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ORIGINAL ARTICLE

Evaluating the Teratogenicity of Ritodrine and Nifedipine using a Frog Embryo Teratogenesis assay (FETAX)

Ayper Boğa Pekmezekmek[1], Uğur Seçil Binokay[1], Mehmet Ata Seçilmiş[1], Eda Kumcu[2], Erhan Şimşek[2], Kübra Akillioğlu[1], Yaşar Sertdemir[3], and Besim Özaykan[1]

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Abstract

The Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX) was used to assess the teratogenic potential of two tocolytics. Embryos of the South African clawed frog, *Xenopus laevis*, were exposed to ritodrine or nifedipine. Exposure media were changed and monitored at 24-hour intervals. The 96-hour LC_{50} (Lethal concentration), the 96-hour EC_{50} (Malformation), and the No Observable Adverse Effect Concentrations (NOAEC) and the Lowest Observable Adverse Effect Concentration (LOAEC) for mortality, malformation and length were determined for each drug. Nifedipine was determined to be the more toxic and teratogenic than ritodrine, with a LC_{50} of 0.606 μg/L, an EC_{50} of 0.006 μg/L, and a teratogenicity Index (TI) value (LC_{50}/EC_{50}) of 101. On the other hand, the LC_{50} of ritodrine was 28.571 mg/L. In addition; the LC_{50}, EC_{50} and TI values for nifedipine in the 5 mg/L ritodrine + nifedipine combination group were determined as 1.050 μg/L, 0.868 μg/L and 1.5 respectively. For ritodrine, the NOAEC and LOAEC values were determined as 2 mg/L and 4 mg/L, respectively. For the nifedipine and the ritodrine + nifedipine groups; while the LOAEC values of these groups were 0.0001 μg/L and 0.1 μg/L, respectively. NOAEC value couldn't be determined. Our results demonstrated that nifedipine administration was associated with higher levels of teratogenic and toxic effects. However, the ritodrine + nifedipine combination form reduced the toxic and teratogenic effects of nifedipine on Xenopus embryos. Further studies should be conducted in order to investigate the optimal combination concentrations of these substances for the treatment of preterm labor.

Keywords

FETAX, nifedipine, preterm labor, ritodrine, *Xenopus*

History

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Introduction

The treatment of preterm labor is a difficult subject for obstetricians across the world. Any labor that occurs before the 37th week of pregnancy is described as preterm labor. Preterm labor can lead to a wide variety of neonatal complications, such as respiratory distress syndrome and necrotizing enterocolitis (Kashanian et al., 2011; Rust et al., 1996).

Tocolytic medications are drugs that are used to inhibit uterine contractions in order to prolong the pregnancy period. These drugs are extensively used in the management of preterm labor. However, several recent reviews and meta-analyses have questioned the evidence on which the use of these drugs are based (Anotayanonth et al., 2004; Aydın, 2005; Gyetvai et al., 1999; Haas et al., 2012; Higby et al., 1993; Higby & Suiter, 1999; King et al., 2003, 2005; Li et al., 2005; Papatsonis et al., 2005; Pryde et al., 2001). In fact, the amount of data demonstrating measurable improvement in perinatal outcomes through the use of the currently available tocolytics is remarkably limited. In this context, there is also emerging data that is increasingly bringing into question the maternal and fetal safety of most tocolytics. Examples of currently used tocolytic drugs include calcium channels blockers, magnesium sulfate, nitrates, prostaglandin inhibitors and betamimetics (Pryde et al., 2001).

The Food and Drug Administration (FDA) uses a category system to classify medication according to the fetal injury risk they present during pregnancy. Based on this system, ritodrine is classified as pregnancy category B. This classification means that there is no available controlled data regarding the effects of this medication on human pregnancy (FDA, 2014).

The beta-sympathomimetic agent ritodrine is currently the most widely used drug for the prevention of preterm labor. Ritodrine's mechanism of effect involves the relaxation of uterine smooth muscles and contractions (Barden et al., 1980; Li et al., 2005; Zhu & Fu, 1996). It is also the only tocolytic drug approved by the Food and Drug Administration (FDA). Although the FDA has approved the use of ritodrine hydrochloride (1980), various studies appear to suggest that ritodrine is not an effective and ideal tocolytic agent.

