Effects of Aluminium on Vitellogenin and its mRNA Induction by Estradiol-17ß in the Primary Culture of Hepatocytes in the Rainbow Trout *Oncorhynchus mykiss*

Un-Gi Hwang, Jin-Il Park, Jung-Min Shim, Chang-Soo Jung and Sung-Yoon Park

East Sea Fisheries Research Institute, National Fisheries Research & Development Institute, Gangneung, Gangwon-do, 210-860, Korea

Effects of Al on vitellogenin (VTG) and VTG mRNA induction by estradiol-17 ß (E_2) were examined in primary hepatocyte culture of rainbow trout. Hepatocytes were precultured for 2 days and then E2 ($2x10^{-6}$ M) and Al (10^{-6}-10^{-4} M) were simultaneously added to the incubation medium. Hepatocytes were cultured for 5 more days. Media and hepatocytes were then analyzed by SDS-PAGE and Northern blotting for VTG and VTG mRNA, respectively. These metal had no appreciable effect on the viability of hepatocytes in culture. However, Al interfered with VTG production and VTG mRNA expression. Al reduced VTG production in a concentration-dependent way and a significant reduction accurred at Al concentrations greater than $5x10^{-5}$ M. VTG mRNA expression also decreased with a negative correlation with Al concentration (r=-0.98). These results suggest that Al inhibit VTG production at the transcriptional level to reduce VTG mRNA expression.

Key words : Al, VTG, VTG mRNA, Hepatocytes, Rainbow trout

1. Introduction

Metal contaminants in the aquatic environment may interfere with reproduction in fish. Al is one of these such metals that impaired vitellogenin (VTG) production. VTG, an egg yolk precursor protein, is synthesized in the liver in response to estrogen and is incorporated into eggs to be processed as yolk proteins in maturing females. Impaired oogenesis due to poor accumulation of egg yolk[1] and a reduction in egg number[2] have been reported in acidic waters, where Al reaches high concentrations of 3.7-34.8 um[3), 4)]. Mugiya and Tanahashi[5] showed directly that Al inhibited the induction of VTG synthesis in hepatocyte culture of rainbow trout. However, the intracellular mechanisms of inhibition are unknown.

Corresponding Author ; Un-Gi Hwang, East Sea Fisheries Research Institute, National Fisheries Research & Development Institute, Gangneung, Gangwon-do, 210-860, Korea
Phone : +82-33-660-8541
E-mail : ungi2222@yahoo.co.kr

The present study was undertaken to determine at which levels these metals interfere with the synthesis of VTG in rainbow trout. Hepatocytes were cultured with a combination of estradiol and Al or Cd, and VTG production and VTG mRNA expression were analyzed by electrophoresis and Northern blotting, respectively.

2. Experimental Methods

2.1 Materials

Rainbow trout, *Oncorhynchus mykiss*, weighing 110-350 g were obtained from a commercial dealer and kept in outdoor ponds with running water at about 14(°C). They were fed trout food pellets once a day but were starved on the last day before sampling to reduce the production of bile. Maturing females were not included.

2.2 Hepatocyte Preparation and Incubation

Hepatocyte were prepared following Hayashi and Ooshiro[6] as described by Kwon et al[7]. Cell

yield and viability were determined by the trypan blue exclusion test.

Cells were plated into a 60-mm plastic petri dish with a positive charge (Falcon) at a density 0.5-1x10^6 cells/dish. William's medium E (Life Technologies, Inc.) containing 0.2 uM bovine insulin (Sigma, St. Louis, MO), streptomycin (100 ug/ml), and penicillin (70 ug/ml) was used for cell culture. All incubations were carried out in 3 ml of the medium at 15(°C) under 5% CO_2. Preculture was conducted for 2 days before each experiment. The whole medium was changed every day throughout the preculture and experimental periods.

2.3 Treatment with Al

After a 2-day precultur, estradiol-17 ß (E_2, 2x10^{-6} M in 3ul of 95% ethanol) and Al ($AlCl^3$ in 3ul of redistilled water) was simultaneously added to the dishes. Final metal concentrations were 10^{-6}, 10^{-5}, 5x10^{-5}, and 10^{-4} M for Al. The effect of this metal on VTG production was examined after 5 days, during which time the media were changed daily. Control cultures received the equivalent amount of the solvents only. After cultre, the media and hepatocytes were analyzed for VTG production and VTG mRNA, respectively.

