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Calcitonin Functions Both as a Hypocalcemic Hormone and Stimulator of Steroid Production and Oocyte Maturation in Ovarian Follicles of Common Carp, *Cyprinus carpio*

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1. Introduction

Calcitonin (CT) is a calcium regulating hormone produced mainly by the parafollicular C cells of the thyroid gland in mammals. In lower vertebrates, these cells are concentrated primarily in a specialized gland, the ultimobranchial gland. The common structure of CT is 32-amino acid residues with a seven-residue cyclic loop formed by disulfide bond between cysteines at position 1 and 7 and prolinamide at carboxyl terminal.

Calcitonin in mammals and birds is acknowledged to be the principal hypocalcemic agent, but the situation in fish is less clear. Several laboratories although showed a hypocalcemic action of CT in fish, conflicting results are often reported. Moreover, fish have other hypocalcemic factor like Staniocalcin, a product of Corpuscles of Stannius, secretion of which is positively regulated by extracellular calcium levels. Control of calcium homeostasis in fish may likely to be different from that in terrestrial vertebrates because of the aquatic environment.

In addition to the involvement of CT in calcium homeostasis in mammals, an endocrine role of endogenous CT at brain, pituitary and gonad has also been suggested. Similarly, a role for CT in calcium ion regulation in fish may be subordinate to other functions which include acting as neurotransmitter or inducer of ovarian steroid hormone synthesis. A major question thus arises- what is the exact role of CT in fresh water teleost? Does it really has any role in calcium homeostasis or involved in many other functions including reproduction? The evolutionary history and functions of CT in aquatic vertebrate therefore require further investigation and as part of ongoing studies in to endocrine/paracrine factors involved in calcium homeostasis and the role of CT, if any, on reproduction in fish, we tried to answer these questions.

2. Work done so far

2.1 Calcium ion regulation

2.1.1 Mammals

The classic concept of CT function in mammals has focused on its effects on calcium homeostasis (Copp et al., 1967). In mammals, CT lowers serum calcium levels by its action

on bone. Cellular activity of the skeleton is largely devoted to an orderly sequence of bone resorption and formation, called remodeling. CT receptors are found in high concentrations in mammalian osteoclast and the hormone exerts its control on serum calcium by inhibiting bone calcium resorption through its direct action on osteoclasts (Nicholson et al., 1986; Raisz et al., 1998; see review Zaidi et al., 2002).

As mammalian bone osteoclasts are characterized by high acid phosphatase activity, particularly, that of tartrate-resistant acid phosphatase (TRACP) and as collagen degradation in bone releases hydroxyproline (HYP) in circulation; these two parameters are used as reliable markers for bone resorption (Vaes, 1988; Raisz et al., 1998; Fujita et al., 1999). Available reports indicate that CT inhibits osteoclast secretory activity, particularly of TRACP in terms of both synthesis and release. It reduces osteoclastic acid secretion by altering Na^+-K^+-ATPase activity, and carbonic anhydrase localization, as well as direct H^+-ATPase inhibition (see review Zaidi et al., 2002). Available information shows that salmon CT is the most potent in hypocalcemic activity in terms of bone resorption determined in human and rat (Zaidi et al, 1988) and least potent is the human CT.

2.1.2 Non-mammalian vertebrates

Calcitonin has been identified from several species of birds including goose, duck, domestic fowl, and Japanese quail (Kenny, 1971; Boelkins & Kenny, 1973). Plasma content of CT is much higher in birds than normally observed in mammals. The role of CT in birds is controversial (Dacke, 2000; DeMatos, 2008) as conflicting accounts regarding the hypocalcemic effect of CT have been reported. Few investigators have found no effect after CT administration to birds (Urist, 1967; Dacke, 1979). On the other hand, positive responses of injected CT to birds have been reported by Calaney and Barket, (1970), on young cock and laying hen (Lloyd et al., 1970), in domestic fowl, Kraintz and Inscher (1969, in partially parathyroidectomized cockrels) and Swarup et al. (1980a, in parrots). Very recently, Yadav and Srivastava (2009) suggested a hypoclacemic role of CT in *Columba livia*. It is also reported that CT is very important during egg laying when it reduce excessive oscillation in blood plasma calcium levels and possibly influence calcium deposition in the egg.

Reptiles are the first vertebrates to be completely independent of the water environment by developing an egg shell and an amnion. This fact shows that reptiles can perform calcium metabolism of the terrestrial type different from that of amphibians. Calcitonin is present in the ultimobranchial gland and in plasma of reptiles (Kline & Longmore, 1986). But hypocalcemic function of CT in reptiles has not yet been demonstrated successfully in a variety of reptiles, including turtles, snakes, and lizards (Cope & Kline 1989). However, using salmon CT, a hypocalcemic effect of CT has been observed in *Iguana iguana* and in young Chuckwallas, *Sauromalus obesus* (Kline, 1981; 1982). Reptiles share with bird the problem associated with the production of large cleidoic eggs, which in many species are covered with a calcareous shell. Very little information is available on the role of calcemic hormones on reptilian bone and intestine.

Amphibians have a special position with regard to our understanding of vertebrate calcium metabolism, as in this group, parathyroid gland first appear on the phyletic scale. Well developed ultimobranchial gland is present in amphibians. Removal of ultimobranchial bodies in *Rana pipiens* results an initial elevation of plasma calcium levels which subsequently subsides and replaces by hypocalcemia (Robertson et al., 1975). Ultimobranchialectomized bullfrog tadpoles display deficiency in their ability to maintain plasma calcium levels when kept in solution containing high-calcium concentrations (Sasayama and Oguru, 1976).

Calcitonin has been identified in the plasma of *Rana pipiens* and the level increased when these frogs were kept in high-calcium water (Robertson, 1987). Srivastav and Rani (1989) reported that CT injection result in hypocalcemia in *Rana tigrina*, but it has no effect on either urinary excretion or plasma calcium concentrations in toad *Bufo arinus* (Bentley, 1983).

Information on the regulation of calcium ion metabolism in cartilaginous fish is very limited. Like most non-mammalian vertebrates, they have ultimobranchial bodies, which contain CT. High levels of CT is present in plasma of several species of sharks (Glowacki et al., 1985). Early observations using tetrapod CT failed to detect any effects of CT on calcium metabolism in cartilaginous fish. Salmon CT however, induced modest hypocalcemia in leopard shark (Glowacki et al., 1985). Information on the regulation of calcium homeostasis in bony fish is almost entirely limited to teleosts. Within this group, few species have been studied and considerable variation exists among the species. Ultimobranchial gland of teleost fish is a rich source of CT and circulatory level of this hormone is very high in this group of fish. CT has been extracted and purified from ultimobranchial gland of many fish (Niall et al., 1969; Noda and Narita, 1976; Ukawa et al., 1991; Sasayama et al., 1993). Several laboratories although have been able to show the hypocalcemic action of CT in fish (Chan et al., 1968; Lopez et al., 1976; Wendelaar-Bonga, 1981; Chakraborti and Mukherjee 1993; Srivastava et al., 1998; Suzuki et al., 1999), conflicting results are often reported (Wendelaar-Bonga and Pang 1991; Singh and Srivastava 1993). Calcitonin producing C cells in ultimobranchial gland of fish showed less distinct responses to changes in extracellular calcium levels than mammals (Ross et al., 1974). As such, establishing a clear and unequivocal role of CT in calcium homeostasis in fish seems to be extremely difficult. Moreover, in those experiments, where CT has been shown to function as a hypocalcemic hormone in fish, the way this is affected has been addressed in only few studies (Milhaud et al., 1977; Milet et al., 1979; Wagner et al., 1997; Mukherjee et al., 2004a, b). There may be two probabilities relating to hypocalcemic action of fish CT. Responses of fish to this peptide could involve either gills and gill-calcium (Ca^{2+}) transport (GCAT) or as in mammals, inhibition of bone calcium resorption or both. Experimental evidence in support of these probabilities is very limited. Calcitonin receptors have been identified in gills of teleosts (Fouchereau-Peron et al., 1987) and this hormone has been observed to decrease influx of calcium across the perfused gills of salmon and eels (Milhaud et al., 1977; Milet et al., 1979). Later on, an *in vivo* measurement of calcium uptake in intact young rainbow trout and fingerlings and adult fresh water air-breathing teleost, *Channa punctatus*, after salmon CT administration lend support in favor of gills and GCAT (Wagner et al., 1997; Mukherjee et al., 2004a). Since the endoskeleton of fish, at least in part, consists of cellular bones, which have the ability to remodel themselves (Mugiya & Watabe, 1977; Dacke, 1979), the probability of CT function on bone can not be ruled out.

2.2 Other function of CT in vertebrates

Calcitonin is reported to have a role to increase the concentration of insulin-like growth factor (IGF-I) in serum free-culture of human osteoblast-like cells (Farley et al., 2000). Calcitonin may also prevent osteoblast and osteocyte apoptosis (Plotkin et al., 1999). Calcitonin actions on brain, pituitary and gonads have been investigated in mammals. CT-like immunoreactivity has been found in human and rat pituitary gland (Cooper et al., 1980). Specific CT-binding sites have been found in brain and pituitary gland (Maurer et al., 1983) and in ovary and testis in mammal (Chausmer et al., 1982). Wang et al., (1994) found that

CT-like peptides, human CT, salmon CT and CT-gene related peptide inhibit spontaneous and gonadotropin-stimulated testosterone secretion by acing directly on testes and also by reducing the release of pituitary luteinizing hormone (LH). Inhibition of salmon CT on secretion of progesterone- and gonadatropin-releasing hormone (GnRH)-stimulated release of pituitary LH in rat also has been documented (Tsai et al., 1999). Very recently, we also reported for stimulation of salmon CT on secretion of 17β-estradiol by the ovarian follicles of common carp *C. carpio* (Paul et al., 2008). All these observations suggest an endocrine role of endogenous CT at brain, pituitary and gonads in vertebrates. Another interesting cellular action of CT involves the early demonstration of its effect on growth of breast cancer cells. CT retards cell division in CT receptor-transfected HEK cells (Piserchio et al., 2000).

3. Objective

The purpose of the present study was aimed: 1) to investigate the action of CT on hypocalcemic regulation in a fresh water teleost, *Cyprinus carpio* and its mode of action through inhibition of gill-Ca^{2+} transport and bone calcium resorption; 2) to examine the effects of CT on basal and hCG-stimulated *in vivo* release of 17β-estradiol (E_2) and *in vitro* production of E_2 and 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-P) by the ovarian follicles; and finally 3) to find out the efficacy of CT alone or in combination with hCG on induction of oocyte maturation in post-vitellogenic ovarian follicles of *C. carpio.*

4. Materials and methods

4.1 Chemicals

Synthetic salmon calcitonin (sCT), all cold steroids and dibutyryl cyclic AMP (dbcAMP) were purchased from Sigma Chemicals. Analytical grade *p*-nitrophenol, *p*-nitrophenyl phosphate, ninhydrin and reference standard amino acid kit were purchased from Sisco Research laboratories, Mumbai, India. hCG was a gift from National Hormone and Pituitary Programm (Torrance, CA, USA). SQ 22536 (RBI, Natrick, MA, USA) and NPC-15437 dihydrochloride (Sigma) was a gift from Dr. Arun Bandopadhyay, Molecular Endocrinology Laboratory, Indian Institute of Chemical Biology, Kolkata, India. TRI reagent was purchased from Ambion Inc., USA. Smart-PCR cDNA synthesis kit was purchased from Clontech. RevertAid M-MuLV reverse transcriptase and deoxyNTPs were purchased from MBI Fermentas and Taq DNA polymerase from Invitrogen. Radiolabelled ^{45}Ca (specific activity 32.5 mCi/mg calcium), radiolabelled [^{3}H] estradiol 17β (specific activity 75.0 Ci/mmol), [^{3}H]testosterone (sp activity 95.0 Ci/mmol), 17α, Hydroxy [1,2,6,7-^{3}H]progesterone (sp activity 75 Ci/mmol) and [^{125}I]sCT (sp activity 2000 Ci/mmol)were purchased from Amersham Biosciences. The E_2 antibody was a gift from Prof. Gordon Niswender, Colorado State University, Fort Collins, USA. Labelled 17α,20β-P was prepared from labeled 17α, Hydroxy [1,2,6,7-^{3}H]progesterone by sodium borohydide reduction followed by chromatographic separation as described by Sen et al., (2002). 17α,20β-P antibody was obtained from Dr. A. P. Scott, Lowestoft, UK. All chemicals used were of analytical grade.

4.2 Calcitonin in calcium ion regulation in fish

4.2.1 Animals

For evaluation of CT action on calcium ion regulation, sexually mature (200-250 g body wt) and immature (30-35 g body wt) female *C. carpio* and fingerlings of this fish (3-4 g body wt)

were obtained from local fish farm and maintained in re-circulating de-chlorinated normal tap water (300 L capacity; Ca, 0.15 mM; 24 ± 1°C) for 10 days prior to use. Groups of mature and immature fish as well as fingerlings were transferred to either low-Ca water (Ca, 0.05 mM) or normal tap water for 7 days prior to treatment. During the period of acclimatization, fish and fingerlings both were maintained on a daily commercial fish food (Shalimer Fish Food, Mumbai, India) and starved 24 h prior to use. Ultimobranchial glands (UBG) were removed from freshly collected female *C. carpio* and extract was prepared following the procedure described early (Chakraborty and Mukherjee, 1993). Briefly, ten glandular bodies were quickly homogenized in ice-cold saline and homogenate was centrifuged in cold condition for 10 min at 5000 x g. The supernatant was taken as UBG extract.

