Journal of Aquaculture Research & Development

Prameswari et al., J Aquac Res Development 2017, 8:6
DOI: 10.4172/2155-9546.1000490

Research Article Open Access

Role of Arachidonic Acid and COX Inhibitors in the Regulation of Reproduction in Freshwater Crab *Oziothelphusa senex senex*

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Abstract

Induced reproduction of cultured species helps to produce quantity and quality seed an important component for upright yield in crustacean aquaculture. The present study was aimed to investigate the role of arachidonic acid in the regulation of ovarian development in the freshwater crab, *Oziothelphusa senex senex*. Injection of AA significantly ($p<0.001$) increased the ovarian index, oocyte diameter and ovarian vitellogenin levels. Injection of COX inhibitors such as indomethacin and aspirin alone, and in combination with AA resulted in significant ($p<0.001$) reduction in ovarian index, oocyte diameter and ovarian vitellogenin levels in crabs. The results of the present study provide evidence that arachidonic acid and COX inhibitors involved in the regulation of female reproduction in the freshwater crab, *Oziothelphusa senex senex*.

Keywords: Arachidonic acid; COX inhibitors; Ovarian development; Crab

Introduction

Arachidonic acid (AA) is a polyunsaturated fatty acid and is the precursor for eicosanoids. It is metabolized by various enzymes and produces eicosanoids through the cyclooxygenase (COX) pathway and produce prostaglandin G_2 (PGG_2) and H_2 (PGH_2). Subsequently, prostaglandin synthase converts PGH_2 to prostaglandins. Eicosanoids have an important role in the regulation of essential functions such as reproduction, haemostasis, growth, and the immune system in vertebrates. The occurrence of eicosanoids and their precursors have been identified in various invertebrates [1,2]. The presence of AA has been also identified in different crustaceans such as kuruma prawns, Penaeus japonicus and *Marsupenaeus japonicus* [3,4], green tiger prawn *Penaeus semisulcatus* [5], shrimps *Penaeus monodon* [6,7], *Litopenaeus vannamei* [8] and *Penaeus merguiensis* [9]. Furthermore, the presence of prostaglandins PGE_2 and $PGF_{2\alpha}$ were reported in *Procambarus paeninsulanus* [10], *Marsupenaeus japonicus* [4] and *Penaeus monodon* [11], whereas, only PGE_2 was reported in *Carcinus maenas* [12]. Along with PGE_2, and $PGF_{2\alpha}$, PGD_2 also has been identified in the rice field crab *Oziothelphusa senex senex* [13]. The presence of PGE_2 has been detected in the muscle and hemolymph of shrimp *Penaeus monodon* [14].

Similar to vertebrates, eicosanoids may also regulate the different physiological functions in invertebrates [15]. The physiological role of prostaglandins in the regulation of female crustacean reproduction was reported in few studies. Involvement of PG in ovarian maturation was studied with penaeid prawn *Metapenaeus affinis* [16], PGE_2 and $PGF_{2\alpha}$ in the regulation of vitellogenesis and the induction of ovulation were reported in *Procambarus paeninsulanus* [10]. In the same species, PGE_2 administration induced significant elevation of cAMP in ovarian tissue [17]. Later, similar results were reported in the prawn *Macrobrachium rosenbergii* and in the crayfish *Cherax quadricarinatus* [18]. Synthesis of PGE_2, $PGF_{2\alpha}$ and PGD_2 reported from isolated ovarian tissue of Oziothelphusa senex senex [13]. This study also reported that injection of PGE_2 and $PGF_{2\alpha}$ increased ovarian index and oocyte diameter in a dose-dependent manner and no changes were observed on ovarian growth with an injection of PGD_2 in *Oziothelphusa senex senex*. Recently, the variation of PGE_2 levels at different vitellogenesis stages were demonstrated in freshwater prawn, *Macrobrachium rosenbergii* and administration of PGE_2 stimulated the ovarian maturation, increasing ovarian somatic index, oocyte proliferation and vitellogenin (Vg) level in the hemolymph of prawn [19]. In contrast to these studies, it was reported that injection of PGE_2 0.1 µg/g body weight did not stimulate the ovarian maturation in *Penaeus esculentus* [20].