The effect of Al on cell viability was also examined using crystal violet as described by Mugiya and Tanahasi[5]. Cell viability was taken as the number of living cells on Day 5 expressed as a percentage of the number of living cells on Day 5 expressed as a percentage of the number of living cells just before metal addition.

2.4 SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis was done as described by Hwang[8]. Briefly, proteins were precipitated from the media by cold trichloroacetic acid, dissolved in sample buffer (0.175 M Tris-HCl, 8 M urea, 1% SDS, and 0.5% 2-mercaptoethanol, Ph 7.4), and subjected to 5-20% gradient SDS-PAGE. The gels were stained with 0.25% coomassie brilliant blue R-250 (CBB). It was difficult to apply a fixed amount of proteins to each lane, because the present experiment was intended to inhibit VTG (protein) synthesis

The identification of the VTG band was based on the results of a previous study[7] in which isolated rainbow trout hepatocytes incubated with E_2 synthesized a proteins with the same electrophoretic mobility (175kDa) as in the present study.

After SDS-PAGE, the intergrated optical density (IOD) of the VTG band was measured by a Bio Image System (Millipore, Bedford) and was expressed as a percentage of the IOD of the total protein including VTG. Minor subunits of VTG were not considered as VTG, because the subunits constituted only a fairly small part of VTG and overlapped with other proteins[7]. An excellent correlation (r= 0.99) was reported between the amount (100-1600 ng/ml) of VTG applied to the electrophoretic lanes and the IOD[9].

2.5 RNA Extraction

Total RNA was extracted from cultured hepatocytes using an RNA extraction kit, Isogen (Nippon Gene), according to the acid guanidinium thiocyanate phenol chloroform method[10]. RNA extracted was washed in 80% ethanol, lysed with water as a total RNA sample, and stored at -75(°C) for Northern blot analysis.

2.6 RT-PCR for VTG cDNA

Rainbow trout were injected ip with estradiol-17 ß (5mg/kg body weight) in 95% ethanol three times at 5-day intervals and total RNA (including VTG mRNA) was extracted from the liver, as described above, 7 days after the last injection.

The reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using an AMV RNA PCR kit (TaKaRa) by a DNA thermal cycler (Perkin-Elmer). Primers were chosen so as to include partial sequences of the rainbow trout VTG cDNA[11]. The upstream primer was a 20-mer sense oligonucleotide (5'-AGCAAACACAAAGTCAATGC-3') and the downstream primer was a 20-mer antisense oligonucleotide (5'-TCCACACTCTTCTCATAGAT-3'). Total RNA (lug) was reverse-transcribed at 96(°C) for 10 min and then PCR amplification were made for 35 cycles 92(°C) for 30s, 55(°C) for 30s, and 72(°C) for 30s). A 1196-bp RT-PCR product (Fig. 1) was analyzed by electrophoresis on a 2% agarose gel and confirmed to be identical to that reported for rainbow trout VTG cDNA[10] using a DNA sequencer (Model-373A, Applied

Biosystems).

2.7 Northern Blotting

Total RNA was quantified by UV spectrophotometry at 260 nm. Ten micrograms of each RNA sample was mixed with 10% 3-morpholinopropane sulfonic acid (Mops) buffer, 16.25% formaldehyde, and 50% formamide, and then was denatured at 65(°C) for 5 min. Northern blotting of the VTG mRNA was based on the results of a previous study[8]. The quantification of VTG mRNA expression by autoradiography using a Bio Image System (BAS2000; Fujix).

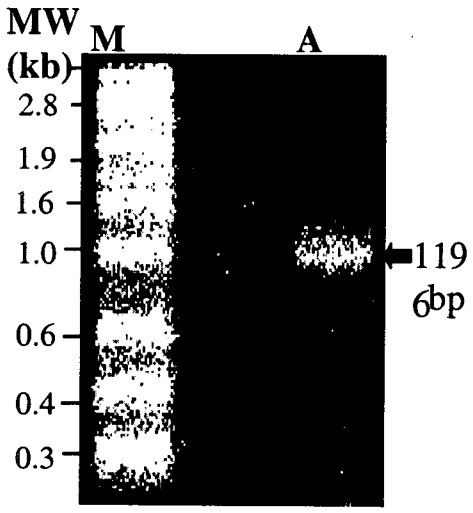

Fig. 1. Agarose gel electrophoresis of RT-PCR products amplified cDNA for VTG mRNA. M: λ Hind size markers; A: cDNA from liver of rainbow trout.

