A schematic diagram of ghrelin regulation on FSHR and ER based on the present results.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgement

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Author's statement

Huan Zhong conceived the project and managed the project; Jiamin Pi, Yi Zhou, Tao Dai and Weiling Qin collected the samples and performed the experiments; Hui Liu, Yi Zhang and Yuling Zhou analyzed the data; Jiamin Pi, Huan Zhong, Yi Zhou and Tao Dai prepared the manuscript; Hui Liu, Yi Zhang and Yuling Zhou revised the manuscript. All authors commented on the manuscript.

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