All beta-adrenergic agents used for tocolysis readily cross the placenta, and trigger similar side effects in both the fetus

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and the mother. The lethal toxicity dosage of ritodrine was determined through experiments involving intravenous and oral ritodrine administration to various animal species, including rats, rabbits, mice, guinea pigs and dogs. In these experiments, rabbits and mice proved to be the species most sensitive to ritodrine, with an LD_{50} value of 64 mg/kg for rabbits following intravenous administration, and an LD_{50} value of 540 mg/kg for mice following oral administration (New drug appl., 1979).

The most common side effect observed in fetuses is tachycardia, which is caused by the direct β_1 stimulation of the fetal myocardium (Shenker, 1965). Uncommon fetal side effects that have been reported to date include fetal cardiac hypertrophy, fetal hyperinsulinemia, hypocalcemia, hypotension, ileus and neonatal intraventricular hemorrhage (Brazy & Pupkin, 1979; Groome et al., 1992; Katz & Seeds, 1989; Leake et al., 1983; Van Assche & Aerts, 1982). The incidence of hypokalemia in the fetuses of mothers administered with ritodrine is not significant (Pryde et al., 2001). In addition, no neonatal intraventricular hemorrhage effects were identified as a result of ritodrine use (Ozcan et al., 1995).

In recent years, many pregnant women have been administered with betamimetic drugs for prolonged periods. Such treatments were found to result in no significant changes in the glycemic balance or hepatic function of newborns (Freysz et al., 1977). Several fetal side effects associated with betamimetic drugs have been described (Gruber et al., 1973; Nochimson et al., 1974; Renaud et al., 1973), for instance, observed that the hearts of certain fetuses exposed to betamimetic drugs beats faster than normal. However, Nochimson et al. (1974) did not observe similar side effects in their study.

Certain researchers describe that ritodrine has not been associated with post-term pregnancy, abnormal labor, postpartum uterine atony or increased neonatal morbidity in any of the controlled studies or clinical reports that have been performed to date (Barden et al., 1980; How et al., 1995; Li et al., 2005; Young et al., 1993).

Due to the inconsistent nature of the results regarding the efficacy and safety of ritodrine, many other compounds are currently being investigated for the prevention of preterm labor. One such compound is nifedipine, a calcium channel blocker used as a tocolytic for the suppression of preterm labor. Calcium channel blockers are characterized by their ability to inhibit calcium influx through potential sensitive channels, and also through receptor operated channels (Creasy et al., 1980). These blockers tend to relax all smooth muscles, including those in the uterus, thereby effectively preventing preterm labor through the inhibition of preterm uterine contractions. Nifedipine has been commonly used for the prevention of preterm labor since the 1980s (Meyer et al., 1990; Ulmsten et al., 1980; Ulmsten, 1984). Nifedipine was assigned a pregnancy category C rating by FDA due to potential side effects identified in animal studies. On the other hand, no specific congenital defects have been reported in human fetuses in association with nifedipine use (Ferguson et al., 1989). In addition, nifedipine also demonstrated improved in utero fetal survival in ovariectomized, estrogen-treated late pregnancy rats (Abel & Hollingsworth, 1986).

Koks et al. studied to compare the efficacy and safety of nifedipine and ritodrine in preventing preterm labor, and to evaluate maternal side effects and neonatal outcome. Maternal side effects were significantly less common in the nifedipine group than in the ritodrine group, however after 7 days of therapy there was no difference between the two groups. Neonatal outcome was similar in the two groups. In conclusion; nifedipine seems to be as effective as ritodrine in the treatment of preterm labor and is associated with less frequent side effects (Koks et al., 1998).

However, various animal studies have suggested that compounds such as nifedipine can detrimentally alter blood flow to the placenta, even in mothers without any prior blood pressure-related conditions (Harake et al., 1987; Larmon et al., 1999). In addition, certain animal studies on nifedipine have shown an increase in the risk of miscarriage and birth defects. However, it is also known that animals do not always respond to drugs in the same way as humans. When administered to pregnant rabbits, mice and rats, nifedipine caused an increase in the ratio of miscarriages and birth defects. These birth defects included finger and toe malformations, rib deformities and cleft palates (Errol & Robinson, 2001).

