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Effect of caffeine, norfloxacin and nimesulide on heartbeat and VEGF expression of zebrafish larvae

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Abstract

The use of pharmaceuticals during pregnancy may causes abnormalities to the embryo. Sometime the drug also effect to the new born if the drug transferred through lactation. We have used zebrafish model to see the effect of some pharmaceuticals on embryos and larvae. Three drugs, caffeine, norfloxacin and nimesulide, were used for this study to see the effect mainly the hatching rate of eggs, heart beat rate and the vascular endothelial growth factor (VEGF) expression of the larvae. VEGF is an important signaling protein that involved generating the new blood vessels during embryonic development. We have used 10, 20, 50, 100 µg ml^{-1} concentrations of all the drugs to see the effect. No significant mortality or malformations were observed in zebrafish embryos. Hatching was stared from 60 hr. In control group, 91% hatching rate was observed. Lowest hatching rate was observed using highest concentration of norfloxacin (100 µg ml^{-1}) and nimesulide (100 µg ml^{-1}) *i.e.* 55 and 56% respectively. In control group, 110 to 115 heart beat rate was counted per minute. Significantly higher heart beat was observed in caffeine treated group which is 125 to140 min^{-1}. Lower heart beat was noted in nimesulide treated group which is 100 min^{-1}. We have tried to observe the possible effect of VEGF of the larvae by these three drugs. Expression of VEGF was very low in caffeine treated group. Almost no VGF expression was observe in 100 µg ml^{-1} caffeine treated group. These studies suggest that there is a possibility that high dosage of caffeine can harm the unborn baby or new born babies, if the mothers use caffeine.

Key words

Caffeine, Norfloxacin, Nimesulide, Heart beat rate, VEGF, Zebrafish

Introduction

The use of pharmaceuticals during pregnancy some time causes abnormalities to the embryo. The drugs use during pregnancy may harm the fetus. Sometime, drugs may be the reason for birth defects, like cleft lip or physiological harms. If the drug is taken during the developmental phase of the fetus, affects the fetus (Rubin, 1998; Hanretty and Whittle, 1995). Not only that, if the mother use the drug during lactation, dugs can transfer through the milk and can also affect the new born.

Caffeine present naturally in certain plant. It can also be produced synthetically. It is also used as an additive of the food products. Caffeine is also most commonly used medicine indicated for headache and other pain relievers. It is sometime prescribed to pregnant woman and it is reported that caffeine increased level of homocysteine of pregnant women that can cause problems to the fetus as well as the mother (Carlsen *et al.,* 2005). A cup of coffee content near about 70 to 80 mg caffeine, however it varies according to different parameters like cup size etc. Usually, coffee prepared by

instant contains less caffeine (near about 60 to 65 mg) and coffee prepared by drip methods contains more amount caffeine (near about 100 to 110 mg). Norfloxacin, a fluoroquinolone antibiotic, is used to treat certain infections to the woman caused by bacteria, such urinary tract infections; which is frequently prescribed by doctors to pregnant woman (Akerele *et al*., 2001). Nimesulide is a non-steroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic properties, which is also prescribed by doctors to the pregnant woman in some geographic location like India, China. However, it is banned in many countries due to cases of jaundice and hepatitis induced by its use (Thawani *et al*., 2003). Vascular endothelial growth factor (VEGF) is an important protein which plays a key role for the development of the blood vessels of the heart and also promotes development of endothelial muscle (Carmeliet, 2000).

Presently, the zebrafish embryos and larvae is one of the most extensively used tools for investigating the negative impact (Kristensen, 1995; Luckenbach *et al*., 2001) as well as disease (Hallare *et al.,* 2004; Hsu *et al*., 2007; Chakraborty *et al*., 2009). As they are very sensitive, several researchers consider embryo or larval stage to use during experiment. Zebrafish, *Danio rerio*, is a small, freshwater, aquarium species which is easy to grow and can maintain in different environments. It has a short generation time, breeds almost all of the year round and the developmental reports are also available (Hisaoka and Battle, 1958; Westerfield, 1995). So, the species provide as an excellent model for studying effect of drugs. Zebrafish appeared to be very suitable for assessing effects of pharmaceuticals. As the developmental stages are transparent; so, effects could be observed clearly and distinctly. Some other reports are also available on the effects of drugs or chemical compound on zebrafish embryos (Holmberg *et al*., 2006; Bello *et al.,* 2004; Brion *et al.,* 2004).

The purpose of the present study was to investigate the effects of caffeine, norfloxacin and nimesulide on heart beat rates and VEGF expression during the developmental stage like zebrafish larval stage and also observe the effects of these three drugs on hatching rate of zebrafish embryos.