4.2.2 Time-course and dose-response effects of sCT and UBG extract on plasma calcium

Sexually mature and immature fish, kept either in normal tap water or low-Ca water received a single i.p injection of sCT (0.5 µg/100 g body wt) at 07.00 h in the morning for conducting time-course study or with increasing concentrations of sCT for dose-response study. Homologous UBG extract treatments were conducted in a similar way only with immature fish kept in low-Ca water. Controls were injected with similar volume of vehicle. A sham-injected control group was always included along with saline–control group to ensure that saline treatment evoked no stress on fish. Fish were sampled at different time intervals or 4 h after CT or UBG extract injection. In a separate experiment, sexually immature fish kept in low-Ca water were injected daily with sCT (0.5 µg/100 g body wt) for 15 days and sampling of fish was done at 4 h after last injection.

4.2.3 Effects of sCT on gill-calcium transport (GCAT)

Time-course and dose-response study of ^{45}Ca uptake in response to sCT was conducted with fingerlings and sexually immature fish, kept either in normal tap water or low-Ca water. After light anesthesia with MS222, both fingerlings and immature fish were given either single injection of sCT (0.5 µg/100 g body wt) for time-course study or with increasing doses of sCT in such a way that each fish received either 0.1 or 0.2 or 0.5, 1 or 2 µg sCT/100 g body wt. Immediately after injection fingerlings were transferred to individual glass tank (2 L capacity) each containing 1 liter ^{45}Ca (200 µCi) water and immature fish to individual glass aquarium (5 L capacity) each containing 3 liter ^{45}Ca (600 µCi) water. In both the treatment groups six individuals per tank or aquarium were kept. Fish were sampled at 1, 2, 4, 6, 8 and 12 h after sCT treatment.

4.2.4 Procedure for determination of whole body calcium uptake and calculation of GCAT

This has been done following the procedure described early (Mukherjee et al., 2004a). Briefly, fish were euthanized in 0.25% solution of benzocane followed by a wash with 0.1M HCl for 10 minutes to remove the externally bound ^{45}Ca. From each fish and fingerling, gut with its content was removed and weight of each fish/ fingerling was recorded. They were then placed in a muffle furnace at 600 °C to convert them to complete ash. The isotope content of ash was determined by scintillation counting of the dissolved ash. The rate of GCAT for each fish was calculated on the basis of isotope content of the fish/ fingerling and specific activity of ^{45}Ca and expressed as mmol Ca^{2+} transport /kg body weight per hour (Wagner et al., 1997; Mukherjee et al., 2004a).

4.2.5 Effects of sCT on plasma TRACP and ALP activities and urinary HYP content

This experiment was conducted with immature fish kept in normal tap water. Fish, after light anesthesia received either a single injection of sCT (0.5 µg/100 g body wt) or single injection of one of four different concentrations of sCT (0.01, 0.1, 0.5 or 1 µg/100 g body wt). Sampling of fish was done at 4 h of sCT injection. Calcium content of skeletal bones as well as plasma TRACP and ALP activities and urinary HYP content of immature fish adapted to normal tap water were measured after daily sCT treatment (0.5 µg/100 g body wt) for 15 days. Fish were sacrificed 4 h after last injection and skeletal bones were processed for quantification of calcium.

4.2.6 Estimation of plasma and bone calcium and determination of TRACP and ALP activities and urinary HYP

Immediately after sampling, blood was collected and processed for plasma separation. Aliquots of plasma samples were subjected to either separation of protein free ultrafiltrate (Chakraborty and Mukherjee, 1993) or determination of TRACP and ALP activities following the procedure described early (Mukherjee et al., 2004b). Ultrafiltrate samples were analyzed by atomic absorbance spectroscopy (Varian SpectrAA 250) for determination of calcium content. Skeletal bones of individual control and treated fish was processed for preparation of bone powder and calcium content was estimated following the procedure described early (Mukherjee et al., 2004b). Urine samples from control and treated fish were collected with the help of fine catheter (0.5 mm i.d) and HYP from the urine was extracted and estimated following the procedure described previously (Mukherjee et al., 2004b).

4.3 Effects of sCT on ovarian steroid production and oocyte maturation in fish

4.3.1 Animals

Adult female *C. carpio* (300-400g body wt) after collection during the month of September to November were maintained in re-circulating de-chlorinated normal tap-water in laboratory concrete tanks (300 L capacity, Ca^{2+}, 0.15 mM) at 23 ± 2 °C at least for 5 days prior to experiment. They were fed with commercial fish food. A group of vitellogenic and post-vitellogenic stage fish was transferred to high-calcium water (Ca, 0.4 mM) for 7 days for *in vivo* and *in vitro* experiments. Reproductive cycle and the method of categorization of different follicles in the ovary of *C. carpio* to different stages have been reported previously (Mukherjee et. al., 2006). To state briefly, during the months of September and October in the plains of West Bengal, India, ovary of female carp comprises mostly vitellogenic follicles (0.3 - 0.4 mm diameter) with oocytes containing centrally located germinal vesicle (GV). The cytoplasm was filled up with yolk granules and cortical granules were shown to cover the entire oocyte. In the month of November, ovaries mostly contain post-vitellogenic follicles (0.5 - 0.7 mm diameter) in which the oocytes were found to initiate coalescence of lipid droplets around a centrally located GV. Follicular developmental stage was determined by stripping out few follicles through the ovipore followed by examination under microscope after fixing them with a clearing solution of acetic acid-ethanol-formalin mixture (1: 6: 3 v/v) for 12 h.

4.3.2 Effects of sCT on plasma 17β-estradiol levels in vitellogenic stage fish

Vitellogenic stage *C. carpio* after maintaining seven days in high-Ca water were divided into two groups and following *in vivo* experiments were conducted: One group of fish received a single i.p injection of increasing concentration of sCT in such a way that each fish received

0.1, 0.5, 1.0 or 2.0 µg sCT/ 100 g body wt. Controls were injected with similar volume of solvent. Sampling of fish was done at 8 h after injection. Fish were lightly anaesthetized with MS222 before treatment. The second group of fish was given a single injection of sCT (0.5µg /100 g body wt) or hCG (0.5µg /100 g body wt) or hCG plus sCT at 07.00 h in the morning. Controls were injected with similar volume of solvent. Fish were sampled at 0, 2, 4, 8, 12, 16 and 24 h after injection. In both the experiments, immediately after sampling, blood was collected from caudal vein of the fish under light anesthesia and processed for plasma separation and kept at –20 °C until steroid analysis.

4.3.3 *In vitro* incubation of ovarian follicles

Vitellogenic and post-vitellogenic follicles after collection from the ovaries of respective stage fish were placed in ice-cold Idler's medium containing streptomycin (100 µg/ml) and penicillin (100 IU/ml) adjusted to pH 7.4 (Mukherjee et al., 2006). Follicles, after collection, were kept separately in ice-cold medium until use. Follicles weighing approximately 100 mg were initially placed in individual well of a 24-well culture plate (Tarson, India) for 2 h that contained 1.0 ml control medium. After 2 h, the medium was replaced by fresh medium containing effectors. Cultures were placed in a metabolic shaker bath at 23 ± 1°C under air. Viability of ovarian follicles was observed to be about 90% as detected using 0.1% Trypan blue dye exclusion. At the end of incubation, medium samples were aspirated, centrifuged (5000x g) and stored at – 20 °C for E_2 and 17, 20β-P measurement by specific RIA. The follicles were fixed for 12 h in clearing solution as mentioned and oocyte maturation was examined by scoring germinal vesicle breakdown (GVBD) under microscope (Bhattacharyya et. al., 2000).

4.3.4 RNA isolation, cDNA preparation and Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted from isolated vitellogenic ovarian follicles using TRI reagent solution following the manufacturer's instruction and method described earlier (Chomzynski and Sacchi, 1987) and cDNA was synthesized using Smart-PCR cDNA synthesis kit. First strand cDNA synthesis was carried out with 2 µg total RNA and RT-PCR was performed following the procedure described early (Paul et al., 2008). The RT-PCR products were cloned, sequenced and used for the expression purpose. The primers (used for RT-PCR) of the respective genes with the accession number and their amplified segments are listed Table 1.

Gene product	Forward primer	Reverse primer	Size of amplicon (bp)
CYP19A (cytochrome P450 aromatase; DQ534411)	5′ TACACATTCTGGAGAGTTTTATCA 3′	5′ GGAAGTTGTCTAGACTGAACTCAT 3′	198
GAPDH (AJ870982)	5′ AGGGGCTCAGTATGTTGTGG 3′	5′ AGGAGGCATTGCTGACAACT 3′	185

Table 1. Primers used in semi-quantitative polymerase chain reaction

4.3.5 Preparation of oocyte membrane for sCT binding assay

Membrane from ovarian follicles was prepared using the method described previously (Mukherjee et al., 1994; Paul et al., 2008). Protein content of the preparation was measured

according to the method of Lowry et al., (1951) using BSA as standard. Salmon CT binding assay was conducted following the procedure described early (Mukherjee et al., 1994; Paul et al., 2008). In brief, for [^{125}I]-sCT binding, membrane preparation (2.0 mg protein) was incubated with 20 µl [^{125}I]/-sCT solution (1x10^5 c.p.m.) in the absence (total binding) or presence of a 1000-fold excess of unlabelled sCT to measure nonspecific binding in a final assay volume of 500 µl at 23 °C. Incubation was terminated at 90 min by addition of γ globulin (0-1% v/v) or NaCl (0.1mol/L). Ice cold polyethylene glycol (PEG; 1 ml, 20% v/v) was then added to each tube. The final pellet was counted in a [^{125}I] gamma counter. Specific binding was estimated by subtracting non-specific binding from total binding.

4.3.6 Extraction and assay of steroids

The method of extraction and assay of E_2 and 17, 20β-P was similar to the previously described procedure for these steroids (Mukherjee et al., 2001, 2006).

4.3.7 Statistical analysis

Data were statistically analyzed by analysis of variance (ANOVA) followed by Duncan's multiple range test for experiments on calcium ion regulation and Bonferroni's multiple comparison tests for reproduction. Differences were considered significant at $p< 0.05$ and $p< 0.01$ as indicated in the text. Comparisons between sexually immature and mature fish were performed with student's t-test.

5. Results

5.1 Calcium ion regulation

5.1.1 Effects of sCT and UBG extract on calcium ion regulation

A single injection of sCT to sexually mature fish, kept in normal tap water (Fig. 1 A) or in low-Ca water (Fig. 1 B) reduced ultrafiltrable plasma calcium content within 2 h with a maximum at 4 h ($p<0.01$). This hypocalcemic effect was continued up to 8 h. There was no change between sham-control and saline-control group. Since there was significant short-duration hypocalcemic effect of sCT in sexually mature fish kept both in normal tap water and low-Ca water, an attempt has been made to examine whether the interference of endogenous sex steroid (s) was responsible. It is evident from a similar experiment with sexually immature fish kept in normal tap water (Fig. 1 C) and low-Ca water (Fig. 1 D) that a single injection of sCT was highly effective in reducing plasma calcium fraction within 2 to 8 h with a maximum at 4 h ($p< 0.01$). No changes in plasma calcium levels were noticed between sham- and saline-control groups. While comparing the degree of inhibitory response after 4 h of sCT injection, the immature fish was shown to be more responsive than mature fish.

Dose-response effect of sCT on plasma calcium was conducted with sexually immature fish kept in normal tap water and low-Ca water. Figure 2 A demonstrate that at increasing doses of sCT, a gradual and significant ($p<0.01$) reduction in plasma calcium fraction was recorded with a maximum at 0.5 µg dose in fish kept both in normal tap water and low-Ca water. Effects of repeated doses of sCT on plasma calcium levels were examined with sexually immature fish kept in low-Ca water and compared with the effects produced after single injection. Results in Fig. 2 B clearly shows that daily injection of sCT (0.5 µg/100g body wt) for 15 days caused significant reduction ($p<0.01$) in plasma calcium fraction as compared to saline control and the rate of inhibition was almost identical with that registered after single injection.

