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EFFECTS OF SYNTHETIC LH-RH AND ANALOG ON PLASMA GONADOTROPIN LEVELS AND MATURATIONAL RESPONSE TO 17α -HYDROXY- 20β -DIHYDROPROGESTERONE

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ABSTRACT

Breton, B., Jalabert, B., Bieniarz, K., Sokolovska, M. and Epler, P., 1983. Effects of synthetic LH-RH and analog on plasma gonadotropin levels and maturational response to 17α -hydroxy- 20β -dihydroprogesterone. *Aquaculture*, 32: 105–114.

The effects of LH-RH and analog on the stimulation of gonadotropin (GtH) secretion have been studied in order to use them as priming, before inducing maturation with 17α -hydroxy- 20β -dihydroprogesterone (17α - 20β P) in the carp *Cyprinus carpio*. One injection of LH-RH (2 μ g/kg body weight) or LH-RH analog (0.3 μ g/kg) induces a transient rise in the plasma GtH concentration, but subsequent 17α - 20β P treatment does not result in an increase in the number of oocytes with germinal vesicle breakdown or in ovulation. Two and three injections of LH-RH or analog at the same dosages are followed by a stimulation of GtH secretion (to 30 ng/ml) which is maintained for 6–9 h, and lasts longer with LH-RH analog than with LH-RH. The same magnitude in the increase of GtH secretion is obtained with lower doses of LH-RH analog. Injection of 17α - 20β P then increases the number of oocytes undergoing germinal vesicle breakdown and sometimes partial ovulation. Further studies on the mechanism of action of GtH during priming hypophysation treatment are needed in order to develop a sequential treatment using: (1) priming by LH-RH or analog, (2) 17α - 20β P induced maturation treatment.

INTRODUCTION

Many investigations have been conducted in order to replace hypophysation using pituitary extracts by treatments which are less expensive and exhibit a wider zoological specificity. Different kinds of natural or synthetic substances which are or could be produced commercially look promising, although all data presently available are not yet conclusive (see reviews by Fontaine, 1976; Donaldson, 1975; Harvey and Hoar, 1980): prostaglandins, progestagens, clomiphene citrate and gonadotropin releasing hormone (Gn-RH). This last substance seems to be the most promising.

LH-RH has been demonstrated to induce in vivo and in vitro release of

gonadotropin (GtH) from carp pituitaries (Breton et al., 1971a, b; Breton and Weil, 1973; Weil et al., 1978). In contrast to gonadotropins, its zoological specificity is low (Breton et al., 1972; King and Millar, 1980). Moreover, its ability to induce successful spawning in several fish species has already been reported: goldfish (Lam et al., 1975, 1976); Japanese medaka *Oryzias latipes* (Chan, 1977); ayu *Plecoglossus altivelis* (Hirose and Ishida, 1974); plaice and goby (Aida et al., 1978); and grass carp (Anonymous, 1977a, b). In most of these experiments the amounts of Gn-RH were so high that the practical use of this product is questionable for economic reasons.

On the other hand, we have demonstrated that a priming pituitary treatment followed by 17α -hydroxy- 20β -dihydroprogesterone injection could induce spawning in the carp (Jalabert et al., 1977), the rainbow trout (Jalabert et al., 1978a), and the coho salmon (Jalabert et al., 1978b). The aim of this work was to investigate the possibility of replacing pituitary extract priming by a Gn-RH priming in order to develop an entirely synthetic treatment. In addition, the efficiency of a syn LH-RH analog (D6 serine ethylamide 10 Gn-RH) was compared with that of LH-RH. Except for the work of Weil et al. (1980), nothing precise is known about the latency of response of the carp pituitary to successive injections of LH-RH or analog in our experimental conditions, nor about the precise delay between GtH surge and 17α - 20β P treatment necessary for successful ovulation. We have chosen a possible, but somewhat arbitrary, injection time schedule, in order to look at the resulting gonadotropin profiles, instead of checking only for ovulation which could fail as a consequence of an inappropriate timing schedule for injections.

MATERIALS AND METHODS

The experiments were carried out during the spring of 1978 at the Midlinicki experimental fish farm near Krakow. Forty-seven 5–6-year-old female carps were used. They weighed between 3 and 5 kg and originated from a commercial fish farm.

They were divided into nine groups: eight groups of five fish and one of seven, kept in concrete 2 m³ tanks in which water was continuously renewed and thermoregulated to the nearest 1°C. Temperature was increased by 1°C per hour from the natural external temperature to the experimental level. Experiments began 24 h after the start of the acclimatization procedure.

