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Induced spawning of kutum, *Rutilus frisii kutum* (Kamenskii, 1901) using (D-Ala^6, Pro^9-NEt) GnRHa combined with domperidone

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Abstract

Kutum, *Rutilus frisii kutum* (Kamenskii, 1901), Cyprinidae is an endemic fish of the Caspian Sea. The Iranian Fisheries Organization (Shilat) produces up to 200 million fry (1–2 g b.w.) to restock the Caspian Sea population annually. These fish are produced by artificial breeding using carp pituitary extract (CPE). The objective of this study was to assay the effectiveness of a gonadotropin releasing hormone analogue (D-Ala^6, Pro^9-NEt GnRH) alone or in combination with the dopamine antagonist domperidone (DOM) on spawning success, latency period, ovulation index (OI), weight of stripped egg mass/weight of stripped egg mass+remnant ovaries, and fertilization success in kutum. Ninety fish were divided into nine groups and injected intraperitoneally as follows: 2 mg kg^{-1} b.w. of CPE as positive control, 20 μg GnRHa kg^{-1} b.w. in single injection, 5 μg+2.5 mg, 10 μg+5 mg and 20 μg+10 mg kg^{-1} b.w. of GnRHa+DOM in single or double injection (10–90%) 24 h apart. Propylene glycol injected fish were used as negative controls. The results showed that the highest doses of GnRHa and DOM in single injection lead to higher spawning success and latency periods in comparison with positive control ($P<0.05$), while no significant differences in the OI and fertilization success were found ($P>0.05$). Only 2/10 fish were ovulated in the group which received GnRHa 20 μg kg^{-1} b.w. alone suggesting dopaminergic tone on gonadotropin (GtH) secretion in this fish at the preovulation stage. Therefore, it can be concluded that like many other cyprinids, dopamine inhibitory tone is active in kutum and it is necessary to combine GnRHa with a dopamine antagonist for spawning induction.

Keywords: Kutum; *Rutilus frisii kutum* (Kamenskii, 1901); Spawning; GnRHa; Domperidone; Carp pituitary extract

1. Introduction

Kutum (*Rutilus frisii kutum* Kamenskii, 1901) live in the Caspian Sea near the coast, from the Terek River in the north to the southern part of the Caspian Sea. This species is a migratory anadromous fish spawning in rivers in March–April. It has a group synchronous, single spawning behaviour (Sharyati, 1993), spawning on aquatic weeds, gravelled and sandy substrates in rivers and lagoons (Abdoli, 1999). This is a very valuable commercial fish in the southern part of the Caspian Sea and has a great demand, due to its good taste and culinary customs of the local people, and is consumed all year round. The average annual catch of

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kutum in Iran was about 9600 tons in 1991–2001 (FAO, 2003).

To restock this valuable species in the Caspian Sea, the Iranian Fisheries Organization (Shilat) produces and releases up to 200 million fry (average weight 1 g) in to the Caspian Sea annually (www.shilat.com). Fry are produced by artificial breeding using the hypophysation technique to induce ovulation. Carp pituitary extract (CPE), the only agent used commonly to induce spawning in kutum, is expensive, not always readily available and with unpredictable potency (Drori et al., 1994). An alternative method for induced ovulation of many fishes is the use of different forms of gonadotropin releasing hormone agonists (GnRHa), which stimulate secretion of endogenous gonadotropin (GtH) (Zohar, 1989; Zohar and Mylonas, 2001). The addition of a dopamine receptor antagonist (DA) to potentiate the response to GnRHa depends on the presence of a dopaminergic inhibitory tone in the target species (Peter et al., 1988; Zohar, 1989).

Induction of spawning in fish using GnRHa together with DA, such as metoclopramide, domperidone (DOM) and pimozide, is known as the Linpe method (Peter et al., 1988). The success of using GnRHa alone or in combination with DA has been described in several species such as common carp (*Cyprinus carpio*) (Drori et al., 1994; Yaron, 1995; Kulikovsky et al., 1996; Arabaci et al., 2004), catfish (*Heteropneustes fossilis*) (Alok et al., 1993), Indian major carps such as rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*) (Halder et al., 1991), nase (*Chondrostoma nasus*) (Szabo et al., 2002), pearl mullet (*Chalcalburnus tarichi*) (Arabaci and Sari, 2004), rainbow trout (*Oncorhynchus mykiss*) (Billard et al., 1984; Breton et al., 1990), lake trout (*Salvelinus namaycush*) (Erdahl and McClain, 1987) and sockeye salmon (*Oncorhynchus nerka*) (Slater et al., 1995). The form of GnRHa, the type of DA, the species of fish and environmental factors may affect the ovulatory response (Zohar and Mylonas, 2001). For these reasons it is necessary to examine the response in each species under local conditions.