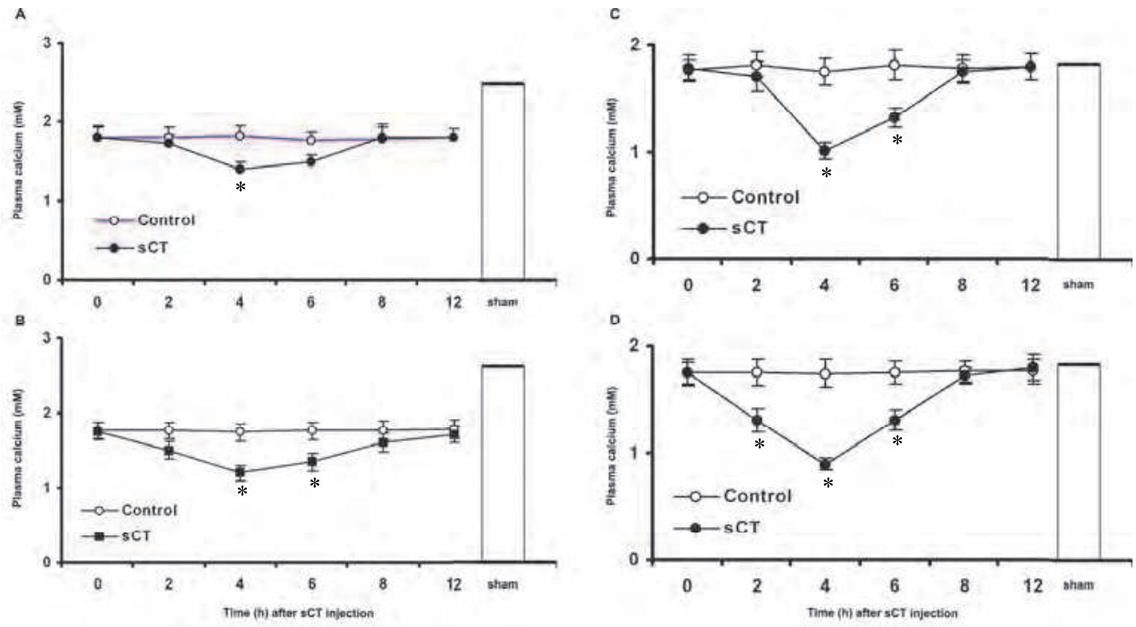

Fig. 1. Plasma calcium levels of sexually mature *Cyprinus carpio* kept in normal tap water (A) or low-Ca water (B) and immature fish kept in normal tap water (C) and low-Ca water (D) after a single sCT injection (0.5 µg/ 100 g body wt.). Data presented as mean ± SEM of five observations from five different fishes. [Asterisks significantly (p<0.01) different from those shown for sham- and saline control (ANOVA, Duncan's multiple range test).]

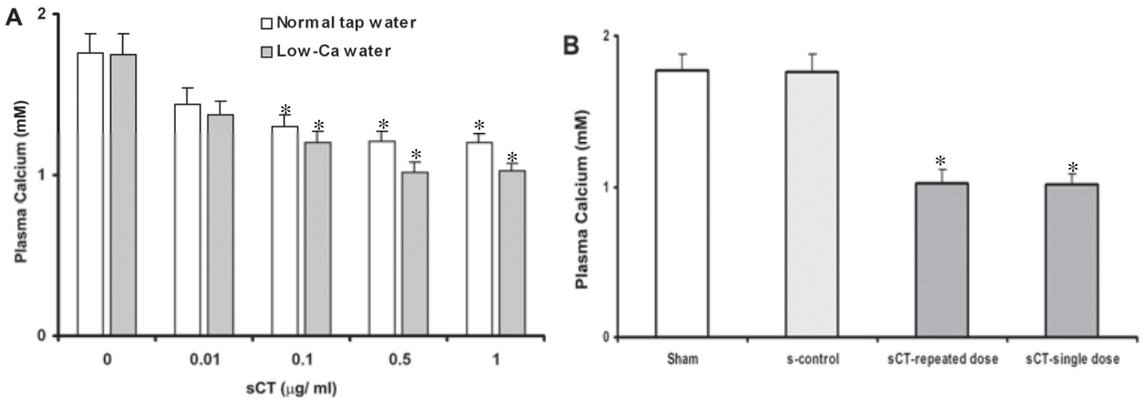

Fig. 2. Dose-response effects of SCT on plasma calcium levels of sexually immature fish kept in normal tap water or in low-Ca water (A) and of immature fish kept in low-Ca water (B) after daily injection of SCT (0.5 µg/ 100 g body wt.) or saline for 15 days. Fish were sacrificed at 4 h after last injection. Each value represents the ± SEM of five observations. Values were compared with those shown after single injection of SCT (B, 0.5 µg/ 100 g body wt.). [Asterisk indicates significant difference at p<0.01 from vehicle injected control. (ANOVA, Duncan's multiple range test).]

Results of the effects of homologous UBG extract on plasma calcium levels are depicted in Fig 3A and B. It appears from the Figure that UBG extract at the dose 0.5 UBG eq. /100 g body wt caused significant (p<0.01) reduction in plasma calcium levels at 4 h after injection both in normal tap water and low-Ca water adapted fish. Dose-response study to observe the effects of UBG extract was conducted in fish adapted only in low-Ca water. Fig. 3 C shows that UBG extract at increasing doses caused a gradual and significant reduction (p<0.05) in plasma calcium levels with a maximum at 0.5 UBG eq/100 g body wt.

5.1.2 Effects of sCT on gill-calcium transport (GCAT)

Results of the time-course tracer uptake study in response to sCT are depicted in Fig. 4. It is evident from the figure that a single injection of sCT (0.5 µg/100g body wt) to fingerlings, kept either in normal tap water (Fig. 4 A) and low-Ca water (Fig. 4 B) significantly (p<0.05) reduced GCAT between 2 to 6 h with a maximum at 4 h compared to their saline controls. No apparent changes in GCAT were noticed between sham-control and saline-control groups. Fig. 4 C depicts that reduction of GCAT was recorded at the dose of 0.1 µg sCT with a maximum at 0.5 µg sCT /100 g body wt compared to their respective control values. Here also no changes were observed between saline control group and sham control group.

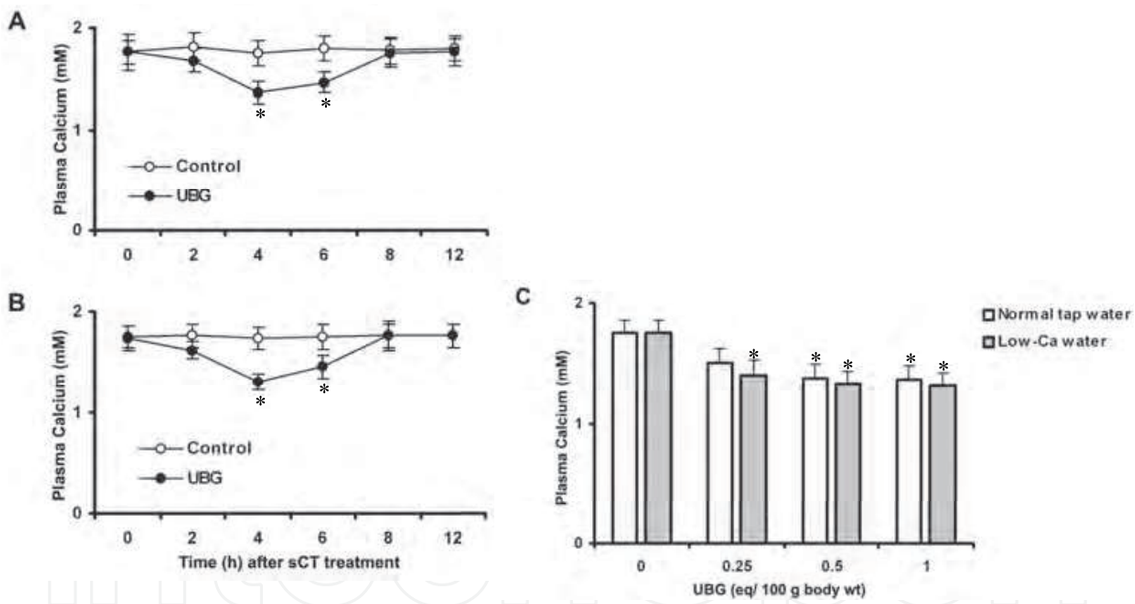

Fig. 3. Time course effects of UBG extracts on plasma calcium levels of sexually immature fish kept in normal tap water (A) or low Ca water (B). Dose response effects of UBG extracts on plasma calcium levels of sexually immature fish kept in normal tap water or low Ca water (C). Fish was sacrificed 4 h after last injection. Each value represents the mean ± SEM of five specimens. [Asterisk indicates significant difference at p<0.05 from sham, and saline injected control.]

It is evident that a single injection of sCT (0.5 µg/100 g body wt) to sexually immature fish, kept in normal tap water (Fig. 5 A) or in low-Ca water (Fig. 5 B) was able to reduce GCAT significantly (p<0.05) between 2 to 6 h with a maximum at 4 h, compared to saline control group. Beyond 6 h, inhibitory effects of sCT on GCAT were absent.

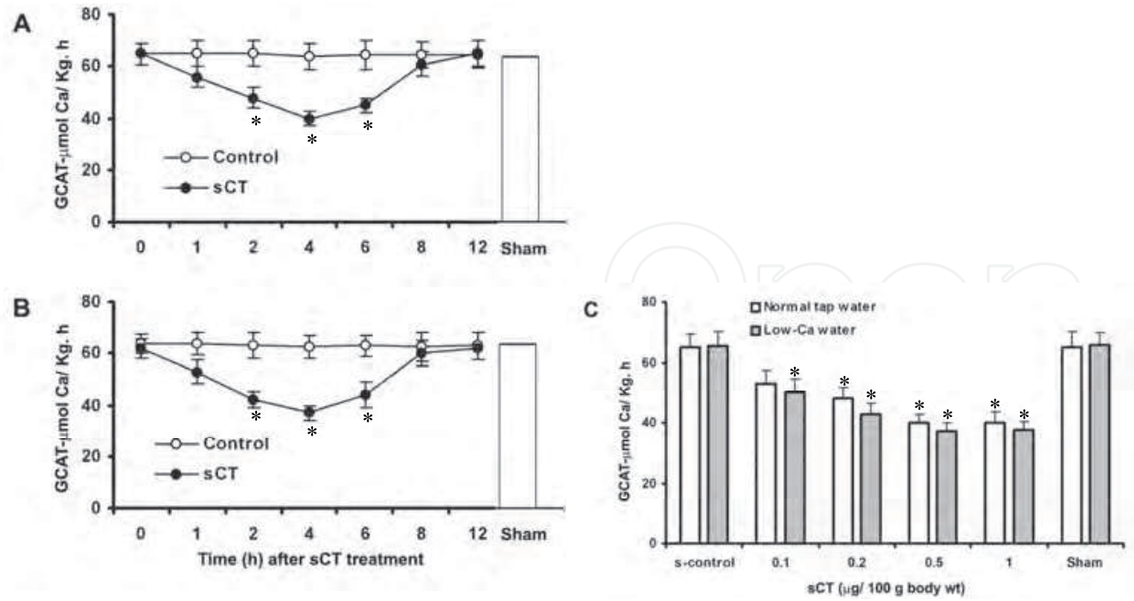

Fig. 4. Time-course study of the inhibition of gill Ca^{+2} transport (GCAT) in fingerlings of *C. carpio,* kept in normal tap water (A) or low-Ca water (B) after a single injection of sCT (0.5 µg/ 100 g body wt.). Dose response effects sCT on inhibition of GCAT in fingerlings kept either in normal tap water or low-Ca water (C). Values are mean of six fingerlings. [Asterisk indicates significantly (p<0.05) low value from these shown for sham and saline control (ANOVA, Duncan's multiple range test)]

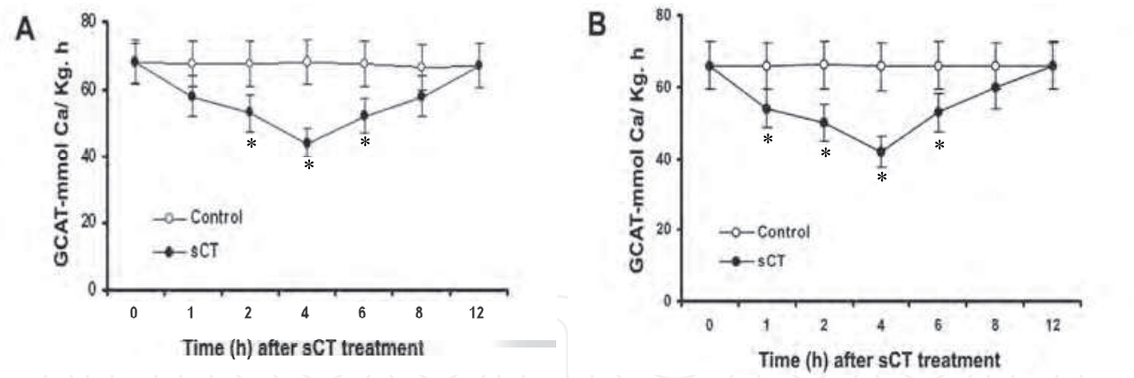

Fig. 5. Time-course study of the inhibition of gill Ca^{+2} transport (GCAT) in immature *C. carpio,* kept in normal tap water (A) or low-Ca water (B) after a single injection of sCT (0.5 µg/ 100 g body wt.). Values are mean of six fingerlings. Asterisk indicates significantly (P<0.05) low value from these shown for sham and saline control (ANOVA, Duncan's multiple range test).