The different experimental groups and treatments are shown in Table I.

All fish from groups 1 to 8 were primed either with syn LH-RH or D6 serine ethylamide 10 LH-RH, and animals from group 9 with partially purified carp gonadotropin. The hormone was prepared using carp acetonetic pituitary powder from Stoller Fisheries (U.S.A.). The procedure for hormone purification was the same as already described for the purification of salmon GtH (Breton et al., 1978). It included buffered extraction—glycoprotein separation after affinity chromatography on concanavaline A sepharose and gel filtration on ultrogel ACA 54. Thus we obtained a glycoprotein fraction of around

TABLE I

Experimental procedure (ppc-GtH = partially purified carp GtH)

Group	Treatment	Time schedule of injection, dosage used ($\mu\text{g/kg}$)				Number of fish	Temperature ($^{\circ}\text{C}$)
		0 h	3 h	6 h	24 h		
1	syn LH-RH	2	—			5	18
2	syn LH-RH	2	2			5	12
3	syn LH-RH	2	2			5	18
4	syn LH-RH	2	2	2		5	18
5	D6 ser des gly 10 ethylamide	0.3				5	18
6	LH-RH	0.3	0.3			5	12
7	LH-RH	0.3	0.3			5	18
8	LH-RH	0.3	0.3			5	18
9	ppc-GtH	150				7	20

30 000 molecular weight. One milligram of partially purified carp GtH was equivalent to 110 μg of pure salmon gonadotropin (Breton et al., 1978) when measured in the in vitro trout oocyte maturation assay (Jalabert et al., 1974). 150 μg partially purified carp GtH, corresponding to 16 μg pure GtH, was injected intraperitoneally directly into the heart.

Twenty-four hours after priming either by gonadotropic or hypophysiotropic hormones all fish received 2 mg 17 α -20 β P/kg body weight. The progestagen was dissolved at a concentration of 20 mg/ml in absolute ethanol and diluted to 1/10 with a 8 $^{\circ}$ / $_{\infty}$ NaCl solution just before use.

Treatments started at 09.00 h. Samples of blood (0.5 ml) were taken from a caudal vessel just before treatment (time 0) and 1.5, 3, 6, 7.5, 9 and 24 h after the first injection. After centrifugation plasma samples were kept frozen until they were used for GtH determination by radioimmunoassay according to the method already described (Breton et al., 1971).

A rough indication of the total amount of GtH secreted during the experimental period was estimated by the determination of a secretion index

$$S = \sum_0^9 \frac{Cti + (Cti + \Delta t)}{2} \times \sum \Delta t$$

Where Cti and $Cti + \Delta t$ are the GtH concentration measured in blood samples taken at successive times ti , $ti + \Delta t$ so that $\sum \Delta t = 9$ h.

All animals were submitted to an ovarian biopsy according to the technique of Bieniarz and Epler (1976) before the beginning of the experiment in order to observe the nucleus position after clearing, according to Bruzka and Bieniarz (1977). A similar procedure was applied 24 h after 17 α -20 β P injection when ovulation was not observed, in order to determine any evolution of oocyte stages.

Fish were considered to be partially ovulated when they produce only some milliliters of eggs, whereas we could expect around 10 to 15% of body weight in the case of total ovulation.

GtH levels were compared using analysis of variance, Duncan test, and t test.

RESULTS

Syn LH-RH effects

The GtH plasma levels following LH-RH treatments are shown on Fig. 1.

At the beginning of the experiment, mean basal levels of immunoreactive gonadotropin were similar, around 5 ng/ml at 09.00 h. First LH-RH injection

Fig. 1. Evolution of the plasma GtH levels ($\bar{X} \pm \text{SD}$) after syn LH-RH treatments (arrows indicate the times of injections). ●—● one injection; ■—■ two injections; △—△ three injections.

induced a significant increase of GtH level in all three LH-RH treated groups. There was a great variability within the responses according to fish which may be because the delay in sampling (1.5 h) did not correspond to the time of maximum increase of Gn-RH injection (Weil, 1981).

Second and third injections induced a new rise in the plasma gonadotropin levels which were maintained at values over 20 ng/ml 9 h after the beginning of the experiment, whereas GtH returned to basal levels 3 h after only one syn LH-RH injection. The time schedule of sampling did not allow the determination of the interval between the second or third injection and GtH decrease to baseline values. It can only be observed that GtH increase lasted longer after repeated LH-RH injections than after only one. But in all three groups GtH levels were similar and not significantly different at the beginning of the experiment and 24 h after.