The objective of the current study was to establish a protocol for spawning induction in kutum, employing GnRHa alone or in combination with DA, which will be based on spawning success, ovulation index (OI), weight of stripped egg mass/weight of stripped egg mass+remnant ovaries (Szabo et al., 2002), latency period and fertilization success.

2. Materials and methods

2.1. Fish stocks and maintenance

The experiments were conducted at Shahid Ansari Cyprinid Fish Complex, Rasht, Guilan, Iran. Kutum were captured from the Sefid Rood River inlets to the Caspian Sea during the spawning migration in April–May 2004 (water temperature 8–12 °C).

Ninety female fish weighing 400–1400 g body weight (b.w.) were selected for ripeness, based on the softness of their abdomens. Prior to injections, fish were individually weighted and marked by placing visible tags on the dorsal fin and randomly were divided into treatment groups.

2.2. Hormones and drugs

The GnRH agonist D-Ala^6, des-Gly^{10} mGnRHa Ethylamide and DOM were supplied as a kit by National Research Institute of Genetic Engineering and Biotechnology (NRIGEB), Tehran, Iran. The GnRHa was

Table 1
The effect of different hormone treatments on spawning success (%), latency period (h), ovulation index OI (%) and fertilization success (%) of kutum, *Rutilus frisii kutum* (Kamenskii, 1901)

Treatment ID	Treatment	Dosage		Spawning success (%)	Latency period (h)	OI (%)	Fertilization success (%)
		1st*	2nd*				
Negative control	Propylene glycol	–	–	10^{a}**	72^{b}	78^{b}	73^{a}
Positive control	CPE	2 mg	–	60^{b}	56±5^{a}	86±3^{b}	65±3^{a}
GnRHa only	GnRHa (G)	20	–	20^{a}	72±0^{b}	66±5^{a}	69±6^{a}
GD 1 (5+2.5)	GnRHa (G)+DOM (D)	5+2.5	–	20^{a}	48±0^{a}	62±4^{a}	70±6^{a}
GD 2 (5+2.5)	GnRHa (G)+DOM (D)	0.5+0.25	4.5+2.25	60^{b}	64±9^{b}	83±3^{b}	73±4^{a}
GD 1 (10+5)	GnRHa (G)+DOM (D)	10+5	–	60^{b}	72±0^{b}	79±3^{b}	77±6^{a}
GD 2 (10+5)	GnRHa (G)+DOM (D)	1+0.5	9+4.5	90^{c}	62±8^{b}	80±3^{b}	68±4^{a}
GD 1 (20+10)	GnRHa (G)+DOM (D)	20+10	–	100^{c}	67±8^{b}	83±4^{b}	67±6^{a}
GD 2 (20+10)	GnRHa (G)+DOM (D)	2+1	18+9	70^{bc}	62±10^{b}	80±4^{b}	64±4^{a}

*The first number indicates the dose of GnRHa (G) in μg kg^{-1} b.w. and the second of DOM (D) in mg kg^{-1} b.w.
**Mean (±S.E.M.) values with a different letter are significantly different ($P<0.05$).

diluted in 40% propylene glycol (P.G.) to achieve a concentration of 50 μg ml^{-1} for the group receiving GnRHa alone; the injected volume was 0.4 ml kg^{-1}.

For other groups which were injected with GnRHa+DOM, at first, DOM was dissolved (100 mg ml^{-1}) in dimethyl sulfoxide (Omeljaniuk et al., 1987) then diluted (1:4) with P.G. which contained 67 μg ml^{-1} GnRHa to achieve a concentration of 50 μg GnRHa plus 25 mg DOM at a final volume 1 ml. The injected volumes were 0.1, 0.2 and 0.4 ml kg^{-1} for fish receiving 5 μg+2.5 mg kg^{-1}, 10 μg+5 mg kg^{-1} and 20 μg+10 mg kg^{-1} of GnRHa+DOM respectively. 2 mg kg^{-1} b.w. of CPE was used in 0.7% saline injected intraperitoneally (i.p.) in a single dose. The injected volume of CPE was 0.5 ml kg^{-1} b.w., according to local hatchery experiences.