The non-steroidal anti-inflammatory drugs (NSAID) may inhibit the biosynthesis of prostaglandins. NSAIDs exhibits competitive inhibition with arachidonic acid the substrate of COX enzyme thereby affects PGs biosynthesis and their physiological functions [21]. The negative role of NSAIDs in the synthesis of PGs also reported in crustaceans. Long-term exposure of ibuprofen (IBU) a NSAID, induced a dose-dependent reduction of reproduction in *Daphnia magna* [22]. In another study, it was reported that ibuprofen primarily affecting oogenesis rather than embryogenesis in daphnids, by interrupting eicosanoid (prostaglandin) metabolism [23]. In contrast, IBU affected reproduction negatively in *Daphnia magna* and *Moina macrocopa* [24]. Recently, Alfaro Montoya [25] reported the significant induced ovarian maturation with IBU treatment to unilateral eyestalk ablated of female *Litopaneus stylirostris*, whereas it had no effect on sperm counts and spermatophore weights in male *Litopenaeus* species. Though there are sporadic reports on the biosynthesis of PGs and their functions in crustaceans, the direct role of AA and COX inhibitors on reproduction in crustaceans are fragmentary. In view of this, the present study was tested the effect of AA and COX inhibitors, indomethacin and aspirin in the regulation of ovarian maturation in freshwater rice field crab *Oziothelphusa senex senex*.

Materials and Methods

Collection and maintenance of crabs

Female rice field crabs with a body weight 30 ± 5 g (intermolt,

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Received May 09, 2017; **Accepted** July 10, 2017; **Published** July 12, 2017

Citation: Prameswari K, Hemalatha M, Kishori B, Sreenivasula Reddy P (2017) Role of Arachidonic Acid and COX Inhibitors in the Regulation of Reproduction in Freshwater Crab *Oziothelphusa senex senex*. J Aquac Res Development 8: 490. doi: 10.4172/2155-9546.1000490

stage C4), uninjured were collected from in and around the Tirupati in Andhra Pradesh, India. The crabs were acclimated to laboratory conditions (temperature: 25-28°C; light/dark 12:12 h) for 3 days before being used for the experiments. Crabs were fed daily with sheep meat *ad libitum*.

Chemicals

Arachidonic Acid (AA) (99% Pure), Indomethacin (IM) and Aspirin (Asp) purchased from the Cayman Chemical company (USA), were used as test chemicals.

Experimental design

Randomly 140 crabs were divided into seven groups and 20 crabs each. Group 1 served as control and the crabs were sacrificed on the first day of the experiment. Crabs in the group 2 served as concurrent control and was received injections of crustacean saline [26] through the arthrodial membrane of the coxa of the third pair of walking legs. Crabs in the group 3 received injections of 10 µg/10 µl of ethanol AA. Crabs in the groups 4 and 5 injected with at a dose of 10 µg in 10 µl of ethanol indomethacin and aspirin respectively. Crabs in groups 6 and 7 injected with AA (as in group 3), IM and Asp (as in groups 4 and 5) on the 1st, 7th, 14th and 21st day and the crabs were sacrificed on day 30. No deaths were recorded in the concurrent controls or in the experimental groups.

Ovarian index

The crabs were weighed and the ovarian tissues were dissected out, blotted with paper towels and weighed wet using electronic balance and the gonad index was determined by using the following formula.

$$Ovarian\ index = Wet\ mass\ of\ the\ ovary / Wet\ mass\ of\ the\ crab \times 100$$

Oocyte diameter

The diameters of twenty-five oocytes (µm) were measured from each freshly isolated ovary, using an ocular micrometer under the compound microscope. The average diameter of 25 oocytes in each ovary was considered as oocyte diameter.

Ovarian histology

The excised and clean ovaries were fixed in aqueous Bouin's fluid. After 24 h of fixation, the ovaries were dehydrated through an alcohol series, cleared in xylene and then embedded in paraffin (melting point 56-58°C). Serial sections (5 µm) were made and stained with haematoxylin and counter stained with eosin. The sections were photographed using phase contrast microscope.