Table II shows the values of the secretion index after LH-RH treatments. There was no significant difference between fish receiving two or three LH-RH injections, but these two groups differed from that receiving only one LH-RH injection in which the total estimated GtH secretion was lower ($P < 0.05$). In fish treated twice with LH-RH, temperature had no effect, even though

TABLE II

Estimated S values of GtH secretion during $\Sigma \Delta t = 9$ h

Treatment	Number of injections	Temperature ($^{\circ}\text{C}$)	Estimated GtH secretion $\bar{X} \pm \text{SE}$ (ng/ml)
syn LH-RH	1	18	108.68 ± 31.5
	2	18	201.82 ± 28.3
	2	12	145.68 ± 26.4
	3	18	165.18 ± 17.4
LH-RH analog	1	18	125.24 ± 21.4
	2	18	187.01 ± 23.5
	2	12	128.81 ± 18.32
	3	18	204.81 ± 30.7

Fig. 2. Evolution of the plasma GtH levels ($\bar{X} \pm \text{SD}$) after two injections of (A) syn LH-RH $\bullet$ — $\bullet$ 12°C ; $\blacksquare$ — $\blacksquare$ 18°C ; (B) LH-RH analog $\bullet$ — $\bullet$ 12°C ; $\blacksquare$ — $\blacksquare$ 18°C .

mean profiles of GtH secretion seemed to be different (Fig. 2A) between 3 and 6 h after treatment.

In all groups 17α -hydroxy- 20β -dihydroprogesterone treatment administered 24 h after the first LH-RH injection induced a significant increase in the

number of oocytes with germinal vesicle breakdown, but the response was significantly higher after two or three LH-RH injections, and did not differ with the temperature in fish receiving LH-RH twice.

LH-RH analog effects

Results are shown on Fig. 3. One injection of 0.3 $\mu\text{g/kg}$ of D6 serine ethylamide 10 LH-RH induced an increase of the plasma GtH levels in the same range of magnitude as one (2 $\mu\text{g/kg}$) injection of LH-RH. A second injection continued to stimulate GtH secretion at similar levels after both analog and syn LH-RH whereas the greatest enhancement of gonadotropin secretion was generally observed after three injections of the analog. In all cases GtH levels seem to remain high longer with the analog than with syn LH-RH. No significant difference between the effects of des gly 10-LH-RH at 12 and 18°C could be observed.

S values are significantly higher in fish receiving two or three syn LH-RH analog injections than in fish receiving only one. The temperature does not affect S after two syn LH-RH analog injections.

All treatments significantly increased ($P < 0.01$) the percentage of oocytes undergoing germinal vesicle breakdown after 17 α -20 β P treatment. This effect was the most pronounced after three analog primings which also induced partial ovulations (two out of five).

Priming with carp gonadotropin resulted in an increase of the plasma GtH levels from basal level of 8.78 ± 3.84 ng to 214.5 ± 88.7 ng/ml 3 h after intraperitoneal injections. Six and 9 h after the beginning of the treatment, immunological assayable GtH was 101.8 ± 56.5 and 112.7 ± 46.22 ng/ml,

Fig. 3. Evolution of the plasma GtH levels after LH-RH analog treatments (arrows indicate the time of injections). ●—● one injection; ■—■ two injections; △—△ three injections.

respectively and did not significantly differ. At least 24 h after, GtH levels returned to normal values, 23.5 ± 15.01 ng/ml, similar to the initial GtH concentration measured before injection. 17α -20 β P treatment following this priming resulted in a significant increase ($P < 0.05$) in the number of oocytes with germinal vesicle breakdown. This increase was significantly less than those obtained after priming with two LH-RH injections and syn LH-RH analog.