In 1989, Xenopus embryos, treated for three days from the early cleavage stage with the calcium channel blocking drugs nifedipine, diltiazem, verapamil or nicardipine continue to develop in water. By the seventh day many developmental abnormalities appear, the most reproducible affecting the central nervous system, failure of forebrain development, synophthalmia and neural tube defect, failure of mandibular growth, malrotation of the gut and severe oedema were observed in some animals. The defects appear to relate to calcium ion antagonism, and provide a pharmacological model for some forms of teratogenesis in which large populations can be studied readily (Burgess & Vere, 1989).

Until now, no tocolytic treatment has resulted in significant prolongation of pregnancy following the onset of true labor (How et al., 1995; Li et al., 2005; Young et al., 1993). In light of the available information on tocolytics, the aim this study was to utilize an alternative bioassay – the Frog Embryo Teratogenesis Assay: Xenopus (FETAX) – to gain further insight into the potential toxicity of these compounds. The FETAX test provides a rapid, simple, and cost-effective method for performing preliminary evaluations of a compound's developmental toxicity. Frogs, and in particular the South African Clawed Frog *Xenopus laevis*, have been used as models of embryonic development for over a hundred years.

The FETAX test used in our study is a test on organ generation and formation. Mechanisms associated with organ formation display a high degree of evolutionary conservation among animals. During the development of *Xenopus laevis* embryos, the initial 96 hours bears numerous resemblances to the developmental processes observed in the embryos of other animals, including humans. For this reason, assessment of drug toxicity and teratogenicity in *Xenopus laevis* embryos with the aid of the FETAX test is highly indicative of potential drug toxicity and teratogenicity for other animals. In this context, the results of the FETAX test are reported to be largely in parallel with the results obtained from other models, with a correlation of up to 89% being described

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in various studies (ASTM, 2004; Fort et al., 2001; O'Brien et al., 2005).

Thus, FETAX can be considered as useful means for predicting potential human developmental toxicants and teratogens (American Society for Testing and Materials (ASTM), 1998). The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) previously validated FETAX as a screening assay for detecting potential human teratogens, and for its use in the ecotoxicological assessment of water/soil/sediment/samples (Boğa et al., 2008; NICEATM, 2004). In this test method, malformations are used to calculate a Teratogenicity Index (TI), which is calculated by dividing the LC_{50} by the EC_{50} (ASTM, 1991, 1998; Bantle et al., 1989). The TI presents a relative hazard ranking or level, with a value ranging from nearly 1 to several thousands. The hazard becomes a concern when the mean TI value of three definitive tests is greater than 1.5 (ASTM, 1991, 1998). More recently, Fort et al. (2000) described a criteria according to which TI values greater than 1.5 indicate increasing teratogenic hazard, while TI values greater than 3.0 indicate concern (NICEATM, 2000).

The TI value shown for mammals is an estimate based on the reproductive and general toxicity testing data reported in the NIOSH Registry of Toxic Effects of Chemical Substances. A value at or near unity for *in vivo* mammalian data represents equal susceptibility to the toxicant between the developing embryo and the mother (Sabourin et al., 1985).

Our study represents the first to comparatively assesses the teratogenic and toxic effects of ritodrine and nifedipine applied separately or in combination to Xenopus embryos by using the FETAX (Frog Embryos Teratogenesis Assay) test.

Materials and Methods

The study tests were performed according to the guidelines and methods described in the Standard Guide (2004) of the American Society for Testing and Materials (ASTM).

Chemicals

Ritodrine hydrochloride (Catalog No: R0758), the reagents for the FETAX solution and De Boers Tris were all purchased from Sigma (St. Louis, MO, United States). Nifedipine was provided as Nidilat capsule by Sanofi Aventis (Istanbul, Turkey). Each gelatin capsule contains 10 mg nifedipine. The follicle stimulating hormone (FSH) was provided by Serono (Istanbul, Turkey). Human chorionic gonadotropin (hCG, Pregnyl, 5000 IU) was provided by Organon (Istanbul, Turkey).

Test organism and bioassay

Xenopus laevis is an aquatic frog species native to Sub-Saharan Africa that is commonly known as the African clawed frog. It is often employed in animal studies as a model organism (Boga et al., 1997).