5.1.3 Effects of sCT on plasma TRACP and ALP activities and urinary HYP content – a time-course and dose- response study

Since no differences were obtained between low-Ca water and normal tap water adapted fish in lowering plasma calcium levels and lowering GCAT after CT treatment, changes of plasma TRACP and ALP activities and excretion of urinary HYP in response to sCT was

observed in fish adapted to normal tap water. A single injection of sCT to sexually immature fish kept in normal tap water was able to suppress both TRACP and ALP activities in plasma and HYP excretion in urine. Salmon CT-induced suppression of TRACP and ALP activities and reduction in urinary HYP extracts were noticed as early as 2 h and reached the lowest values (p< 0.01) at 4 h after treatment (Fig. 6 A, B and C). No changes in the activities of TRACP and ALP and excretion of urinary HYP were observed between sham-control and saline group.

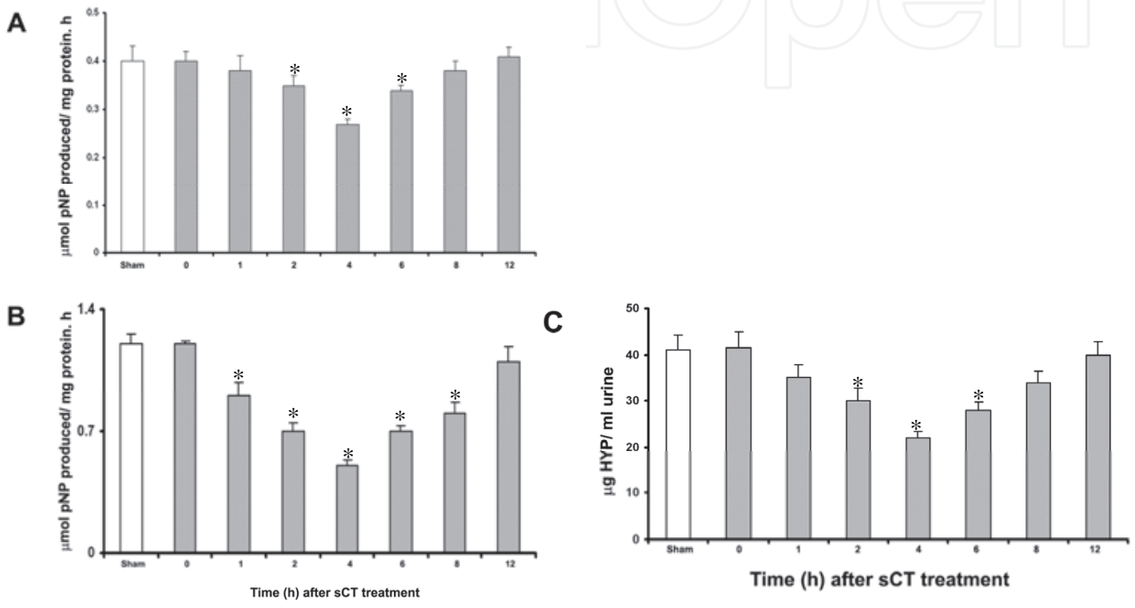

Fig. 6. Effects of sCT (0.5 µg/ 100 g body wt) and vehicle on TRACP (A) and ALP (B) activities in plasma and HYP (C) content in urine in sexually immature fish kept in normal tap water. Values of (A) and (B) are means of five specimens and (C) is of four observations. For each observation urine samples from three individual fish were pooled. [Asterisk indicates significant difference at p<0.01 from vehicle-injected control (ANOVA, Duncan's multiple range test)].

Result of increasing concentration of sCT administration to sexually immature fish is shown in Fig. 7. At a very low concentration (0.01µg /100 body wt) sCT was able to suppress plasma TRACP and ALP activities (Fig. 7 A and B) as well as urinary HYP excretion (Fig. 7 C). Increasing doses suppressed activities of both the enzymes and urinary HYP content gradually with a maximum (p< 0.01) at the dose 0.5 µg sCT/100g body wt.

Results of plasma TRACP and ALP activities and excretion of urinary HYP and calcium content of skeletal bone of sCT-, treated and -untreated fish are depicted in Fig. 8. It appears from the figure that daily injection of sCT for 15 days to sexually immature fish kept in normal tap water caused significant suppression (p<0.01) of the activities of both the enzymes in plasma and HYP content in the urine (Fig. 8 A, B and C). On the other hand, there was a significant increase (p<0.01) in the calcium content of skeletal bones of sCT-injected fish compared to control values (Fig. 8 D).

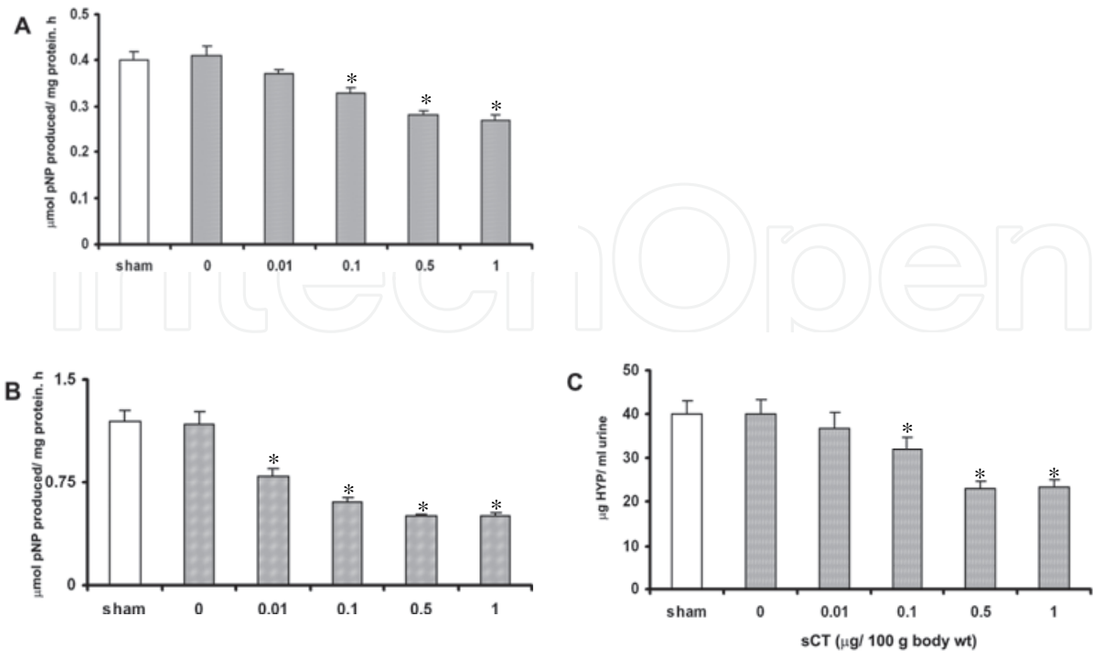

Fig. 7. Effects of increasing doses of sCT on TRACP (A) and ALP activities in plasma and HYP (C) content in urine in sexually immature fish kept in normal tap water. Each value of TRACP and ALP represents ± SEM of five specimens and HYP of four observations. [Asterisk indicates significant difference at $p<0.01$ from sham and saline control (ANOVA, Duncan's multiple range test)].

5.2 Effects of sCT on 17β-estradiol production by the vitellogenic follicles

5.2.1 Plasma 17β-estradiol levels in response to sCT and HCG

Results shown in Fig. 9 A demonstrates that sCT at increasing doses caused a gradual increase in plasma E_2 levels in carp, 8 h after injection. The maximum effective dose of sCT was 0.5 µg/ 100g body wt. The minimum dose at which sCT was able to induce increase in plasma E_2 levels was 0.1 µg. Injection of solvent did not alter the level of plasma E_2 in fish (Fig. 9 B). Injection of hCG (0.5 µg/ 100 g body wt) stimulated E_2 secretion and highest plasma concentrations of this steroid was recorded at 12 h (Fig. 9 B). Injection of hCG plus sCT (each 0.5 µg /100g body wt) resulted in a significantly ($p<0.05$) higher level of plasma E_2 at 8 h and 12 h after challenge compared with that induced by hCG alone (Fig. 9 B). Mean levels of ultrafiltrable plasma calcium in vitellogenic stage fish at all time points were 1.6 ± 0.17 mM, 1.6 ± 0.16 mM and 1.65 ± 0.17 mM respectively for the vehicle-, hCG- and sCT-injected group.

5.2.2 sCT binding to fish ovarian tissue

Fig. 10 shows that with increasing concentrations of [^{125}I] sCT, the specific binding increases until 2 nM and then saturation was reached. Scatchard plot analysis of the data (inset of Fig. 10) showed that Bmax (MBC) of ovarian follicular membrane preparation for [^{125} I] sCT was 1.2 pM/mg protein and *K*d was 48.8 pM/ L.

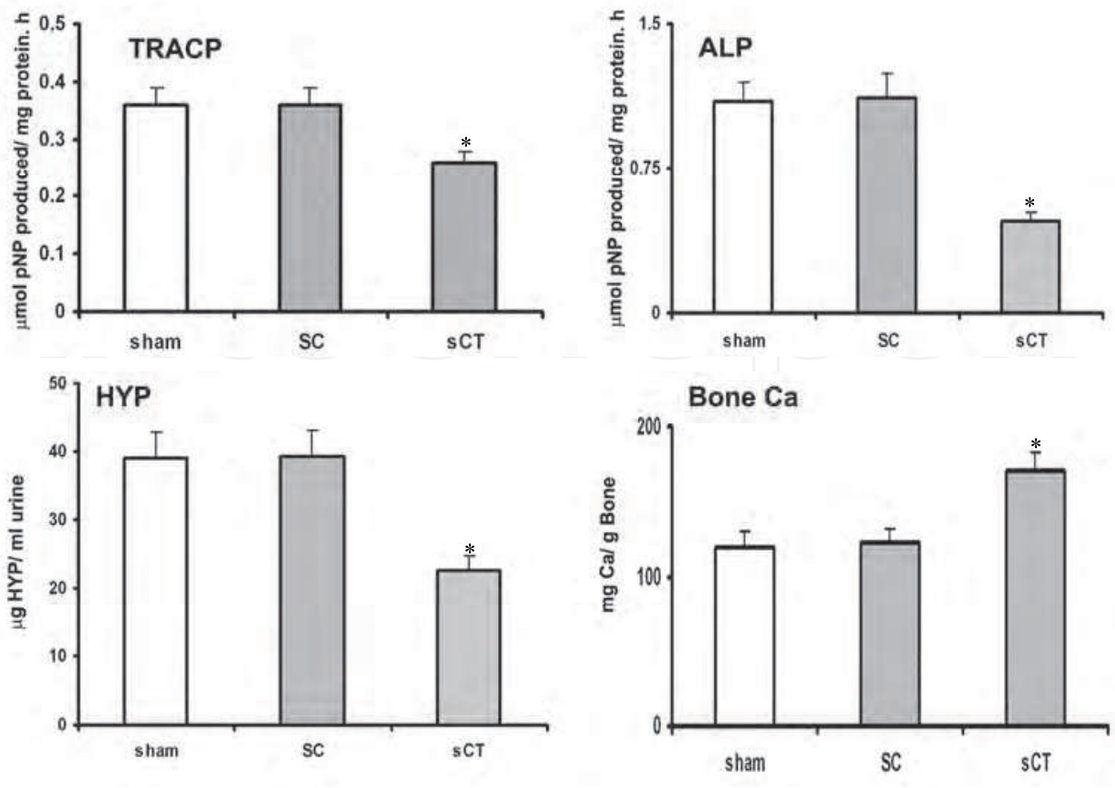

Fig. 8. Changes in plasma TRACP and ALP activities in plasma, HYP content in urine and calcium content of skeletal bones of sexually immature fish kept in normal tap water after daily injection of sCT (0.5 µg/100g body wt) for 15 days. Each value of TRACP and ALP of plasma and the bone calcium content represents the mean ± SEM of five and HYP of four observations. [Asterisk indicates significant differences (p<0.01) compared with the values in the normal control (ANOVA, Duncan's multiple range test)].

5.2.3 Effects of sCT on *in vitro* E_2 production by ovarian follicles

Since there was a significant increase in plasma E_2 levels in vitellogenic stage fish after sCT treatment, and since sCT binds with the ovarian membrane preparation with high-affinity, physiological importance of sCT binding to ovarian follicles was assessed by incubating follicles with varied concentrations of sCT with or without hCG and steroid production was measured.

The effect of sCT ranging from 25 to 200 ng /ml incubation on E_2 release by the ovarian follicles is illustrated in Fig. 11A. During a 12-h incubation, sCT at 50 ng dose released maximum quantity of E_2 in the medium (p<0.05). Incubation with increasing doses of hCG for 12 h caused a significant increase (p<0.05) of E_2 release at 25 ng dose and highest was recorded at the dose of 50 ng/ml incubation (Fig. 11 B). Ovarian follicles were incubated with sCT (0 to 100 ng/ ml) in presence of hCG (25 ng ml) for 12 h. Fig. 11 C shows that hCG-induced release of E_2 was significantly increased (p<0.05) by sCT ranging from 25 to 100 ng dose/ ml incubation. Ovarian follicles were incubated with sCT or hCG (each 50ng/ ml) for various length of time up to 16 h. It appears from Fig. 11 D that after addition of hormones, E_2 release increased steadily from 2 h and the maximum was recorded at 6 h by sCT and 12 h by hCG.