DISCUSSION

The activity of synthetic LH-RH on the stimulation of gonadotropin secretion had already been well demonstrated in salmonid and cyprinid fishes (see review by Peter 1978). More recently Peter (1980) also demonstrated the activity of a substituted LH-RH analog (des gly 10 ala 6 ethylamide) in the stimulation of gonadotropin secretion in the goldfish. Conversely, in mammals (Fujino et al., 1974) the potency of the LH-RH analog was not greater than that of LH-RH, maybe because of the time schedule of administration and blood sampling together with the intraperitoneal route of injection. However, syn LH-RH analog increased GtH levels for longer than LH-RH. Our results indicated a similar situation in carp, but, in addition, the same magnitudes of GtH increase were obtained using 6 to 7 times less D6 ser ethylamide 10 analog than LH-RH. This suggests that, although no fish received the same dosage of both LH-RH and analog, there is a superactivity of the latter in carp. Similar results were recently obtained in the coho salmon (Donaldson et al., 1981). However, in trout, other 6 and 10 LH-RH substitutes have been recently demonstrated to be as potent as native LH-RH in vivo and in vitro (Crim et al., 1981). Although trout seemed to be less receptive to LH-RH than carp (Crim and Cluett, 1974; Weil et al., 1978), reasons for such a difference between these two species are not elucidated in regard to the phylogenetic conservation of LH-RH biological activity among vertebrates (Breton et al., 1975; King and Millar, 1980). These last authors postulated the isology of the amino acid sequence responsible for the biological activity and differences in the immunological determinants of LH-RH during evolution. Crim's results (1981) seemed to indicate that substitution in position 6 of the glycine residue either by a leucine, tryptophane or alanine did not affect Gn-RH activity in trout. This is the contrary in cyprinid fishes in which the same substitution by alanine or serine resulted in a more potent Gn-RH derivative. Two hypotheses seemed possible. First, differences in the affinity of Gn-RH and its analogs for Gn-RH receptors have been demonstrated in mammals (Catt et al., 1980); this situation might also exist between Gn-RH receptors in cyprinids and salmonids. Second, comparisons of the results obtained in trout and carp could raise new questions concerning the earlier hypothesis of the biological homology between fish and mammalian Gn-RH (Breton et al., 1975; King and Millar, 1980) and among fishes (Idler and Crim, 1981).

In our experiments GtH levels decreased by 7.5–9 h after the first injection, but always returned to basal levels after 24 h. On the contrary, in goldfish,

after two LH-RH or analog injections at 12 h intervals, plasma GtH concentrations remained high after 24 h (Peter, 1980). One of the most important differences between the two experiments is the route of injection. LH-RH may be resorbed less rapidly when injected intraperitoneally than intravenously, thus increasing the duration of its action.

These results also suggested that the maturational action of 17α -20 β P should not need priming by high GtH levels; between 30 and 40 ng/ml would suffice for longer than 9 h when classical hypophysation techniques resulted in immunological GtH levels 50–100 fold higher (Bieniarz et al., 1980).

However, we did not observe complete ovulations in our experiment. There may be several reasons for this:

- (1) Classical hypophysation when performed by means of two successive injections requires a precise schedule between the first priming pituitary injection and the second, depending upon temperature, in order for ovulation to occur in good injections.
- (2) In our experiment, the latency between LH-RH injections and plasma profile of GtH was unknown, depending upon the number of injections, the time between injections and the temperature.
- (3) Plasma profiles of GtH after GtH and LH-RH priming were completely different; the plasma GtH levels after LH-RH were lower than those generally observed during classical hypophysation using pituitary extracts (Bieniarz et al., 1980). For the improvement of such techniques some basic knowledge is lacking, especially the *in vivo* profiles of gonadotropin and steroid secretion during natural spawning compared to that obtained after artificial induction by hormonal treatments. Another important gap is the fundamental study of the differential priming effects of low and high GtH dosage for further action of hormonal treatments. There is also a lack of definition of criteria of maturity, which could be responsible for most of the unsuccessful treatments reported until now and maybe for the results obtained in these experiments.

From the economic point of view, all the data available so far seemed to show that Gn-RH treatments cannot yet entirely replace the classical hypophysation technique because either the amounts required appear very high, such as in the ayu (Hirose and Hishida, 1974) and the plaice (Aida et al., 1977) (around 5 mg/kg), or successful treatments need repeated injections and handling, such as in cyprinid fishes (Lam et al., 1975, 1976; Shanghai Cooperative group 1977a, b). The administration of LH-RH in a form allowing delayed release could probably partially solve this last problem. LH-RH analogs combined with partially purified salmon gonadotropin seem to be effective (Donaldson et al., 1981), but even at the Gn-RH A dosage used (11 μ g/kg body weight), this priming remains too expensive compared to the price of crude pituitary preparations. On the contrary, we think that improvement of a Gn-RH induced ovulation method requires preliminary characterisation of piscine Gn-RH and specific analogs whose actions seem to differ from those of mammalian LH-RH according to the recipient species.

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