2.8 Statistical Analysis

Data were analyzed by one-way ANOVA (Fisher PLSD test). Fisher's *r*-test was also used to examine the significance of correlation coefficients. Significance was accepted at $P<0.05$. Percentage data were statistically analyzed after being arcsine transformed.

3. Results and Discussion

The collagenase perfusion method yielded about $3x10^8$ hepatocytes per fish. Cell viability was estimated to be over 90% by trypan blue staining. After being transferred to a positively charged dish, hepatocytes firmly attached to the dish in the serum-free medium and started to spread within 2 days of preculture. Although hepatocytes maintained good viability for at least 15 days, they spread rather slowly and formed a few aggregations. When expressed as a percentage of the number of living cells on Day 5 after addition of the metal to the number of living cells just before addition, Al had no appreciable toxic effect on cell viability showing survival rates of 90.5-79.1% regardless of the concentrations used

After a 2-day preculture, hepatocytes were incubated in the serum-free medium with E_2 and Al for 5 days and the spent medium was analyzed by SDS-PAGE. A newly synthesized protein band was detected at a molecular weight position of 175 kDa (Fig. 2).

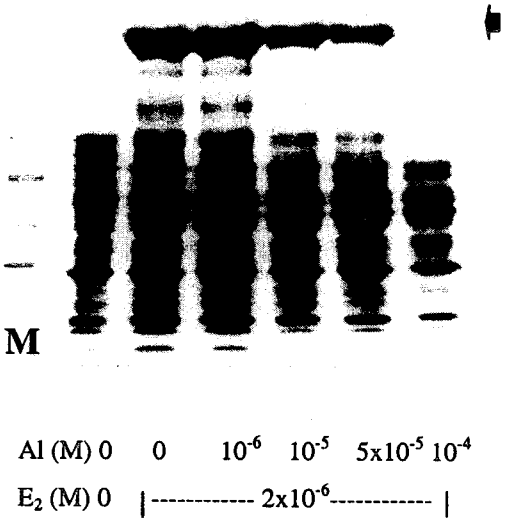

Fig. 2. SDS-PAGE showing the inhibition of VTG (arrowhead) production by Al in hepatocyte cultures with E_2 in the rainbow trout. Spent media were analyzed on Day 5 after Al addition. M: molecular weight (MW). CBB stain.

This band was identified as a main VTG band[7] on the basis of immunoblotting and immunoelectrophoresis. The control culture without E_2 did not produce an equivalent protein.

The addition of Al to the incubation medium

reduced the intensity of CBB staining for the VTG band in a concentration-dependent fashion (Fig. 2). Protein bands other than VTG were less affect by Al. In the present study, SDS-PAGE was used to separate VTG, before potical quantification. VTG production in the presence and absence of metals was expressed as a percentage of total proteins. This type of expression has the benefit of excluding the effects of amount of proteins applied to the gel. It also has the advantage of checking the effects of metals on the production of proteins other than VTG..

The relative amount of VTG was determined by IOD. VTG accounted for 10.3% of the total proteins in the control culture, but this value decreased with increasing Al concentrations (Fig. 3).

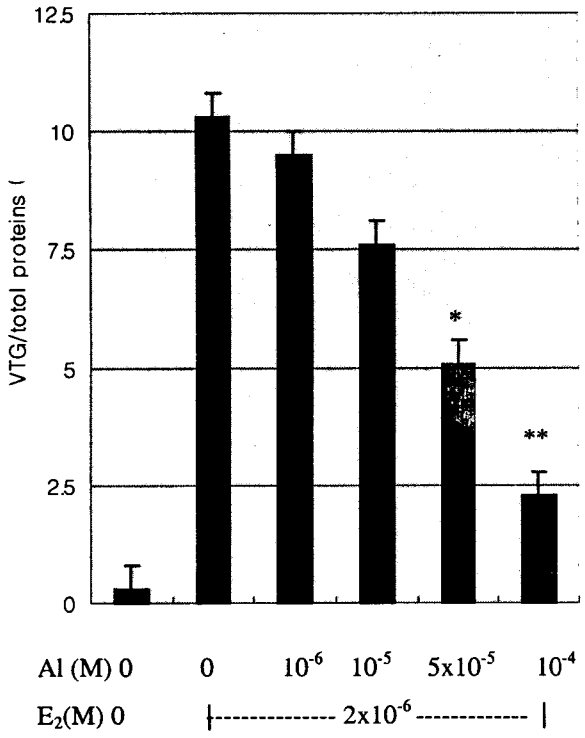

Fig. 3. Concentration-dependent inhibition of VTG production by Al in hepatocyte cultures with E2 in rainbow trout. The activity of VTG production was estimated as a percentage of VTG to total proteins after SDS-PAGE on Day 5 after Al addition. Vertical bars represent the SE of the mean for three individuals. *P<0.05 and **P<0.01 for control (E_2+0 M Al).

