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# COMPARISON OF IN VITRO AND IN VIVO EFFECTS OF DIFFERENT SPECIES SPECIFIC GnRH AND THEIR ANALOGS

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SUMMARY: Mammalian, salmon and chicken gonadotropin-releasing hormones (mGnRH, sGnRH, cGnRH) and their analogs were synthesized and tested for their ability to stimulate in vitro LH and FSH release from cultured and superfused rat pituitary cells and also their in vivo effect were investigated on the artificial propagation of fishes. The LH and FSH releasing activity of sGnRH, cGnRH and their analogs were lower than the appropriate mammalian ones from cultured rat pituitary cells, but two of the cGnRH analogs showed increased LH and FSH secretory activity from superfused rat pituitary cells compared to the mGnRH. At the same time these two analogs are very potent to stimulate reproductive function of fishes and using these peptides we were able to fulfill the artificial propagation of fishes which could not be artificially propagated before. 

The existence of structural variation in gonadotropin hormone releasing hormone (GnRH) in vertebrates has been known only from the last few years (1,2). The first non-mammalian GnRH was isolated from chicken hypothalamus and its chemical structure was established as /$Gln^8$/-GnRH (3,4). Later a second form of GnRH ($His^5$, $Trp^7$, $Tyr^8$/-GnRH was found in chicken hypothalamus (5). Another type of GnRH was isolated from salmon hypothalamic tissue. Its structure was characterized as /$Trp^7$, $Leu^8$/-GnRH (6).

These findings raised the question to what extent these peptides are interchangeable among the vertebrates being on the different level of evolution. We have synthesized mammalian,

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salmon and chicken GnRH and several analogs of them and examined their in vitro LH and FSH releasing activity in cultured and superfused rat pituitary cells. On the other hand the biological activities of the species specific analogs were tested on the artificial propagation of fishes to check if there are any distinction among the in vivo effects of these analogs. In the case of the in vivo studies our final purpose was to find GnRH analogs to be potent inducers of ovulation in fishes which are normally difficult to propagate in captivity.

## MATERIALS AND METHODS

### Synthesis and purification of peptides

All the peptides were synthesized in our laboratory on a Beckman-990 automatic peptide synthesizer using a standard solid phase procedure (7). Benzhydrylamine resin. HCl (0.49 meq/g) (Beckman) or Merrifield Polymer (0.7 meg/g) (Fluka) and tert-butyloxy-carbonyl (Boc)-protected amino acids were used as starting materials. Reactive side chains of amino acids were protected as follows: Arg, $N^G$-tosyl; Ser and Tyr, O-benzyl; His, $N^{im}$-tosyl. All amino acids were coupled using N,N'-diisopropyl--carbodiimide (Aldrich). The completeness of the coupling reaction was measured by ninhydrin test (8). If the test was positive, the coupling was repeated. In the case of Benzhydrylamine resin the decapeptides were cleaved from the resin with simultaneous deprotection by anhydrous hydrogen fluoride (9). The protected peptide-resin (0.5 mmol) was treated with 30 ml of redistilled HF in the presence of 10% anisole and 100 mg dithiothreitol for 60 min at 0°C. The hydrogen flouride was eliminated in dry nitrogen gas stream. The resin was then suspended in absolute ether and filtered off. The solid residue was washed with 5o % acetic acid, then the solution was evaporated in vacuum at 37°C. In the case of Merrifield resin the nonapeptides were split off from the resin as their ethylamine forms (10). For this purpose the peptide resin was stirred with 15 ml of condensed ethylamine at 0°C for 3 hours. The excess of the ethylamine was eliminated in nitrogen gas stream then the residue was washed with dimethyl-formamide and filtered. The filtrate was evaporated in vacuum then, for the complete deprotection, the peptide was treated with HF. The crude peptides were subjected to gel filtration on a Sephadex G-25 column (2.5x100 cm) in 20% acetic acid. Further purification was carried out using reversed-phase $C_{18}$-bounded silica gel LRP-1 (13-24 μm) (Whatman) preparative HPLC column (2,5x45 cm) eluted with n-PrOH-2o % AcOH linear gradient system (10-60 % n-PrOH) at a flow rate of 2 ml/min and pressure of 60 psi. The separation was monitored at 280 nm and by TLC on Silica Gel plates. The purity of the peptides was tested by TLC in different solvent systems by HPLC and by amino acid analysis.