2.3. Experiments

Groups of 10 fish were injected i.p. with different preparation as follows: P.G. as a negative control (0.2 ml kg^{-1} b.w), CPE as a positive control (2 mg kg^{-1} b.w.), GnRHa alone 20 μg kg^{-1} b.w. in a single injection, GnRHa combined with DOM in a single or double injection as follows: 5 μg+2.5 mg kg^{-1}, 10 μg+5 mg kg^{-1} and 20 μg+10 mg, respectively. Double injections were done in 10–90% ratio, 24 h apart (Table 1).

After injection, the fish were placed in an indoor concrete tank with running water, temperature 11–12 °C. The fish were checked for ovulation 48 h after first injection every 8–10 h interval up to 24 h. Although, they can spawn spontaneously in the tanks after hormonal induction, but because of a large number of broodfish in hatchery and stickiness of eggs (Razavi Sayyad, 1984), it is better to strip them manually after hormonal induction to enhance gamete quality and quantity. So when ovulation was observed, the eggs were stripped manually and fertilized with milt from at least two males and 20–30 g of fertilized eggs from each female was incubated in jar incubators up to hatching.

Assessment of ovulation was carried out by determining the ovulation success (no of ovulated females/no of injected) and by ovulation index (OI), weight of stripped egg mass/weight of stripped egg mass+remnant ovaries (Szabo et al., 2002). The OI can be a rapid and suitable index for ovulation estimation just for fish like kutum which scarified after spawning. The fish must be scarified after ovulation for calculating the remnant ovaries.

Fertilization success was determined under a dissecting microscope 3 days after fertilization, when eggs were at the stage of gastrulation (Razavi Sayyad, 1984). The latency period was defined as the time between the first injection and fish ovulation (Drori et al., 1994).

2.4. Statistical analysis

Spawning success was analysed by the Chi-square test. Differences in latency period, OI and fertilization success were analysed by one way analysis of variance (ANOVA) followed by Duncan's New Multiple Range test at minimum significant of $P<0.05$. Results are presented as means±standard error of the mean (S.E.M.).

3. Results

Only one out of 10 fish ovulated in the negative control group 72 h after injection (Table 1). In the positive control group, six out of 10 fish ovulated (60%). The lowest spawning success (20%) in the hormone-treated groups was observed in GnRHa alone and GD 1 (5+2.5). Other combinations of higher dose were more effective for spawning induction and the highest spawning success (100%) was observed in GD 1 (20+10) with similar result achieved by the GD 2 (10+5) treatment.

The latency periods were in the range of 48–72 h after the first injection. The negative control, GnRHa-only and GD 1 (10+5) groups showed the longest (72±0 h) latency period, while the shortest period (48±0 h) was observed in the GD 1 (5+2.5). The mean latency period was 56±5 h in the positive control which was lower than all other groups ($P<0.05$) except the GD 1 (5+2.5).

The OI was in the range 62–86% and showed significantly differences among groups (Table 1). The lowest OI were in the GD 1 (5+2.5) and GnRHa-only groups, while the other groups showed higher OI value ($P<0.05$).

Fertilization success in treated fish was in the range of 64–77% (Table 1) and was within the normal range of hatchery practice for kutum. There was no significant difference in fertilization success among groups ($P>0.05$).

4. Discussion

One fish ovulated without any hormonal treatment, showing that kutum caught from the estuary of the Sefid Rood River during the spawning migration were relatively ready to spawn. However, like other anadromous fish in the Caspian Sea, they need to migrate a

relatively high distance in rivers to be completely ready for spawning (Sharyati, 1993; Vossoughi and Mostajeer, 1994; Abdoli, 1999).

In Iran, CPE is a traditional agent for spawning induction in kutum at the dose of 2 mg kg^{-1} b.w., based on local hatchery experiences although, it is well documented that CPE must be injected at higher doses (3–6 mg kg^{-1} b.w.) in double injections to stimulate spawning in female carps such as common carp (Arabaci et al., 2004), grass carp (*Ctenopharyngodon idella*) (Billard, 1990) and nase (Szabo et al., 2002). Maybe because of a relatively high number of broodfish in the hatchery (up to 2000 brood fish in each spawning season) and the high cost of CPE, it is used at a dose of only 2 mg kg^{-1} b.w., in a single injection.