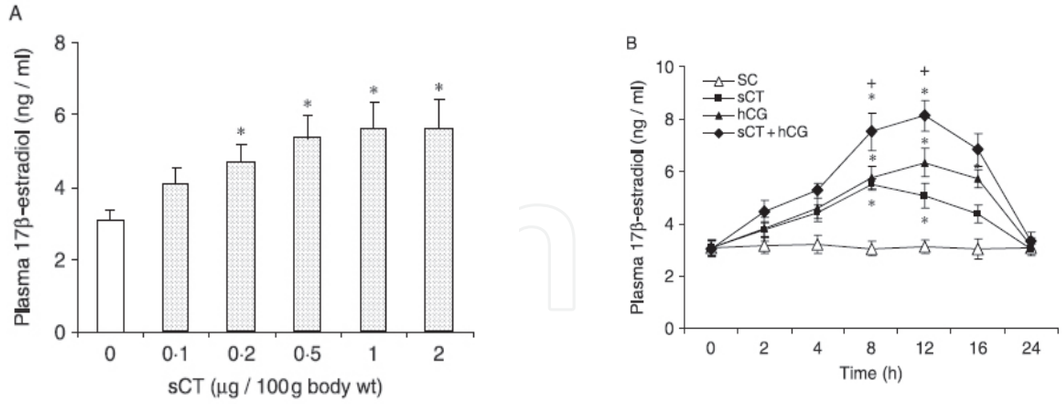

Fig. 9. Dose-response effects of sCT (A) and a time-course study on the effects of sCT (0.5 µg /100g body wt), human chorionic gonadotropin (hCG, 0.5 µg/100g body wt) and hCG + sCT (each 0.5 µg /100g body wt) (B) on plasma 17β-estradiol (E_2) levels in vitellogenic stage *C. carpio*. Fish were injected with increasing doses of sCT as indicated and sacrificed 8 h after injection (B). Fish were given a single intraperitoneal (i.p.) injection of sCT, hCG or sCT + hCG. Blood samples were collected at time indicated after hormone challenge (B). Each value (A and B) is mean ± SEM of five observations. SC, saline control, *p< 0.05 vs. sCT at 0 µg /100g body wt, (A) + ^{+}p<0.05 vs. hCG at 0.5 µg /100g body wt (B).

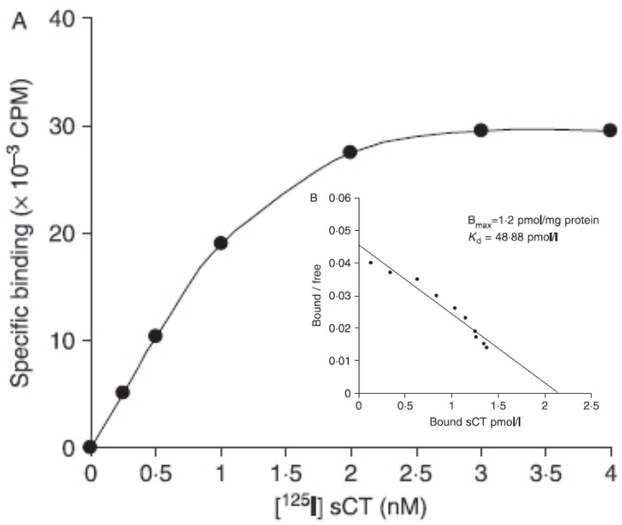

Fig. 10. Saturation curve of [^{125}I] sCT binding to membrane preparation of ovarian follicles of vitellogenic stage fish. Specific binding was determined by subtracting the nonspecific binding from the total binding. Inset shows Scatchard plot of [^{125}I] sCT binding to fish ovarian follicular membrane preparation. Values are mean ± SEM of four observations taking ovarian follicles in duplicate from four donor fish.

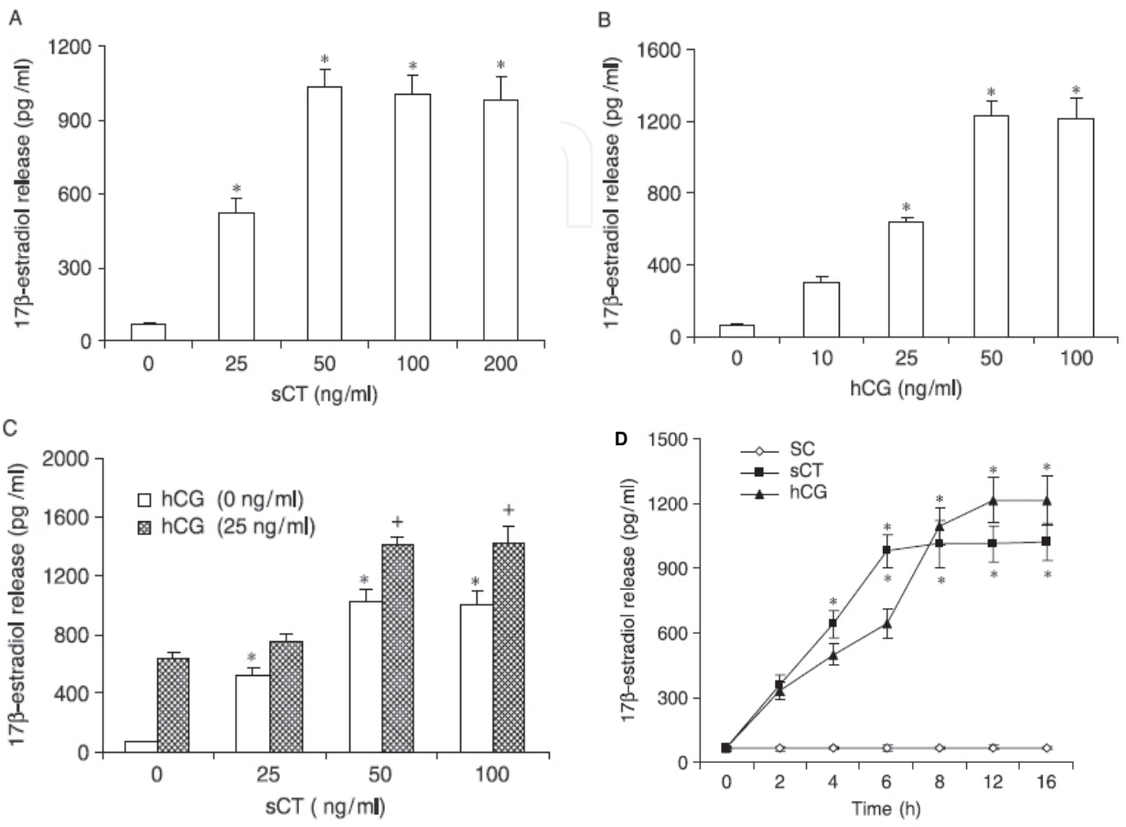

Fig. 11. Dose-response effects of sCT (A) and hCG (B) on *in vitro* production of 17β-estradiol by the vitellogenic follicles. Dose-response effects of sCT on basal (hollow bar) and hCG-stimulated (solid bar) *in vitro* production of 17β-estradiol by the vitellogenic follicles (C). Time-course effects of sCT and hCG (each 50 ng/ ml) on *in vitro* 17β-estradiol production by the vitellogenic follicles (D). All incubations (A, B and C) were terminated 12 h after addition of test compounds. Each value represents mean ± SEM of five incubations taking follicles in triplicate from five donor fish. *p< 0.05 vs. tissues incubated without hormone (0) (A and B), *p<0.05 verses hCG at 0 ng /ml (C). *p< 0.05 verses saline control (SC) at respective time period (D).

5.2.4 Effects of sCT on activity of P450arom

When ovarian follicles were incubated with [^{3}H] testosterone for 8 h in the presence of either sCT or hCG (each 50 ng/incubation), a significant increase (p<0.05) in [^{3}H]E_2 formation were noticed both in hCG and sCT administered incubations relative to their control values (Fig 12). These finding indicate that sCT-induced E_2 production in fish ovarian follicles like that of hCG was due to its stimulation of aromatase activity.

5.2.5 Effects of sCT and hCG on P450arom gene expression

Total mRNA was extracted from ovarian follicles exposed with or without sCT or hCG (each 50 ng/ml) for different time intervals and RT-PCR was performed using P450arom primer (CYP19A). Fig 13 A shows that both hCG and sCT stimulated P450arom gene expression in ovarian follicles incubated for 2 h and increased gradually and significantly (p<0.05) from 4 to 8 h. The expression of GAPDH was used as loading control (Fig. 13 B).

5.2.6 Effects of dbcAMP on sCT-stimulated E_2 release

To evaluate the role of intracellular cAMP in the regulation of E_2 release by sCT, the effects of dbcAMP and SQ22536 (a cell permeable selective adenylate cyclase inhibitor) on ovarian follicles were examined. Dibutyryl cyclic AMP at both the concentrations (0.5 mM and 1.0 mM) stimulated the release of E_2 by the ovarian follicles in a dose-dependent manner and addition of sCT (25 ng/ incubation) potentiated the effects of dbcAMP on E_2 release at all concentrations tested (Fig. 14 A). Administration of SQ22536 at increasing doses attenuated sCT-stimulated E_2 production in a concentration-dependent manner (Fig. 14 B). SQ22536 at increasing doses also gradually and significantly (p<0.05) attenuated hCG-stimulated E_2 production by the follicles (Fig. 14 C).

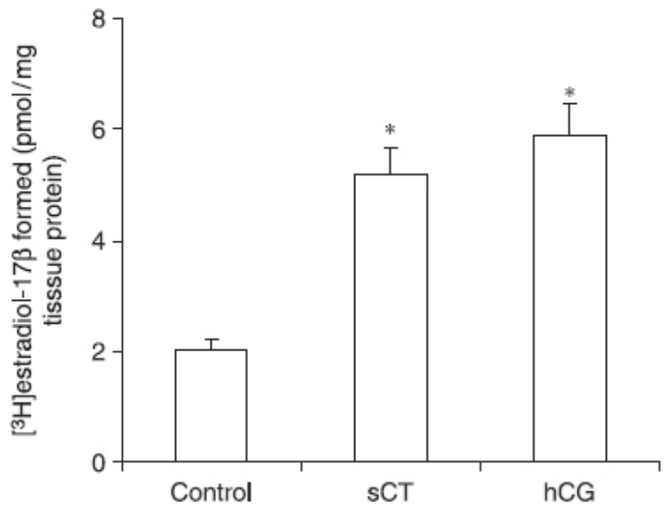

Fig. 12. Salmon CT- and hCG-stimulated conversion of [^{3}H]testosterone to [^{3}H]estradiol-17β in vitellogenic follicles. Follicles were incubated in the presence of [^{3}H]testosterone (1x10^6 c.p.m, 140 mmol) without or with sCT or hCG for 8 h. Each value is the mean ± SEM of five incubations taking follicles in triplicate from five donor fish). *p< 0.05 vs. saline control.

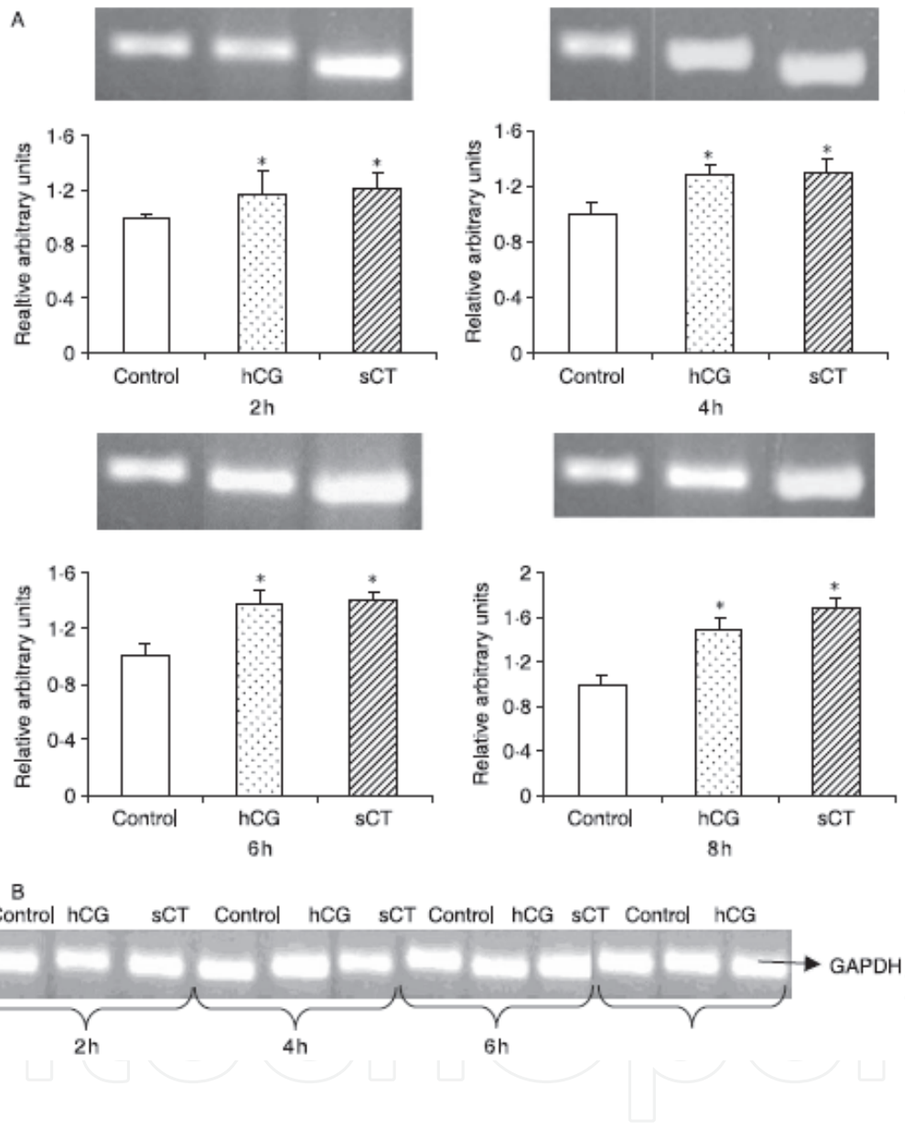

Fig. 13. Effect of hCG and sCT on the expression of P450arom mRNA in vitellogenic ovarian follicles. Follicles were incubated for 2, 4, 6 and 8 h without or with sCT or hCG (each 50 ng / ml). Total RNA was isolated from ovarian follicles and RT-PCR was performed using CYP19A gene specific primer. Amplified product was loaded in agarose gel as control, sCT and hCG treated samples. The pixel densities of the bands were quantified with ImageJ software[National Institute of Health (NIH)] and have been represented in bar diagram as relative arbitrary units considering the control value as 1 (A). The expression of GAPDH was used as a loading control (B). The experiments were performed three times in duplicate, and the values are mean ± SEM, * p<0.05.