### Superfused rat pituitary cell system

Anterior pituitaries were obtained from twelve adult (male or female) Wistar strain rats for each experiment according to the method previously described (13). The pituitaries were cut into small pieces, incubated with collagenase than dispersed

mechanically to single cells. The cell-suspension was mixed with Sephadex G-10 beads as support material and transferred into a superfusion chamber. The cells were continuously perfused with oxygenated medium (Medium 199) or with the medium containing the peptides to be tested. The LH and FSH content from each 1 ml fraction of the superfusate was measured by radioimmunoassay (RIA) using rat LH and FSH RIA kits provided by NHPP (NIADDK, NIH)(12). The biological potencies of the analogues were determined based on the pituitary hormone responses (peaks) to the peptide-stimulation over the baseline secretion.

Rat pituitary cell culture

Pituitary cells from female Spraque-Dawley rats were isolated and cultured according to the method previously described (11). On the fourth day of culture the cells were incubated with various concentrations of the GnRH analogs for 2 hours. The peptides were dissolved in 0.1 M acetic acid and further diluted with culture medium. LH and FSH released into the medium were determined by RIA.

Induced artificial propagation of fishes

In any season of the year and at any temperature mature breeders were selected and then transported to the place of propagation. Sexes were segregated and the stage of ripening of eggs from females was determined. Fish were considered to be ripe for ovulation if the nucleus were situated on the periphery of the egg cell. Sperms of males were not examined as in the population of a given habitat the sexual products of males and females are practically in the same stage of ripening. If fish were ripe for ovulation, GnRH analog in a single dose of 10-100 µg/body weight (depending from the fish species) was injected into each fish, including males. If fish were not ripe for ovulation, they were kept at the temperature of natural spawning and the GnRH analog was administered in a daily dose of 1-5 µg/body weight till the nucleus moved from the center to the periphery. The number of treatments depended on the type of fishes and on the actual stage of maturity of sexual products in comparison to the stage "ripe for ovulation". Ovulation takes place 24-32 hours or 240-260 hours (depending on the fish) after the administration of the last dose. Thereafter sexual products were obtained in 40-60 minutes.

## RESULTS

A series of species specific GnRH analogs were synthesized. (Table 1). Superfused rat pituitary cell system was used to characterize the in vitro LH and FSH releasing potencies of mGnRH, cGnRH, sGnRH and superactive analogs of mGnRH as well seven new "species specific"-GnRH analogs. All of the peptides were measured at the dose ($10^{-9}$M) which is a convenient dose for mGnRH. The LH and FSH contents of the superfusate were measured by RIA. Total gonadotropin released above baseline during each experiment is presented in Table 2. Two of the new GnRH analogs did show higher gonadotrop releasing activity than mGnRH, the others had no activity at the dose tested.

Table 1

SPECIES SPECIFIC GnRH ANALOGS AND THEY DERIVATIVES

| | 1. | 2. | 3. | 4. | 5. | 6. | 7. | 8. | 9. | 10. | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Glp | His | Trp | Ser | Tyr | Gly | Leu | Arg | Pro | Gly-$NH_2$ | mammalian |
| | Glp | His | Trp | Ser | Tyr | Gly | Leu | Gln | Pro | Gly-$NH_2$ | chicken |
| | Glp | His | Trp | Ser | Tyr | Gly | Trp | Leu | Pro | Gly-$NH_2$ | salmon |
| 1./ | Glp | His | Trp | Ser | Tyr | DPhe | Leu | Gln | Pro | Gly-$NH_2$ | |
| 2./ | Glp | His | Trp | Ser | Tyr | DPhe | Trp | Leu | Pro | Gly-$NH_2$ | |
| 3./ | Glp | His | Trp | Ser | Tyr | Gly | Trp | Gln | Pro | Gly-$NH_2$ | |
| 4./ | Glp | His | Trp | Ser | Tyr | Gly | Phe | Gln | Pro | Gly-$NH_2$ | |
| 5./ | Glp | His | Trp | Ser | Tyr | Gly | Phe | Leu | Pro | Gly-$NH_2$ | |
| 6./ | Glp | His | Trp | Ser | Tyr | Gly | Trp | Leu | Pro | NH-$C_2H_5$ | |
| 7./ | Glp | His | Trp | Ser | Tyr | DPhe | Leu | Gln | Pro | NH-$C_2H_5$ | |
| 8./ | Glp | His | Trp | Ser | Tyr | Gly | Trp | Gln | Pro | NH-$C_2H_5$ | |

For comparison, we measured the in vitro activities of four species specific GnRH analogs on rat pituitary cell culture too. The dose dependent LH and FSH releasing ability of mGnRH, cGnRH, sGnRH and their DPhe$^6$ analogs are shown in figures 1 and 2. On the fourth day, of culture the cells were incubated