Materials and Methods

Adult zebrafish, purchased locally, were about 4-6 months old and 3 cm in length and were kept in 5-10 lit glass aquariums with continuous re-circulating system. Additional oxygen was provided by placing air-stones in the water and 1/3 of water was replaced weekly. Aquariums water temperature was maintained at 28.5-32°C and a constant 14 10-hr^{-1} light $dark^{-1}$ cycle. Zebrafish laid their eggs by the natural spawning in the aquarium. After natural fertilization in the aquarium, the fertilized eggs were collected for the experiment through the standard method (Westerfield,1995).

Caffeine (200 mg), norfloxacin (400 mg) and nimesulide (400 mg) tablets was purchased form the local pharmaceutical stores. The tablets ware grinded and make powder. Then directly used into water. The solutions of all the drugs were prepared in distilled water to obtain various dilutions (10, 20, 50, 100 µg ml^{-1}).We have wormed on a water bath to accelerate the dissolution process as describe previously (Maheshwari *et al.,* 2006). The pH of the solution water was in between 7 to 7.5.

Effect of caffeine, norfloxacin and nimesulide on hatching rate of eggs: The eggs were collected and rinsed several times with double distilled water. We have transferred the eggs various petridishes where the eggs has exposed to the drugs with water. At around 4-6 hr postfertilization, only the fertilized eggs were selected and transferred to the petridishes (20 $plate^{-1}$) containing different concentrations (10,20,50,100 µg ml^{-1}) of caffeine, norfloxacin and nimesulide of at 32°C. The entire experiment was conducted in twice to verify results with a total of 100 eggs for each treatment group. We have chosen the four concentrations of the drugs (10, 20, 50, 100 µg ml^{-1}) as reported previously (Siu *et al.,* 2002).

Effect of heartbeat rates of larvae: Larval group with 48 to 72 hr age was used for this study. In all treatment groups, larvae were exposed into the drugs near about 10 hr, then the heart rate was observed. The effect of caffeine, norfloxacin and nimesulide on heartbeat of the larvae was noted after treating different concentrations like 10, 20, 50, 100 µg ml^{-1}. We have placed the petridishes under a stereomicroscope connected with a computer and camera device to count the heartbeat. Then we have counted the heartbeat rates per minute using a stop watch (visually determined as the average for 10 embryos, counted for 1 min under a temperature-controlled stage) (Zhu *et al.,* 2007).

Measurement of VEGF expression level: Expression level of VEGF from all treatment groups was measured using western blotting followed by SDSPAGE. Larvae from all traded group were collected and frozen in liquid nitrogen and stored at - 80°C for further analysis. The frozen embryos (10 embryos pooled for one sample) were homogenized for 5 sec extraction buffer (pH 7.0). The homogenized samples were then centrifuged (10 min in 20,000 g at 4°C) and protein were loaded (10 µl of protein per lane) were loaded on SDSPAGE (10% acrylamide, 90 min at 120 V). Protein was transferred to nitrocellulose by semi-dry blotting, and the filter was blocked for 2 hr in 50% horse serum in tris [hydroxymethyl] amino methane (Tris)-bufferered saline (TBS) (50 mM Tris pH 5.7, 150 mM NaCl). After washing in TBS, an anti-(His-tag) monoclonal antibody was used as the primary antibody and signals were detected, following the addition of the secondary antibody, with the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) according to the manufacturer's instructions (Chakraborty *et al.,* 2006). All chemicals were purchased from Sigma.