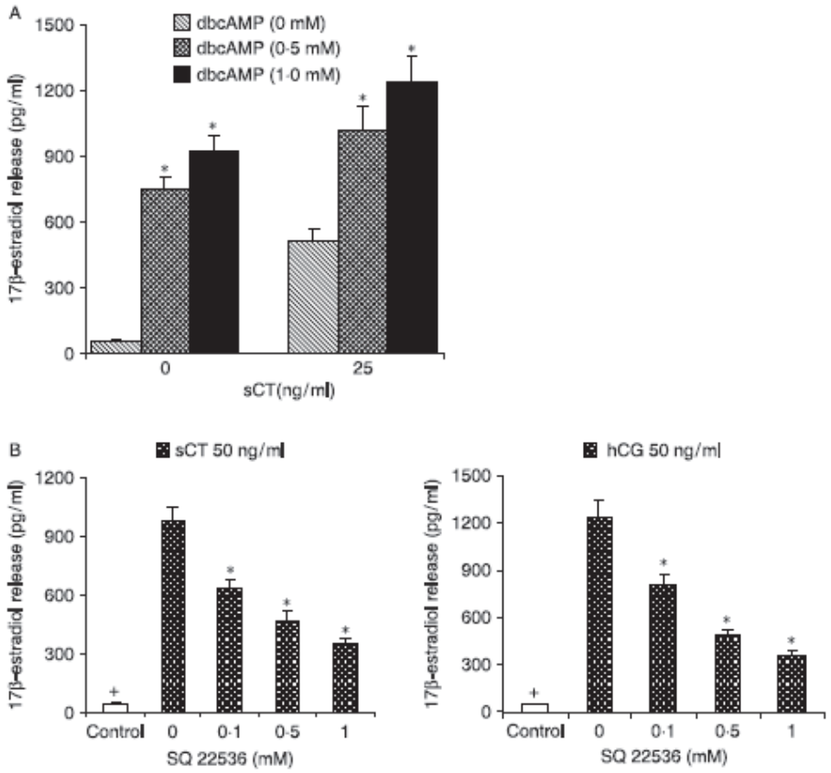

Fig. 14. Effects of sCT on *in vitro* production of 17β-estradiol by the vitellogenic ovarian follicles in absence (hatched bar) or presence of 0.5 mM (cross hatched bar) and 1.0 mM (solid bar) dbcAMP (A). Effects of SQ22536, a selective inhibitor of adenylate cyclase, on sCT- and hCG-stimulated *in vitro* 17β-estradiol production by the ovarian follicles (B). Ovarian follicles were incubated in the absence (control) or presence of sCT or hCG with increasing doses of SQ22536 for 12 h. Each value represents ± SEM of five incubations taking follicles in triplicate from five donor fish. (*p< 0.05 vs. sCT corresponding control group. *p<0.05 vs. hormone alone. +p<0.05 from those shown for tissues incubated with hormone alone).

5.2.7 Effects of PKC inhibitor on sCT- stimulated E_2 production

To ascertain the involvement of protein kinase C in the regulation of E_2 release by sCT, the effects of NPC-15437 dihydrochloride, a selective protein kinase C inhibitor, was examined. NPC-15437 at all concentration tested (0.1 mM to 1.0 mM) failed to attenuate sCT stimulated E_2 release in the medium (Fig. 15 A). PKC inhibitor, however attenuated hCG-stimulated E_2 release in a concentration-dependent manner (Fig 15 B; p<0.05).

5.2.8 Effects of sCT on 17, 20β-P production *in vitro*

Experiments were conducted to observe whether sCT is able to stimulate 17, 20β-P production *in vitro* in post-vitellogenic ovarian follicles of *C. carpio*. It appears from Fig. 16 A that sCT at increasing concentration during 24 h incubation was able to induce production of 17, 20β-P in a dose-dependent manner with a maximum at the dose 50 ng/ml. Higher doses over 50 ng/ml had no additive effect. hCG at increasing doses also produced 17, 20β-P almost in a dose-dependent manner with a maximum at the dose of 50 ng/ml (Fig. 16 B). In the next experiment, ovarian follicles were incubated with sCT (0-100 ng/ml) in presence

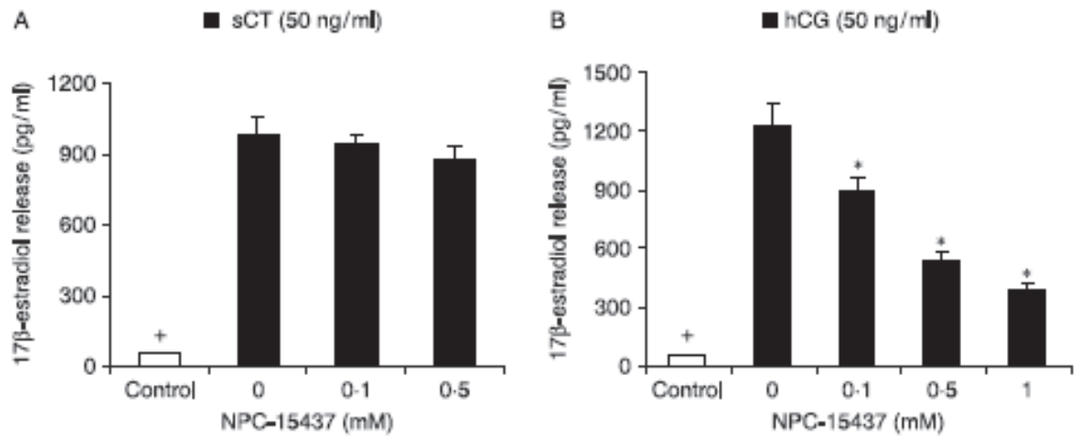

Fig. 15. Effects of NPC-15437 dihydrochloride, a selective inhibitor of PKC, on sCT- (A) and hCG-stimulated (B) *in vitro* production of 17β-estradiol by the ovarian follicles of *C. carpio*. Ovarian follicles were incubated in the absence (control) or presence of sCT or hCG with increasing doses of NPC-15437 dihydrochloride for 12 h. Each point represents mean ± SEM of five incubations, taking follicles in triplicate from five donor fish. (*p< 0.05 vs. hormone alone. +p<0.05 from those shown for tissues incubated with hormone alone).

of HCG (25 ng /ml) for 24 h and results depicted in Fig. 16 C shows that HCG-stimulated 17, 20β-P production was significantly increased (p<0.05) by sCT ranging from 25-100 ng dose/ml incubation. Ovarian follicles were incubated with sCT and hCG for various length of time up to 24 h. It appears from Fig. 17 that after addition of hormones, 17, 20β-P production increased steadily from 6 h and maximum was recorded at 16 h by sCT and hCG.

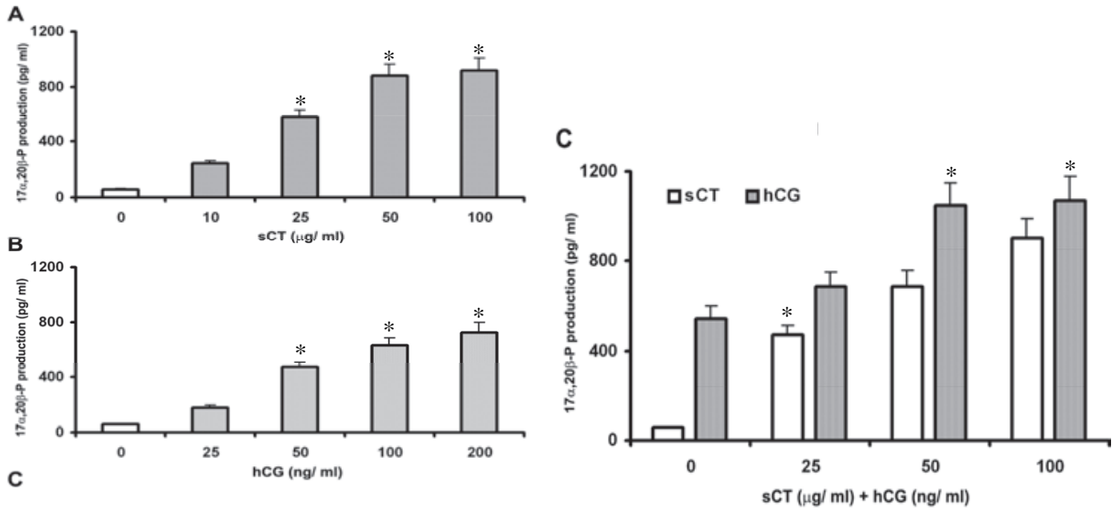

Fig. 16. Dose-response effects of sCT (A) and hCG (B) on *in vitro* production of 17, 20β-P by the post-vitellogenic follicles. Dose-response effects of sCT on basal (hollow bar) and hCG-stimulated (solid bar) *in vitro* production of 17, 20β-P by the follicles (C). All incubations were terminated 24 h after addition of test compounds. Each value represents mean ± SEM of five incubations, taking follicles in triplicate from five donor fish. (*p< 0.05 vs tissues incubated without hormone (0) (A and B), *p< 0.05 vs sCT at 0 ng /ml and *p<0.05 vs hCG at 0 ng /ml; C).

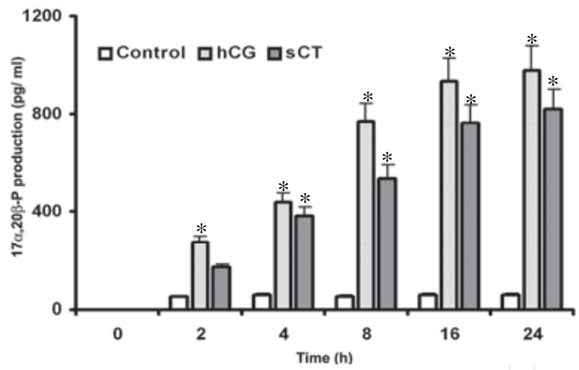

Fig. 17. Time-course effects of sCT and HCG (each at 50 ng/ml) on *in vitro* production of 17, 20β-P by the post-vitellogenic follicles. Each value represents ± SEM of five incubations taking follicles in triplicate from five donor fish. [*p< 0.05 vs tissues incubated without hormone (0)].

5.3 Effects of sCT on oocyte maturation

Experiments were also conducted to ascertain whether sCT can induce oocyte maturation in fish. For this, both intact follicles and denuded oocytes were incubated with test compounds. Increasing concentrations of either hCG (0-100 ng/ml) or sCT (10-100 ng /ml) for 24 h resulted induction of GVBD in intact follicles almost in a dose-response manner with maximum induction at the dose of 50 ng hCG/ml and 100 ng sCT/ml (Fig. 18 A, B). Higher doses had no additive effects in the induction of GVBD. Time-course effects of sCT and hCG on induction of GVBD shows that maximum induction of GVBD was recorded after 16 h after hormone treatments (Fig. 19). Interestingly, almost similar effects of sCT on GVBD induction was recorded in fully denuded oocyte with increasing concentrations of only sCT after 16 h incubation (Fig. 20). hCG had no effect on GVBD induction in fully denuded oocytes (data not shown).