Table 2

Total gonadotropin release above baseline in superfused rat pituitary cells in response to GnRH analogs

| No. | Peptide ($10^{-9}$M ) | ngLH | ngFSH |
|---|---|---|---|
| 1. | (DPhe$^6$)GnRH-EA | 490.0 | 36.0 |
| 2. | (DTrp$^6$)GnRH | 176.0 | 19.5 |
| 3. | GnRH | 69.6 | 7.8 |
| 4. | (Gln$^8$)GnRH | 1.7 | 0 |
| 5. | (DPhe$^6$,Gln$^8$)GnRH | 85.4 | 8.8 |
| 6. | (DPhe$^6$,Gln$^8$)GnRH-EA | 115.0 | 17.0 |
| 7. | (Trp$^7$,Leu$^8$)GnRH | 0 | 0 |
| 8. | (DPhe$^6$,Trp$^7$,Leu$^8$)GnRH | 0 | 0 |
| 9. | (Phe$^7$, Leu$^8$)GnRH | 0 | 0 |
| 10. | (Phe$^7$, Gln$^8$)GnRH | 0 | 0 |
| 11. | (Trp$^7$, Gln$^8$)GnRH | 0 | 0 |
| 12. | (Trp$^7$, Gln$^8$)GnRH-EA | 0 | 0 |

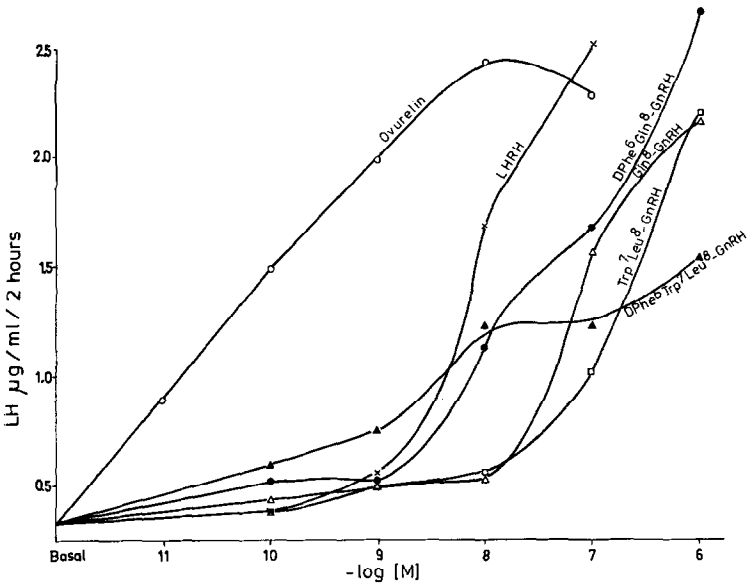


Fig. 1.: Effect of various GnRH analogues on LH release in cultured rat pituitary cells. Pituitary cells were incubated on the 4th. day of culture with various concentrations of the hormones for two hours in Medium 199 containing 2o% fetal calf serum. Aliquots of the medium were assayed for LH content by RIA.

with various concentrations - including the dose used in the superfusion system - of the GnRH analogs for two hours. The LH

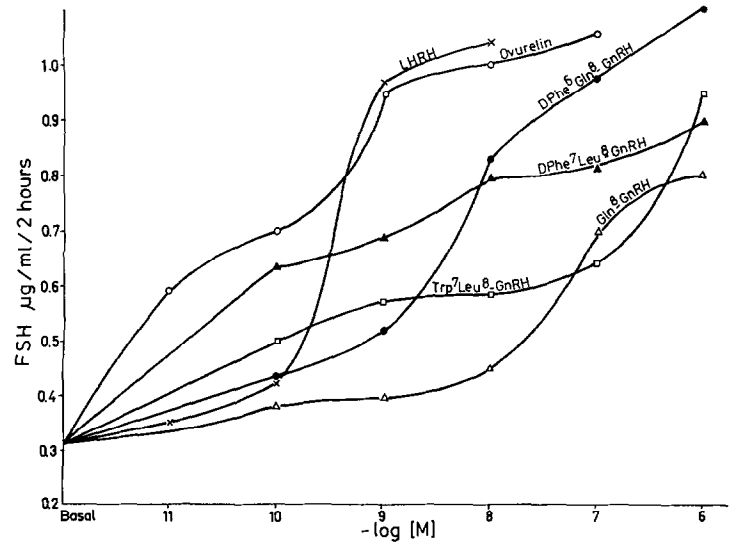


Fig. 2.: Effect of various GnRH analogues FSH release in cultured rat pituitary cells. Pituitary cells were incubated on the 4th. day of culture with various concentrations of the hormones for two hours in Medium 199 containing 2o% fetal calf serum. Aliquots of the medium weew assayed for FSH content by RIA.