The Linpe method is an alternative technique to stimulate spawning in fish based on using different kinds of GnRHa and DA (Lin et al., 1986). The GnRHa, D-Ala^6, des-Gly^{10} mGnRHa Ethylamide and DOM are the most popular compounds for induction of ovulation and spermiation in different fish species (Donaldson and Hunter, 1983; Donaldson, 1996, 2003). Although the effectiveness of such treatment is variable depending on species and local conditions (Zohar and Mylonas, 2001), the typical effective dose for commercial cyprinid species such as common carp, grass carp and silver carp (*Hypophtalmichthys molitrix*) is 10 μg kg^{-1} b.w. of GnRHa and 5 mg kg^{-1} of DOM in a single injection (Billard, 1990). Therefore this was used as the median dose in the present study.

The spawning success was different among groups (Table 1), with 100% spawning success observed in the GD 1 (20+10), a value significantly higher than the positive control. It is probably due to low dose (2 mg kg^{-1} b.w.) of CPE applied as a positive control in this study.

In lower doses, double injections showed better results than single injection; it maybe related to rapid degradation and short half-life activity of GnRHa (Zohar et al., 1989), because of cytosolic enzyme activity in pituitary, kidney and liver of the fish (Zohar and Mylonas, 2001). Double injections are preferred for some other fish species such as walleye (*Stizostedion vitreum*) (Pankhurst et al., 1986), brown trout (*Salmo trutta*) (Mylonas et al., 1992) and the yellow perch (*Perca flavescens*) (Dabrowski et al., 1994).

The latency periods were greater than reported for catfish (Alok et al., 1993), common carp (Yaron, 1995; Dorafshan et al., 2003; Arabaci et al., 2004) and other major cultivated cyprinids (Billard, 1990), probably because of the lower temperature in the spawning season of kutum (7–15 °C) compared to other well known cyprinids. However, the latency periods in GnRHa-alone or GnRHa+DOM-treated fish were generally longer than those in the CPE-treated group. Similar results were obtained in common carp (Drori et al., 1994; Kulikovsky et al., 1996; Dorafshan et al., 2003) and other major carps (Billard, 1990). A probable explanation can be that GnRHa and DOM act at the level of the pituitary, while CPE acts directly on gonads (Donaldson and Hunter, 1983), thus eliciting a quicker response. Another probable reason could be the use of P.G. as solvent for GnRHa, which can cause slower release of the hormone in comparison with the saline solvent of CPE. Sato et al. (1995) described that salmon pituitary extract (SPE) administration in water-in oil-water type emulsion by cotton seed oil caused the gradual increase in serum GtH, peaking at 24 h, contrary to saline solution of SPE which caused the rapid increase in GtH, peaking at just 3 h after injection.

Assessment of effectiveness of hormonal treatments can be done by examining spawning success, fecundity and fertilization success after hormonal treatments. Fecundity estimation is a time and labour consuming practice especially in large scale hatchery conditions. An alternative is the OI which provides a rough estimation of ovulated eggs. It can be calculated easily and rapidly under hatchery conditions, but its application is limited to fish species which die or are sacrificed after spawning (Szabo et al., 2002). The lowest OI was calculated in fish given GnRHa only and the GD 1 (5+2.5) groups. The lowest OI and spawning success in the GnRHa-only group suggest the existence of dopamine inhabitation on GtH release from the pituitary of kutum. Dopamine inhibits basal as well as GnRH-stimulated GtH release in many fish species especially cyprinids (Peter et al., 1991; Mylonas and Zohar, 2001). As a result, it is proposed to combine GnRHa with a dopamine antagonist such as DOM, pimozide or metoclopramide for successful spawning induction in cyprinid fish (Peter et al., 1993; Zohar and Mylonas, 2001).

Fertilization success did not show any significant differences among groups, suggesting that inducing ovulation with CPE, GnRHa alone or combined with DOM did not have any adverse effect on egg viability. Similar results were obtained in common carp (Drori et al., 1994; Kulikovsky et al., 1996; Dorafshan et al., 2003) and pearl mullet (Arabaci and Sari, 2004).

In conclusion, this study demonstrated that a combination of GnRHa and DOM is an effective and reliable method for induction of ovulation in kutum and can be very useful for hatchery and broodfish management in the kutum spawning and restocking programs. The

advantages over the use of CPE include its greater availability and lower cost (GnRHa + DOM is 2–3 times less expensive than a CPE treatment).

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