Estimation of vitellogenin levels using ELISA

Vitellogenin was isolated from crab ovaries [27] and estimated the Vg content by Enzyme Linked Immuno Sorbent Assay (ELISA). Wells were coated with 20 µl of sample diluted in 1:10 ratio with coating buffer (carbonate buffer: 1.59 g sodium carbonate, 2.93 g sodium bicarbonate in 1000 ml distilled water, diethyl dithio carbonate (DIECA) 56 mg per 25 ml buffer). Blank values were obtained from wells coated with buffer alone. The plate is covered with a lid and placed in a humid chamber at 37°C for 2 h. After discarding the contents, the plate was washed five times with 0.1 M Phosphate Buffer Saline containing 0.05% Tween-20, pH 7.2 (PBST). Then, 200 µl of diluted (1:1000) primary antibody (antibodies raised in rabbits against crab vitellogenin isolated from vitellogenic stage III ovary from crab) in 0.1 M PBST with 2% polyvinyl pyrrolidine, 0.2% ovalbumin (PBST-PO) is added to each well. The plate is covered with a lid and placed in a humid chamber at 37°C for 2 h. The plate was washed 5 times with PBST after discarding the contents. After that 200 µL of horseradish peroxidase (HRP) conjugated anti IgG antibody (1:1000 dilution with PBST-PO; purchased from Genei, Bangalore) was added to each plate and kept in dark for 1 h at 37°C. The plate was washed 5 times with PBST and 200 µL tetra methyl benzidene (TMB in 0.015% hydrogen peroxide) was added to each well. The plate was kept in dark for 1 h at 37°C. The reaction was stopped by adding 50 µL of 1 M phosphoric acid per well. Absorbance of each well was measured using an ELISA-plates reader (Bio-Rad Lab., Model 680) at 450 nm. All standard and sample measurements were performed in duplicate.

Statistical analysis

The significance of differences between the means was determined by performing one-way ANOVA (Tukey: Compare all pairs of columns) were carried out using SPSS 16.0 version software. Statistical significance was considered at $p<0.001$.

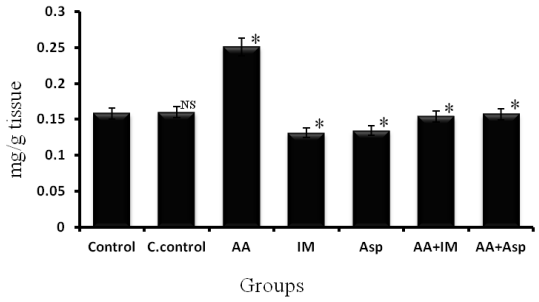
Results

The ovary of the initial controls is in immature stage with a mean ovarian index of 0.322 ± 0.024 and oocyte diameter of 26.65 ± 0.78. No change in the mean ovarian index (0.327 ± 0.021) and oocyte diameter (27.01 ± 0.63) were found in the concurrent control group (Table 1) and are similar to controls. Injection of AA significantly ($p<0.001$) increased the mean ovarian index (1.568 ± 0.11) and oocyte diameter (68.36 ± 1.19) with a maturation stage III (vitellogenic stage III) compared to control crabs (Table 1). Injection of COX-1 inhibitors indomethacin (IM) and aspirin (Asp) significantly ($p<0.001$) decreased the mean ovarian index (0.228 ± 0.023 and 0.231 ± 0.014 respectively) and mean oocyte diameter (22.12 ± 1.02 and 23.98 ± 1.06 respectively) compared to control crabs (Table 1). Co-injection of AA with either indomethacin or aspirin is also significantly decreased the ovarian index (0.226 ± 0.021 and 0.230 ± 0.024 respectively) and oocyte diameter (22.06 ± 1.01 and 23.66 ± 1.05 respectively) when compared with controls (Table 1). The ovarian vitellogenin levels of the initial control crabs were 0.158 ± 0.088 and similar (0.160 ± 0.055) in concurrent controls. The injection of Arachidonic acid significantly ($p<0.001$) increased the ovarian vitellogenin (0.251 ± 0.008) whereas