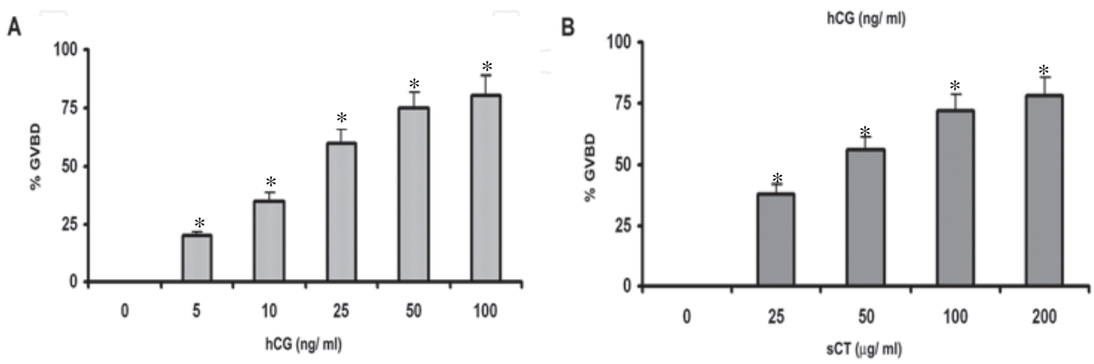

Fig. 18. *In vitro* oocyte maturation (GVBD) in post-vitellogenic follicles of *C. carpio* exposed to graded doses of hCG (A) and SCT (B) for 24 h. Each point represents mean of five incubations taking follicles from five donor fish. [Asterisks denote values significantly (p<0.05) different from those shown for tissue incubated without hormone (0)].

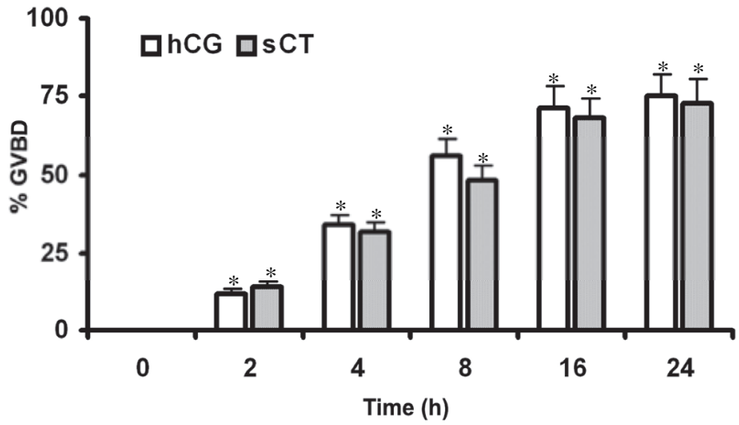

Fig. 19. Time-course effects of sCT (100 ng/ml) and hCG (50 ng/ml) on oocyte maturation in post-vitellogenic ovarian follicles. Each point represents the mean of five incubations taking follicles from five donor fish. [Asterisks denote values significantly (p<0.05) different from those shown for tissue incubated without hormone (0).]

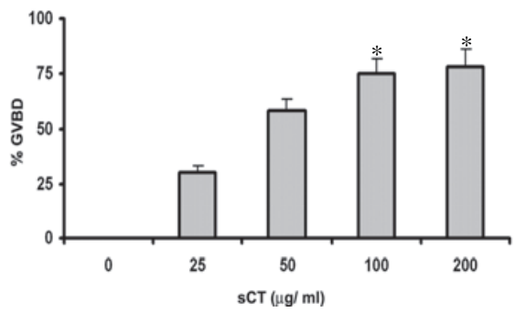

Fig. 20. Percent GVBD in denuded oocyte of post-vitellogenic follicles exposed to graded doses of sCT for 24 h. Each point represents the mean of five incubations taking follicles from five donor fish. [Asterisks denote values significantly (p<0.05) different from those shown for tissue incubation without hormone (0)].

6. Discussion

The present study demonstrate that administration of sCT and homologous UBG extract to sexually mature and immature *C. carpio* caused hypocalcemia within few hours. Salmon CT is capable of inhibiting both gill-Ca^{2+} transport (GCAT) and bone calcium resorption *in vivo* in this fish and the inhibitions were dose-, and time-related. We further described that carp ovarian follicles are equipped with CT receptors and demonstrated that administration of sCT to carp at vitellogenic stage significantly stimulated spontaneous and hCG-induced secretion of E_2 *in vivo* and *in vitro*. We also reported that sCT stimulated both aromatase activity and P450arom (CYP19A) gene expression and stimulatory action of sCT on ovarian E_2 secretion was mediated through cAMP pathway. In addition, sCT also stimulated 17, 20β-P (maturation inducing hormone of this fish) production *in vitro*. The most interesting aspect of this study was that sCT can induce carp oocyte maturation independent of MIH.

Calcitonin-induced short-duration reduction in plasma calcium levels in carp is in full agreement with earlier observations with some fresh water teleosts (Chan et al., 1968; Lopez et al., 1976; Wendelaar-Bonga, 1981; Chakraborti and Mukherjee 1993; Srivastava et al., 1998; Mukherjee et al., 2004a, b), marine bony fish (Glowacki et al., 1985) and elasmobranch *Dasyatis akajei* (Srivastava et al., 1998). Ultimobranchial gland of fish is a rich source of CT. In our present study we used crude extract of homologous UBG extract and observed good hypocalcemic potency. Since, the content of CT in UBG extract was not estimated; findings could only suggest effectiveness of native UBG extract of carp as a hypocalcemic factor and warrant further investigation on the nature and quantity of hypocalcemic factor present in UBG of this fish.

The present study demonstrates that sCT is capable of inhibiting gill Ca^{2+} transport (GCAT) *in vivo* in carp. This observation is in agreement with earlier studies on some other teleosts and also on carp (Wagner et al., 1997; Mukherjee et al., 2004 a). We, in our earlier studies and also in the present study observed that hypocalcemic effect of sCT is to be dependent on the levels of dissolved calcium in the surrounding water; in the sense that greater effects are observed if the fish are maintained in low-Ca water (Chakraborti and Mukherjee 1993; Mukherjee et al., 2004 a, b), findings that directly implicate the gills and changes in GCAT as an integral part of the response. Urist (1976) reported that teleosts have the ability to absorb most of the calcium from environment through gills. Salmon CT is reported to contribute to the calcium homeostasis in salmon and eel by preventing gill calcium influx (Milhaud et al., 1977; Milet et al., 1979). Wagner and his co-workers in 1997, considering whole body calcium uptake as a reliable indicator of GCAT, showed that sCT can inhibit GCAT in young rainbow trout. Our findings clearly indicate a dose- and time-related action of sCT in the inhibition of GCAT both in fingerlings and immature fish and this has been correlated with lowering of plasma calcium under identical situations. The dose and time required to produce maximum inhibition of GCAT as well as lowering plasma calcium levels in mature and immature fish was 0.5 µg/100 g body wt and 4 h respectively. In our study the estimated ED50 value for CT to evoke inhibition of GCAT and reduction of plasma calcium levels were ≈ 0.12 µg/100 g body wt). This value seems to be high as compared to the normal circulatory levels of CT in other teleosts studied (5 – 15 ng/ml) (Deftos et al., 1974; Sasayama et al., 1996). To get an exact idea of the pharmacological dose required for CT function in this fish, plasma CT value needs to be evaluated.

In the present study, tracer uptake rate in untreated fish has been found to be almost constant from 1 to 12 h indicating an efficient and steady-state maintenance of calcium homeostasis by the endogenous hormone (s), which may be either CT or STC or both. In CT-treated fish, a gradual inhibition of GCAT started from 1 h and achieved a maximum at 4 h irrespective of age and maturity. Such a short-duration inhibitory responses of this fish to sCT might be due to rapid metabolism of this hormone in fish blood. Similar short-duration GCAT inhibitory response of CT has been reported in young rainbow trout (Wagner et. al., 1997).

Calcitonin-induced tracer uptake in our study could not be attributed to changes in the intestinal absorption of ^{45}Ca, as fish were not fed during experiment and gut content of all the fish were excluded. Even externally bounded ^{45}Ca in fish was also removed. The present study therefore indicates that calcium measured in the fingerlings or sexually immature fish was the calcium uptake through gills. In our previous study (Chakraborti and Mukherjee 1993, Mukherjee et al., 2004 a) and also in the present study it has been shown that CT is

more hypocalcemic when kept in either normal tap water or low-Ca water, than fish kept in high-Ca water. The findings therefore indicate an anti-hypercalcemic role of CT in this fish and suggest that GCAT is an integral part of this response to CT. GCAT inhibitory response in this fish in the present study seemed to be independent of age, since both fingerlings and sexually immature fish showed similar response. It may therefore be concluded that salmon CT is a potent inhibitor of GCAT in fresh water teleost *C. carpio* adapted to both low-Ca water and normal tap water and fish adapted to both the calcium environment are equally responsive to calcium regulating hormone.

In the present study using sexually immature and mature female fish, we observed a significant dose- and time-dependent suppression of plasma tartrate-resistant acid phosphatase (TRACP) and alkaline phosphatase (ALP) activities and excretion of urinary hydroxyproline (HYP) after a single injection of varied doses of sCT. It is known that in mammals TRACP is secreted by the osteoclasts in to serum and blood TRACP activity measurement is often used as a marker of bone resorption (Raisz et al., 1998). Collagen degradation in bone releases HYP in to circulation. As bone resorption is by far the largest contributor of collagen breakdown, urinary HYP excretion has also been considered as a measure of bone resorption (Raisz et al., 1998). In mammals, CT exerts its control on serum calcium and phosphate by inhibiting bone resorption and thus decreasing the loss of calcium from bone (Raisz et al., 1998). In our previous study with a fresh water air-breathing teleost, *Channa punctatus*, we reported significant suppression of plasma TRACP and urinary HYP after sCT treatment and suggested that like mammals, in *C .punctatus,* CT exerts its hypocalmecic function through bone Ca resorption (Mukherjee et al., 2004 b) and this was the first report of the action of CT through bone calcium resorption in any fresh water teleost. Our present study using sexually immature and mature carp also demonstrated that CT inhibited bone osteoclastic activity. The suppression of TRACP activity and urinary HYP content by sCT in carp was noticed from 2 h with a maximum at 4 h after sCT injection. Even effective dose and time at which sCT started its suppressive effects on plasma TRACP activity and excretion of urinary HYP were the same at which it caused reduction of plasma calcium levels and inhibition of GCAT of this fish. From all these findings it is clear that CT-induced reduction in plasma calcium may in part due to inhibition of bone calcium resorption.

Total plasma ALP was measured to monitor the osteoblastic activity and is used as a marker of bone formation in mammals (Raisz et al., 1998). In sCT-injected carp we observed dose-, and time-dependent suppression of the activity of plasma ALP, indicating an action of CT on osteoblast in fish bone. From histological observation by Wandelaar-Bonga and Lamers (1982), it seemed that CT has a stimulatory effect on the growth of bones and scales of fish, even though CT exerts no action on calcium and phosphate concentrations in the skeletal bone. In our earlier study by observing suppression of plasma ALP activity simultaneously with reduction in plasma Ca concentration in *C. punctatus* we reported for a osteoblastic activity after CT treatment (Mukherjee et al., 2004 b). In the present study a similar suppression in plasma ALP activity in carp also indicate an action of CT on osteoblast of this fish. Furthermore, we recorded considerably higher calcium concentration in skeletal bone in CT-treated fish, and from our findings it can be assumed that a relationship between bone osteoblast activity and plasma calcium levels exist in this fish and that is altered in presence of CT.

An important question may emerge from our findings as to what could be relevance of CT-induced inhibition of bone resorption when there are evidences that fish regulate hypocalcemia through inhibition of GCAT (Milhaud et al., 1977; Wagner et al., 1997; Mukherjee et al., 2004 b)? It is true that the skeleton of fresh water teleosts have cellular bones (Mugiya and Watabe, 1997; Dacke 1979), which have the ability to remodel themselves. In the light of these findings it appears most likely that in fish there might be some scope to find out a regulatory mechanism in bone remodeling. Since CT in carp as well as in *C. punctatus* is an effective regulator of plasma calcium, its action on bone calcium resorption is not unlikely.

In the present study, we found that administration of sCT to carp during vitellogenic stage significantly stimulated spontaneous and hCG-induced secretion of E_2 both *in vivo* and *in vitro.* We described that ovarian follicles are equipped with CT receptors as evidenced from the specific binding of sCT to the membrane preparation. We also reported that sCT stimulated the activity of cytochrome P450 aromatase and P450arom gene expression in the ovarian follicles. Furthermore, we suggested that stimulatory action of sCT on ovarian E_2 secretion was mediated through cAMP pathway.

Evidence for a physiological role of CT during teleost sexual maturation has now been available. Histological and ultrastructural studies of ultimobranchial glands of a number of fish show that this gland is maximally active in sexually mature preovulatory females (Oguri 1973; Yamane 1977, 1978; Yamane and Yamada, 1977). Plasma CT levels in coho salmon, Japanese eel and rainbow trout are higher in females during spawning season and reached a peak just before ovulation (Deftos et. al. 1974; Yamauchi et. al., 1976, 1978; Bjorsson et al., 1986; Norberg et al., 1989). 17β-estradiol (E_2) increases plasma CT levels in rainbow trout (Bjorsson et al., 1986, 1989) and a direct induction of estrogen on CT secretion from ultimobranchial glands in goldfish has also been suggested (Suzuki et al., 2004). Possible explanation for the hyperactivity of ultimobranchial gland and the rise in plasma CT levels during peak reproductive season in female fish has been put forwarded by many workers (Brown and Bern 1989; Bjorsson et al., 1989; Suzuki et al., 2004), but none of them were able to suggest an exact relationship between CT and reproduction in fish.