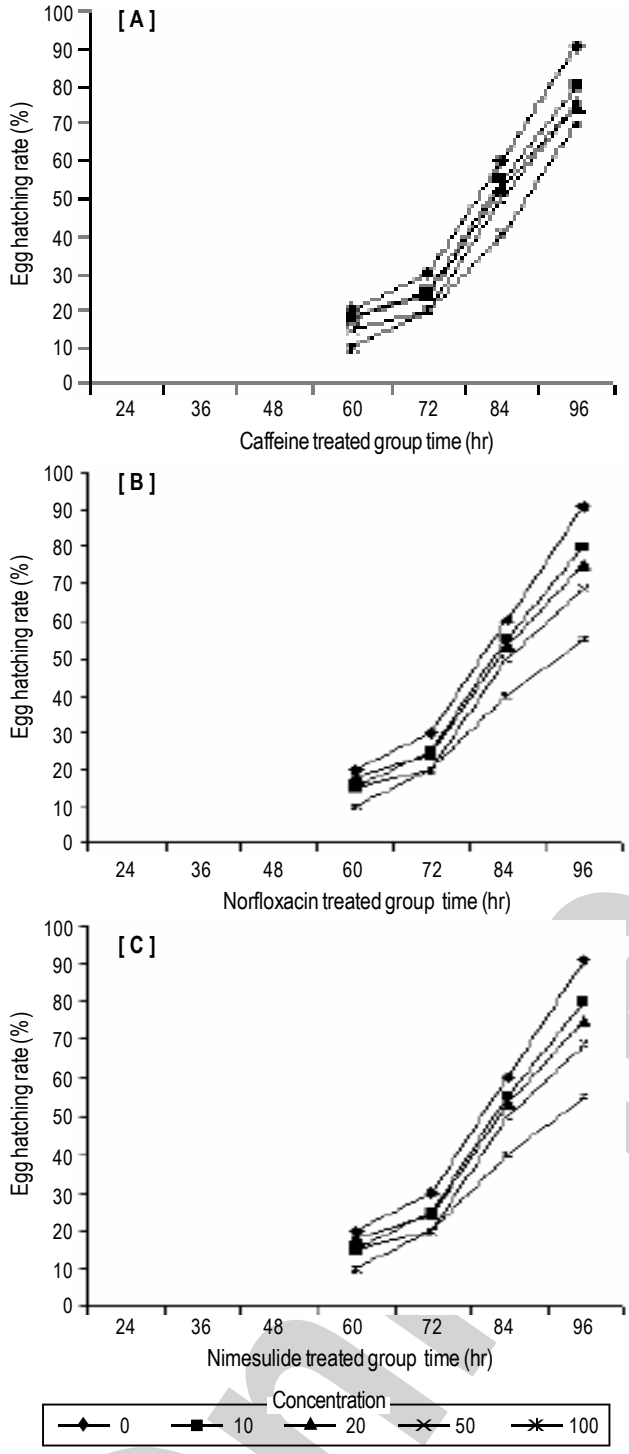

Fig. 1: Egg hatching rate of zebrafish treated with the three drugs. (A) caffeine treated group (B) norfloxacin treated group and (C) nimesulide treated group

Statistical analysis: Treatment effects on the hatching rate and heartbeat rates were determined using one-way analysis of variance (ANOVA). Significant difference occurs for a given parameter when $p<0.05$. The entire statistical analysis was carried out using Mathematica 5.0 (statistical software).

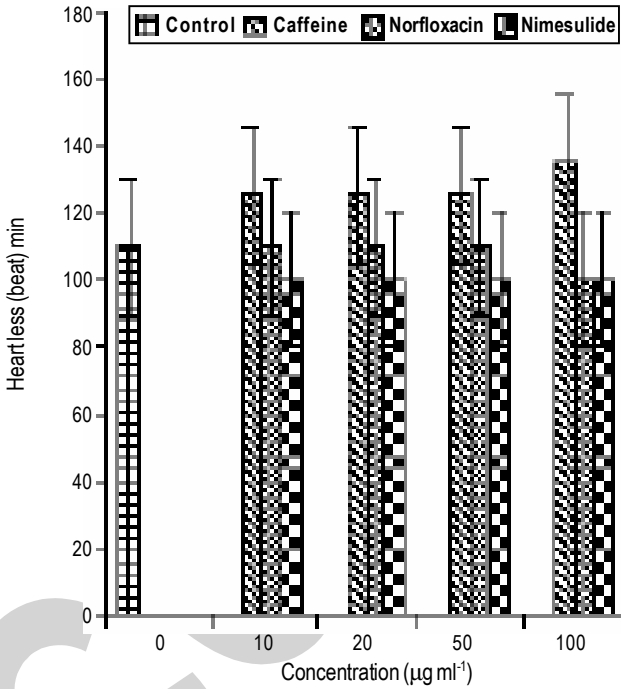

Fig. 2: Heart beat rate of zebrafish larvae treated with three drugs

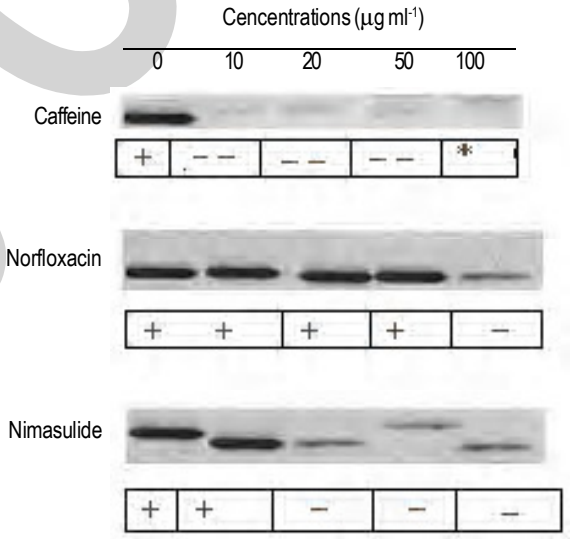

Fig. 3: Western blotting with VEGF antibody from the larval protein showed VEGF expressed very low in caffeine treated group. '+' means normal expression of VEGF; '-' means low expression of VEGF; '- -' means very low expression of VEGF; '*' means no expression of VEGF