Table 3

Effect of various analogues on induced artificial propagation of sterlet

| Hormone | Treatment | Apl. | Treated | Reacted /Ovulated/ | % | Fertility rate of the ovulated eggs+ % |
|---|---|---|---|---|---|---|
| D-Phe$^6$-Gln$^8$-GnRH | 5 x 30 µg/24 hours | ip. | 10 | 10 | 100 | 80-90 |
| D-Phe$^6$-Gln$^8$-GnRH-EA | 5 x 30 µg/24 hours | ip. | 10 | 10 | 100 | 80-90 |
| Trp$^7$-Leu$^8$-GnRH | 5 x 30 µg/24 hours | ip. | 10 | 3 | 30 | 70-80 |
| Trp$^7$-Gln$^8$-GnRH | 5 x 30 µg/24 hours | ip. | 10 | 1 | 10 | 60 |
| Phe$^7$-Gln$^8$-GnRH | 5 x 30 µg/24 hours | ip. | 10 | 3 | 30 | 70-80 |
| Phe$^7$-Leu$^8$-GnRH | 5 x 30 µg/24 hours | ip. | 10 | 0 | 0 | 0 |
| D-Phe$^6$-Trp$^7$-Leu$^8$-GnRH | 5 x 3o µg/24 hours | ip. | 10 | 4 | 40 | 70-80 |
| D-Phe$^6$-GnRH-EA | 5 x 3o µg/24 hours | ip. | 10 | 0 | 0 | 0 |
| GnRH | 5 x 30 µg/24 hours | ip. | 10 | 0 | 0 | 0 |
| Gln$^8$-GnRH | 5 x 30 µg/24 hours | ip. | 10 | 0 | 0 | 0 |

+ Fertility rate during in vitro fertilisation of the ovulated eggs

and FSH released into the medium during the incubation were measured by RIA. In this system the cGnRH, sGnRH and their DPhe$^6$ counterparts - except the extremely high dose - showed lower LH and FSH releasing activity than mGnRH, though the DPhe$^6$ substitution preserved it's ability ro rise the biological effect of the given peptide.

In the case of the in vivo experiments, we measured the effect of sGnRH, cGnRH and their analogs on the artificial propagation of fishes (Table 3). Surprisingly among the tested peptides the agonistic analogs of cGnRH were the most potent materials for the artificial propagation of fishes. In every cases, they had 100 % activity for inducing ovulation or spermiation; however the sGnRH and it's DPhe$^6$ analogs were less potent in stimulating reproductive functions. The untreated fish because they do not reproduce in captivity didn't show any changes. The DPhe$^6$ and the DPhe$^6$-ethylamide analogs of cGnRH were very potent in different dosages and type of treatment. With the aid of these analogs we were able to propagate artificially those fishes which couldn't be artificially propagated before, for example sterlet and the process was succesfully applied during and out of season too.

## DISCUSSION

Since the existence of different species specific GnRH analogs are known a lot of experiment were made to clarify the biological role of these structural differences. From earlier studies it was well known that in mammalian GnRH Arg in position 8 is essential for full biological activity, its substitution with other amino acids resulted in decreased gonadotropin releasing activity (14). So it was understandable, that in the case of rat pituitary cell culture relative potencies of chicken GnRH were 2-3 % that of mammalian GnRH. At the same time LH secretion from chicken anterior pituitary cells were practically identical stimulating the cells by a GnRH agonist, /DLeu$^{6}$/-GnRH, mGnRH and cGnRH (15). The response of superfused goldfish pituitary fragments to mGnRH, sGnRH and /DAla$^{6}$/-GnRH ethylamide were also equivalent (16) while sGnRH was twice as active than cGnRH in stimulating LH release from chicken pituitary cells (17). From these relatively few data, it appears that to some extent these peptides are interchangeable among the vertebrates being on a lower level then the mammals. We have synthesized several analogs of cGnRH and sGnRH in order to develop GnRH analogs which can be used for the artificial propagation of fishes. We tested our analogs both in vitro and in vivo.

In the case of rat pituitary cell culture our results showed correlation with the literature. The substitution of position 7 and/or 8 resulted in a decreased biological activity which is due to the sensitivity of mammalian GnRH receptors to the C-terminal region of the molecule. The substitution for Gly to DPhe in position 6 increased the LH and FSH releasing activity but it wasn't enough to compensate for the effect of the substitutions in positions 7 and/or 8 (Fig. 1,2).

In the more sensitive and more physiological superfused rat pituitary cells the /DPhe$^{6}$, Gln$^{8}$/-GnRH and the /DPhe$^{6}$, Gln$^{8}$/--GnRH-ethylamide behaved like a superactive mammalian GnRH analog.

The most reliable and most important system to check the potency of these analogs was the measurement of their ability to stimulate reproductive functions in fishes. We found that two of the analogs were very active in stimulating reproductive functions of fishes and using these analogs we were able to fulfill the induced artificial propagation of several kinds of fishes which could not be artificially propagated before.

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