Significant inhibition was confirmed at Al for control (concentrations of $5x10^{-5}$ and 10^{-4}, in which the VTG production was reduced to 48% (P<0.05) and 20% (P<0.01) of the control, respectively. A significant decrease in the percentage means that the synthesis of VTG is more susceptible to metals than are other hepatocyte-derived proteins.

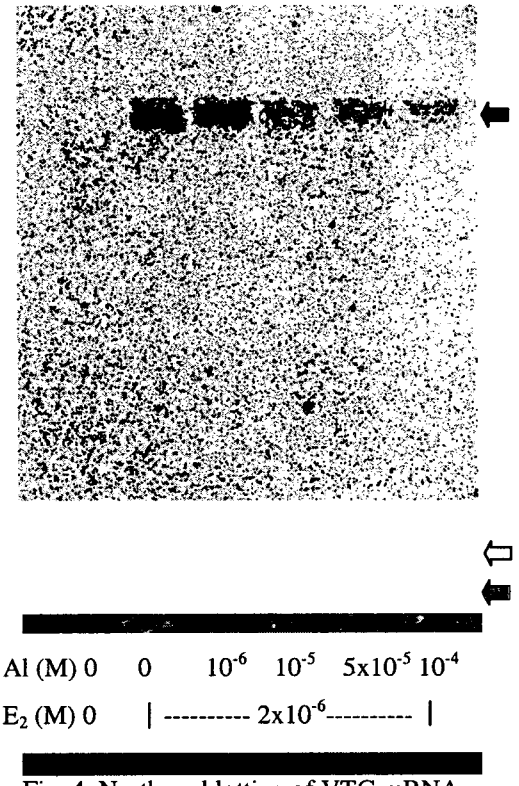

Fig. 4. Northern blotting of VTG mRNA. (A) The inhibition of VTG mRNA (arrowhead) expression by Al in hepatocyte cultures with E_2 in rainbow trout. Hepatocytes were extracted for total RNA on Day 5 after Al addition. (B) .and 18 S (arrowhead).

Al is nonessential biological elements and therefore fish may have no regulatory mechanisms for them. Al and Cd are known to be toxic to fish, interfering with ion regulation[12] and respiratory function[13] in the gill. Fish reproduction was also

impaired by this metal[14]. Al reaches high levels in acidic waters and aggravated unfavorable effects of low pH on reproduction. For example, the spawning of perch was delay in acidic lakes with high Al levels[14].

After a 5-day culture with E_2 and Al, hepatocytes were analyzed by Northern blotting. VTG mRNA bands were detected at about 6.6kb in cultures with E_2, while the culture without E_2 did not induce an equivalent band (Fig. 4).

Al reduced the intensity of the VTG mRNA bands in a concentration-dependent way (Fig. 4). The level of VTG mRNA expression was quantified by autoradiography (Fig. 5). VTG mRNA expression decreased with increasing Al concentration (r= -0.98, P<0.01).