Results and Discussion

Hatching rate of eggs: Hatching was occurred during 60 to 96 hr. No significant mortality or malformations were observed in zebra fish embryos exposed to the different treatment groups. In control group, 91% hatching rate was observed. Lowest hatching rates were observed of norfloxacin and nimesulide groups treated with in 100 μg ml^{-1} concentration which was 55 and 56% respectively. Hatching rate was decrease in all groups with the increasing amount of concentration for all three drugs (Fig. 1).

Heart beat rates: In control larval group, 110 to 115 heart beat rate min^{-1} was noted. Significantly higher hear beat was observed in caffeine treated group which was 125 to 140 min^{-1}. On the other hand, lower hear beat was noted in nimesulide treated group which was 100 min^{-1} (Fig. 2).

VEGF expression level: In control larval group, proper expression of VGF was observed. Expression level was very low in caffeine treated group. No VGF expression was observed in 100 µg ml^{-1} caffeine treated group. In nimesulide treated group, lesser amount VGF expression was observed in 10, 20 and 50 µg ml^{-1} concentration. In norfloxacin treated group, no variation of expression was observed; however, in 100 µg ml^{-1} concentration norfloxacin treated group, the expression level of VEGF was low. It is very prominent that VEGF expression was very low in 100 µg ml^{-1} concentration in all treated groups (Fig. 3).

Our data clearly indicate that low concentration did not cause any detrimental effects on the early life stages of zebrafish and also hatching rates. However, higher concentration (100 µg ml^{-1}) can effect on hatching rate also. The over-all hatching success rates did not differ significantly among the different exposure groups. Furthermore, no differences were observed in either mortality or incidence of malformations between the treated and control embryos. The concentration threshold did not effect morphologically to the embryo. So, the question of why zebrafish embryos showed concentration threshold that did not effect morphologically to the embryo needed to be satisfied. As stated earlier, the concentrations used in the present investigation were not sufficient to cause morphological or developmental effects. There is a possibility that they can tolerate this amount of concentration. They might develop natural tolerance capacity of toxicity from the natural systems (Kristensen, 1995; Luckenbach *et al*., 2001). The present study with caffeine suggested that, the expression of VEGF was low due to the effect of caffeine and that may cause the high heart beat rate. But, we are unable to describe the low heart beat rate in nimesulide treated group. However, conclusion can be made that zebrafish is a tropical water fish species and thus have a much faster embryonic development, which is also observed by some other investigator (Chou and Dietrich, 1999).

In human, the concentration of drug reaches into the serum level varies which is depending on the types of the drugs and the dosage. In these cases, three dugs, the concentration range varies from 10 to 100 µg. We have chosen the concentrations like 10, 20, 50, 100 µg ml^{-1} because Siu *et al.* (2002) reported that naproxen, a non-steroidal anti-inflammatory drug, detected from a pregnant woman serum at a concentration of 70 µg ml^{-1}. In this point of view, our experiment concentrations are very relevant. To the best of our knowledge, this is the first study that demonstrating the effects of caffeine, norfloxacin and nimesulide on heart beat rates and VEGF expression during the developmental stage like zebrafish larval stage and also observes the effects on hatching rate of zebrafish embryos.

To conclude, since the concentrations of caffeine, norfloxacin and nimesulide used in the present study, is same as the concentrations detected in the serum of the lactating woman. So, use these drugs or much drink of coffee by a mother can cause physiological harm to the new born baby because of VEGF expression is affected by caffeine and VEGF is directly related to the heart blood vessel development and mussel development. So, low expression of VEGF may cause the abnormalities of heart or heart blood vessel development. In this point of view, this study shows an alarming for the new born whose mother is addicted with coffee. Furthermore, our study model using zebrafish is very much relevant. In order to take this factor into account, we are currently conducting similar studies to answer the question that why VEGF expression is low and why the heart beat rate is high if the new born are exposed into the caffeine and also if there is any relation between the heart beat rate and VEGF expression.

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