Groups	Ovarian index (g%)	Oocyte diameter (µm)	Color of the ovary
Control	0.322 ± 0.024	26.65 ± 0.78	White
Concurrent control	0.327 ^{NS} ± 0.021 (1.55)	27.01 ^{NS} ± 0.63 (1.35)	White
Arachidonic acid (AA)	1.568* ± 0.11 (386.95)	68.36* ± 1.19 (156.51)	Dark brown
Indomethacin (IM)	0.228* ± 0.023 (-29.19)	22.12* ± 1.02 (-16.69)	White
Aspirin (Asp)	0.231* ± 0.014 (-28.26)	23.98* ± 1.06 (-10.01)	White
AA+IM	0.226* ± 0.021 (-29.81)	22.06* ± 1.01 (-17.22)	White
AA+Asp	0.230* ± 0.024 (-28.57)	23.66* ± 1.05 (-11.21)	White
p value	<0.001	<0.001	
F value	2319.7	5882.4	

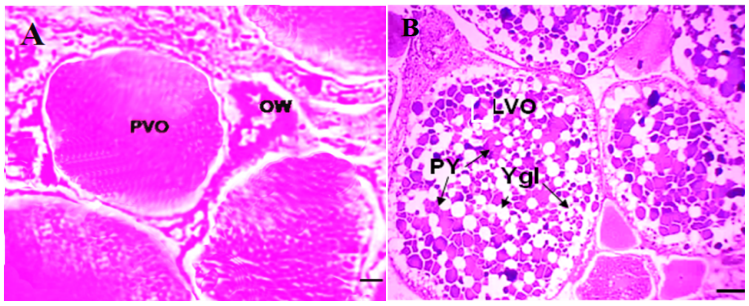
Values are mean ± S.D. of 20 individuals.
Values in parentheses are percent change from control.
Values with * are significantly different from controls; NS: Not Significant.

Table 1: Effect of arachidonic acid and COX-1 inhibitors, indomethacin and aspirin on ovarian in fresh water crab, *Oziothelphusa senex senex*.



Bars with mean ± S.D. of 20 individuals.
Bars with * are significantly changed from controls at p<0.001; NS: Not significant.

Figure 1: Effect of arachidonic acid (AA), COX-1 inhibitors-indomethacin (IM) and aspirin (Asp) on ovarian vitellogenin levels in freshwater crab, *Oziothelphusa senex senex*.



PVO: Pre-Vitellogenic Oocytes; OW: Thick Ovarian Wall; Ygl:Yolk Globules;
LVO: Late Vitellogenic Oocytes; PY: Protein Yolk;
Scale line=10 µm.

Figure 2: (A) Histological appearance of the immature stage ovary (control) and (B) Vitellogenic stage III ovary (arachidonic acid injected) of the freshwater crab *Oziothelphusa senex senex*.

injection of indomethacin or aspirin significantly (p<0.001) decreased the ovarian vitellogenin (0.131 ± 0.005 and 0.134 ± 0.006 respectively) (Figure 1). Co-injection of AA with either indomethacin or aspirin also significantly (p<0.001) decreased ovarian vitellogenin levels (0.154 ± 0.004 and 0.157 ± 0.002 respectively) compared to controls (Figure 1).

At the end of the experiment, only the ovaries of AA injected crabs were in vitellogenic stage III (dark brown in colour). Histological sections of vitellogenic stage III contain a large accumulation of yolk globules occupies in the whole oocyte (Figure 2A) against immature ovary contain a thick ovarian wall with a centrally located germanium surrounded by no of oocytes (Figure 2B).

Discussion

In crustaceans, the ovaries undergo a series of developmental changes in color and increase in size during the reproductive cycle. Measurement of the ovarian index, oocyte diameter and histological changes of the ovary were the best indicators of measuring the female reproductive status in crustaceans [28,29]. Vitellogenesis is the synthesis and ovarian accumulation of yolk lipoprotein vitellogenin, a major nutritive component of embryo development after fertilization [30,31]. The vitellogenin concentration in various tissues correlates with the yolk accumulation in the oocyte [32,33]. The measurement of vitellogenin levels at different stages in the hemolymph and the ovary is used as additional criteria to determine the ovarian maturation in recent past [34].