The present study provide evidence that sCT is effective in increasing plasma E_2 levels in vitellogenic stage fish and stimulating both spontaneous and hCG-induced secretion of E_2 by the ovarian follicles *in vitro*. For our *in vivo* experiment, we used fish kept in high-Ca water. As shown previously, hypocalcemic effects of sCT was less in fish kept in high-Ca water than in normal water (Chakraborti and Mukherjee, 1993; Mukherjee et al., 2004 a, b). In the present study, after sCT injection to vitellogenic fish, plasma calcium levels altered a little but the release of plasma E_2 was significantly increased. We concluded that the increase of plasma E_2 was independent of Ca-decreased effect of sCT. Effects of sCT on increased plasma E_2 levels might be due to its action either on high pituitary GtH release or its direct action on ovarian follicles. Intravenous infusion of CT in human caused a calcium-independent reduction in thyrotropin and LH secretion in response to hypothalamic releasing hormone (Leicht et al., 1974). Inhibition of sCT on secretion of progesterone and GnRH-stimulated pituitary LH has also been reported (Tan et al., 1994). Moreover, receptors of CT-designated C1a and C1b receptors have been identified in rat brain (Sexton and Hilton, 1992; Albrandt et al., 1993). All these information indicates a physiological role of CT at pituitary and ovarian levels in mammals. However, no such information is available in fish. Indeed we, in our present study, did not observe the pituitary LH release after sCT injection to fish. Therefore, possibility of a CT-regulated GtH release by the pituitary of fish

can not be ruled out. In our *in vitro* observation as low as 25 ng sCT peptide per incubation is effective in stimulating spontaneous and hCG-induced release of E_2 by the vitellogenic ovarian follicles of fish. Therefore, the reason for sCT-induced rise in plasma E_2 levels is that sCT stimulated E_2 production by acting directly on ovarian follicular cells in fish.

Result of the present study shows that sCT can bind specifically to membrane preparation from ovarian follicles of carp indicating the presence of receptor molecules in the carp ovarian follicles which recognize sCT. Binding of CT with membrane receptors was found to be saturable with high affinity (*B*max, 1.2 pmol/mg protein, *K*d 48.8 pmol/L). Available information on the presence of CT-binding sites and sCT-induced inhibition of progesterone secretion in rat granulosa cells indicates a physiological role of CT at ovarian levels in mammals (Tsai et al., 1999). Our finding is the first report of the presence of sCT receptor in fish ovary apart from its presence in gills. Therefore, the presence of functional receptors for CT in the ovarian membrane preparation in vitellogenic follicles and sCT-induced *in vitro* production of E_2 by the ovarian follicles clearly indicate a functional link between binding and specific biological response.

In the present study, significant augmentation of aromatase activity both in hCG- and sCT-treated ovarian follicles is supported by a high rate of conversion of aromatizable androgen (testosterone to E_2) and enhanced synthesis and release of E_2 under stimulation of both the hormones. We also showed for the first time in teleost that sCT stimulated expression of P450arom gene in the ovarian follicles. It has been documented that the fish ovarian follicles possess aromatase enzyme participating in the conversion of aromatizable androgen to E_2 and P450arom mRNA levels are increased in association with the increase of enzyme activity under the stimulation of hCG (Gen et al., 2001; Kagawa et al., 2003, Paul et al., 2008, 2010).

It has been well established that in fish ovary, gonadotropin stimulates steroid production involving both PKA and PKC pathways (Nagahama, 1987; Srivastava and Van der Kraak, 1994). In our experiment, we found that in addition to lone effect of sCT on E_2 production, stimulatory effects of hCG on E_2 production *in vitro* was potentiated in presence of sCT in carp ovarian follicles. Results indicate that administration of dbcAMP stimulated sCT-induced E_2 production and cell permeable selective inhibitor of adenylate cyclase, SQ22536 attenuated both hCG- and sCT-induced E_2 production by the ovarian follicles in a concentration-dependent manner. Specific PKC inhibitor NPC-15437 dihydrochloride on the other hand had no inhibitory effects on sCT-stimulated E_2 release. We, therefore, suggest that signal for sCT-stimulated E_2 release might be transduced through cAMP pathway and interaction between sCT and hCG on the signal transduction in fish ovary is still open to elucidate. Increased production of cAMP caused by CT has been demonstrated in perfused rat bone, osteoblast like cell line, osteoclast, atria and aortic smooth muscle (Kubota et al., 1985; Sugimoto et al., 1986; Nicholson et al., 1987; Wang and Fiscus 1989; Iida-Klein 1992), and in rat testicular and anterior pituitary gland (Wang et al., 1994) substantiate the action of CT through cAMP path way in fish ovarian follicles.

Our present finding on the stimulatory role of sCT on basal and hCG-induced E_2 production by fish ovarian follicles is completely opposite to the observed action of CT in mammalian ovary and testes in the regulation of steroid production. Our unpublished data with ovarian follicles of perch *Anabas testudineus* also showed the same stimulatory action of sCT on E_2 production. Although the action of CT is well characterized in mammals, its action in fish, particularly with regard to calcium regulation is still controversial. Therefore, the observed stimulatory effect of CT on fish ovarian steroidogenesis, in contrast to mammals, is not

unusual. It is most likely that CT has evolved distinct function in different lineage, which probably relates to its aquatic life and this needs further studies on higher group of vertebrates. It is also not clear the exact physiological relevance of the stimulation of E_2 production by sCT in fish ovary when GtH-stimulated E_2 production is normally operative. Researchers have become increasingly aware that the traditional concept of the action of GtH in the regulation of ovarian growth, maturation and steroidogenesis may no longer be tenable. Localization of several neuropeptides in the nerve that innervate the ovary, neuropeptide Y, substance P (SP), vasoactive intestinal polypeptide (VIP) and somatostatin in the ovary of mammals have already been reported (Ojeda et al. 1985; Ahmed et. al., 1986; McDonald et al., 1987). Function of most of these peptide in the ovary although remain unknown but stimulatory effects of VIP on estrogen and progesterone release from cultured granulosa cells have been reported. Reports are also available that the stimulatory action of VIP appears to be exerted at least, in part, through a direct stimulatory action of neuropeptide on the synthesis of cholesterol-side chain cleavage enzyme (Trzecizk et al., 1986; 87). Recently, Clark et al., (2002) reported for the expression of CT gene in the ovary of a teleost, *Fugu rubripes* and suggested that CT may act as a potential neuropeptide. Considering all these, it would appear that CT in fish may take some role, at least in part, to support the action of GtH on the ovary during vitellogenic growth by acting independently or synergistically with GtH. This assumption supports the high plasma CT levels in fish during this phase of gonadal growth (Deftos et al., 1974; Yamauchi et al., 1978; Bjorsson et al., 1986).

The present study show that sCT is also capable of stimulating 17, 20β-P production in vitro in postvitellogenic ovarian follicles and this fish and stimulation is dose- and time-dependant. Results show that 25 ng sCT peptide per incubation is effective in stimulating spontaneous and hCG-induced 17, 20β-P production by the postvitellogenic ovarian follicles. 17, 20β-P is considered to be the maturation-inducing hormone in most teleosts including *C. carpio* and is released by the postvitellogenic ovarian follicles by the induction pituitary gonadotropin (GtH-II) immediately before oocyte maturation (Nagahama et al., 1987, Paul et al., 2010). Apart from many other intra-ovarian factors shown to increase 17, 20β-P production in the fish ovarian follicles (Maestro et al., 1997; Weber and Sulivan, 2000; Mukherjee et al., 2006; Chourasia et al., 2008), CT-induced production of 17, 20β-P in fish ovarian follicles in our study is a new finding and warrant further study.

Meiotic cell division in fish oocyte is arrested at G2/M border of cell cycle and reenters cell division in response to MIH produced in the follicular cells by the induction of gonadotropins (see review Nagahama et al., 1997; Mukherjee et al., 2006; Paul et al., 2010). Apart from GtH, many other intra-ovarian peptides including IGFs also have the potentiality to induce oocyte maturation in fish (Kagawa et al., 1994; Weber and Sullivan, 2000; 2001; Mukherjee et al., 2006; Paul et al., 2010). Incidence of CT-induced oocyte maturation in carp ovarian follicles in the present study suggest the function of new peptide involved in fish oocyte maturation, origin of which is not the ovary itself. Most interestingly, sCT-induced occurrence of GVBD in carp oocytes also observed in fully denuded oocytes indicating that the action of CT may be via a steroid-independent pathway. Similar steroid-independent pathway of the action of IGF-I in fish oocyte maturation has been reported earlier (Mukherjee et al., 2006; Paul et al., 2010). Involvement of CT in oocyte maturation is thus a new finding and deserves further study to unfold the real picture on this aspect. One interesting cellular action of CT involves the demonstration of its effects on the growth of

breast cancer. CT retards cell division in CT receptor infected HEK cells. It can affect cyclin-dependent kinase inhibitor, P21/WAF1/CIPI, which arrest cell cycle in G1 and G2 phases (Evdokion et al., 2000 and see review Zaidi et al., 2002). We do not know functional relevance of CT-induced oocyte maturation and its mechanism of action at the present moment and work is progressing to find out the mechanism of CT action in oocyte maturation in this fish.

7. Conclusion

In summary, the present findings suggest that salmon calcitonin would be an effective regulator of plasma calcium levels in fresh water teleost *C. carpio* adapted to water with different calcium concentrations, except a very high calcium level. Such effects of CT registered in *C. carpio* were shown to be dose- and time-dependent and may mediate at least in part, through inhibition of bone calcium resorption. Salmon CT is a potent inhibitor of GCAT in fresh water teleost adapted to both low-Ca and normal tap water. Fish were equally responsive to hormone at ages before attaining sexual maturity. From *in vivo* and *in vitro* study it may also suggest that sCT stimulates E_2 production in vitellogenic ovarian follicles and 17, 20β-P production in the post-vitellogenic ovarian follicle of *C. carpio* by acting directly on ovary, without altering plasma calcium level. Membrane preparation of ovarian follicles is equipped with CT receptor with high affinity. Salmon CT could stimulate both basal and hCG-stimulated E_2 and 17, 20β-P production. The stimulatory effect of sCT on E_2 production is associated with an increase in P450aromatase activities and P450arom gene expression in ovarian follicles. The signal transduction of the stimulatory effect of CT is mediated through cAMP pathway. The stimulatory effect of sCT on 17, 20β-P production is associated with oocyte maturation. Most interestingly, sCT-induced oocyte maturation also occurred independent of MIH action.

8. Acknowledgement

The authors are thankful for the supports from Council of Scientific and Industrial Research (CSIR), Govt. of India, New Delhi and from University of Kalyani, Kalyani, Nadia, India. There is no conflict of interest that would prejudice the impartiality of this work.

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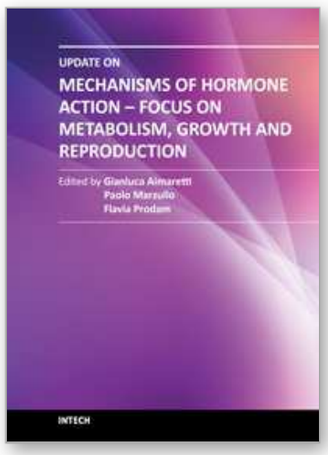

Update on Mechanisms of Hormone Action - Focus on Metabolism, Growth and Reproduction

Edited by Prof. Gianluca Aimaretti

ISBN 978-953-307-341-5
Hard cover, 470 pages
Publisher InTech
Published online 26, October, 2011
Published in print edition October, 2011

The purpose of the present volume is to focus on more recent aspects of the complex regulation of hormonal action, in particular in 3 different hot fields: metabolism, growth and reproduction. Modern approaches to the physiology and pathology of endocrine glands are based on cellular and molecular investigation of genes, peptide, hormones, protein cascade at different levels. In all of the chapters in the book all, or at least some, of these aspects are described in order to increase the endocrine knowledge.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Dilip Mukherjee, Sourav Kundu, Kousik Pramanick, Sudipta Paul and Buddhadev Mallick (2011). Calcitonin Functions Both as a Hypocalcemic Hormone and Stimulator of Steroid Production and Oocyte Maturation in Ovarian Follicles of Common Carp, Cyprinus carpio, Update on Mechanisms of Hormone Action - Focus on Metabolism, Growth and Reproduction, Prof. Gianluca Aimaretti (Ed.), ISBN: 978-953-307-341-5, InTech, Available from: http://www.intechopen.com/books/update-on-mechanisms-of-hormone-action-focus-on-metabolism-growth-and-reproduction/calcitonin-functions-both-as-a-hypocalcemic-hormone-and-stimulator-of-steroid-production-and-oocyte-

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