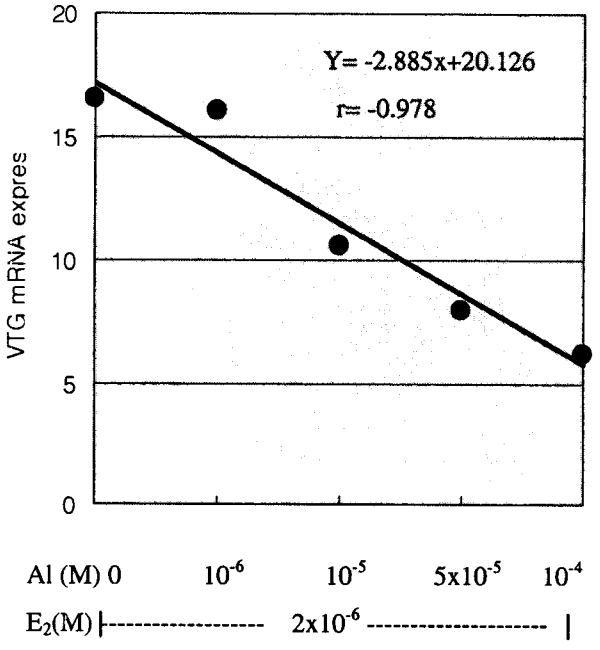

Fig. 5. Concentration-dependent inhibition of VTG mRNA expression by Al in hepatocyte cultures with E_2 in rainbow trout. The activity of VTG mRNA expression was estimated on Day 5 after Al addition by autoradiography of Northern blotting. The regression was significant (P<0.05).

4. Conclusions

This metal had no appreciable effect on the viability of hepatocytes in culture. However, Al interfered with VTG production and VTG mRNA expression. Al reduced VTG production in a concentration-dependent way and a significant reduction accourred at Al concentrations greater than $5x10^{-5}$ M. VTG mRNA expression also decreased with a negative correlation with Al concentration (r=-0.98). These results suggest that Al inhibit VTG production at the transcriptional level to reduce VTG mRNA expression.

References

1) Lee, R.M. and S.D. Gerking, 1980, Survival and reproductive performance of the desert pupfish, *Cyprinodon n. nevadensis* (Eigenmann and Eigenmann), in acid water, J. Fish Biol., 71, 507-515

2) Runn, P., N. Johansson and G. Milbrink, 1977, Some effects of low pH on the hatchability of eggs of perch, *Perca fluviatilis L.* Zoon, 5, 115-125.

3) Brumbaugh, W.G. and D.A. Kane, 1985, Acidification and toxic.ity of metals to aquatic biota, Can. J. Fish. Aquat. Sci. 42, 2034-2049.

4) Borg, H., 1986, Metal speciation in acidified mountain streams in central Sweden, Water Air Soil Pollut., 30, 1007-1014.

5) Mugiya, Y. and A. Tanahashi, 1998, Inhibitory effects of aluminium on vitellogenin induction by estradiol-17 ß in the primary culture of hepatocytes in the rainbow trout Oncorhynchus mykiss, Gen. Comp. Endocrinol., 109, 37-43.

6) Hayashi, S. and Z. Ooshiro, 1975, Gluconeogenesis and glycolysis in isolated perfused liver of the eel, Bull. Jpn. Soc. Sci. Fish., 41, 201-1988.

7) Kwon, H.C., S. Hayashi, S. and Y. Mugiya, 1993, Vitellogenin induction by estradiol-17 ß in primary hepatocyte culture in the rainbow trout, *Oncorhynchus mykiss*, Comp. Biochem. Physiol., B 104, 381-386.

8) Yeo, I.Y. and Y. Mugiya, 1997, Effects of extracellular calcium concentrations and calcium antagonists on vitellogenin induction by estradiol-17 ß in primary hepatocyte culture in the rainbow trout *Oncorhynchus mykiss.*, Gen. Comp. Endocrinol., 105, 294-301.

9) Hwang, U.G., 2001, Vitellogenin and its mRNA

induction by estradiol-17 ß in the primary culture of hepatocytes in the rainbow trout, *Oncorhynchus mykiss*, J. Fish. Sci. Tech., 4(4), 186-191.
10) Chomczynski, P. and N. Sacchi, 1987, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem., 162, 156-159.
11) Le Guellec, K., K. Lawless, Y. Valotaire, M. Kress and M. Tenniswood, 1998, Vitellogenin gene expression in male rainbow trout (Salmo gairdnerii), Gen. Comp. Endocrinol., 71, 359-371.
12) Booth, C.E., D.G. McDonald, B.P. Simons and C.M. Wood, 1988, Effects of aluminium and low pH on net ion fluxes and ion balance in the brook trout, *Salvelinus fontinalis*, Can. J. Fish. Aquant. Sci., 45, 1563-1574.
13) Wood, C.M., R.C. Playle and D.G., McDonald, 1988, Blood gases, acid-base status, ions, and hematology in adult brook trout, *Salvelinus fontinalis*, under acid/aluminum exposure, Can. J. Fish. Aquat. Sci., 45, 1575-1586.
14) Rask, M. and J. A. McCarter, 1990, Delayed spawning of perch, *Perca fluviatilis* L., in acidified lakes. J. Fish. Biol., 36, 317-325.