In the present study, injection of AA resulted in a significant increase in the ovarian index, oocyte diameter, and ovarian vitellogenin levels with vitellogenic stage III ovary with enormous deposition of yolk histologically indicating the involvement of AA on ovarian maturation in crab. Several investigators hypothesized that AA regulates female reproductive development in crustaceans and presence of AA have been identified in several crustaceans [11]. Injection of arachidonic acid significantly induces maturation, in shrimp to *Penaeus semisulcatus* at a dose of 5 µg/g body weight and 10 µg/g body weights [35]. Earlier it was reported that arachidonic acid supplementation through diet significantly promotes reproductive performance in tank domesticated *Penaeus monodon* [36], in *Daphnia magna* [37] and improved gonad somatic index, hepatosomatic index, egg clutch weight and fecundity in prawn, *Macrobrachium rosenbergii* [38]. In several fishes, also Arachidonic acid supplementation increases reproduction [39-42].

AA is a precursor of PGE_2 and PGF_2, the role of AA in shrimp maturation has been suggested as a precursor for prostaglandins [43]. Further, this was supported by several studies in different crustaceans where PGs, stimulates ovarian maturation in freshwater prawn *Metapenaeus affinis* [16], in crayfish [17], in freshwater crab, *Oziothelphusa senex senex* [13], in the shrimp *Penaeus monodon* [11] and in the freshwater giant prawn *Macrobrachium rosenbergii* [19]. Recently, it was demonstrated that administration of PGE_2 increases the vitellogenin levels in hemolymph of *Macrobrachium rosenbergii* [19]. Similar to earlier studies, in the present study, the regulation of reproduction in crab, *Oziothelphusa senex senex* by AA may connect to its role as a precursor to prostaglandins.

The prostanoid pathway converts polyunsaturated fatty acids into PGs by COX enzymes from substrate AA. The presence of COX pathway in crustaceans is also available [18,44]. To date, COX enzymes isolated, sequenced and characterized in two crustacean species, *Gammarus*

pulex and *Caprella mutica* [45]. COX has also been identified and sequenced in the shrimp *Penaeus monodon* [11]. Similar to mammals, NSAIDs affect the synthesis of PGs in invertebrates NSAIDs blocks the COX pathway [18,45], thereby the physiological roles of PGs in the present study the injection of COX inhibitors, indomethacin and aspirin individually and co-administration with AA resulted in a significant decrease in ovarian index, oocyte diameter and ovarian vitellogenin levels. There are no direct studies available to support the present results, but previous studies supported the COX-inhibiting role of non-steroidal anti-inflammatory drugs in crustaceans [21-25,19,46]. The exposure to IBU reduces the reproductive performance in *Daphina magna* and *Moina macrocopa* [24]. Similarly, the treatment of IBU (0.01 µg/g and 0.1 µg/g) reduced ovarian maturation in unilaterally eyestalk ablated female and male *Litopaneus* species. It is also reported that 0.1 µg/g of IBU significantly induced ovarian maturation in *Litopenaeus stylirostris*, but not in *Litopenaeus vannamei* whereas administration of IBU had no effect on sperm counts and spermatophore weights in *Litopenaeus* species [25]. COX inhibitor-indomethacin blocks the synthesis in *Macrobrachium rosenbergii* [47].

Conclusion

Administration of AA results in significant induction in ovarian development, whereas administration of COX inhibitors alone or in combination with AA inhibited the ovarian development in the crab. AA is deserved to act as a precursor induces synthesis of PGs. There by maturation takes place, on the other hand COX inhibitors blocks the PGs synthesis thus inhibits the maturation process. This study provides potential evidence that AA play a vital role in the regulation of reproduction. However, the regulatory mechanism of AA and COX inhibitors in crustaceans is yet to be known. Further emphasis is required to know the mechanistic action of arachidonic acid and PGs in ovarian development.

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