In this study, we utilized *Xenopus* laevis specimens previously obtained from the USA and China in the early 1990s. The breeding and embryo collection procedures were performed at the Cukurova University Physiology Department (Adana, Turkey). These procedures were carried out in accordance with the guidelines described in the Standard Guide For Conducting FETAX (ASTM E1439-98) (2004). The *Xenopus laevis* were kept in aquaria with dimensions of $95 \times 60 \times 44$ cm, and at temperatures maintained at 23 ± 1 °C. The *Xenopus laevis* were kept under a 12-hour light and dark cycle, and fed ad libitum twice weekly (Boga et al., 2009).

In vitro fertilization

The methods outlined by Lindi et al. (2001) were employed for *in vitro* fertilization purposes. In each experiment, hCG injections were performed to two or three *Xenopus* females at a dose of 700–1000 IU approximately 16 hours prior to egg laying. Insemination of the eggs was performed immediately after they were laid onto plastic Petri dishes by using sperm from minced *Xenopus* testes suspended in 1–2 mL cold De Boers Tris (DBT: 119 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 15 mM Tris-HCl, with a pH of 7.5). The greater osmolarity of the DBT solution prevented the spermatozoa from engaging in flagellar movements (Prati et al., 2000).

One minute after insemination, 10 mL of FETAX solution (625 mg/L NaCl, 96 mg/L $NaHCO_3$, 30 mg/L KCl, 15 mg/L $CaCl_2$, 60 mg/L $CaSO_4$. $2H_2O$, and 70 mg/L $MgSO_4$, with a pH of 7.8–8.0) was poured into the Petri dishes. Insemination was confirmed through the orientation of the eggs: the upwards orientation of the darker, animal pole several minutes after application of the sperm suspension served to indicate that insemination had taken place. Eggs displaying irregular segmentation were disposed. Embryos demonstrating normal development were selected when they reached their late blastula stage (in other words, their stages 8–11). FETAX was then performed on the selected embryos. The FETAX Guideline (2004) of ASTM specifies that 95% of the selected embryos must be viable (Boga et al., 2012).

FETAX procedure

The study procedures with ritodrine and nifedipine were performed on embryos (N: 20, n: 9000 embryos) prepared by using the abovementioned fertilization method. 10 mg nifedipin in each gelatin capsule was dissolved in 1 ml distilled water (Dengiz & Akpınar, 2007). This solution was then diluted with FETAX solution to obtain the concentrations used in the experiments between 0.0001–1.5 μg/L. Ritodrine was dissolved in distilled water, then concentrations of assay was prepared with FETAX solution between 2–60 mg/L.

The ritodrine and nifedipine solutions were then applied onto petri dishes with the aid of a pipette. Stock solutions were stored at 4 °C. Embryos were allowed to develop for nearly five hours; upon reaching phase 7 or 8 of development, the embryos were transferred onto the Petri dishes with the ritodrine and/or nifedipine solutions. Incubation of the embryos was performed at 23 ± 1 °C for 96 hours; during incubation, solutions were replaced every 24 hours. At hour 96 of incubation, embryos were counted with the aid of a stereo microscope (Kyowa) in order to determine the numbers of normal, abnormal, and dead embryos. The counting and evaluation of the embryos was performed in accordance with procedures outlined in Nieuwkoop's Normal Table Atlas (Nieuwkoop & Faber, 1994). Embryos that were alive yet

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unhealthy/malformed were considered as abnormal embryos. Embryo death by the end of incubation (hour 96) was identified by the absence of cardiac activity, which could be easily observed due to the transparence of the *Xenopus laevis* embryos. Dead embryos with malformations were not taken into consideration when determining the number of abnormal embryos (ASTM, 2004; Boga et al., 2000).

Embryos that were still alive following the 96-hour incubation with ritodrine and/or nifedipine were fixed using 3% (w/v) formalin. Abnormal embryos were counted with the aid of a dissection microscope (Bantle et al., 1994). All embryos were scored by measuring their length from head-to-tail using a millimeter ruler. A length lower than normal was indicative of growth inhibition (Boga et al., 2012). For embryos in flexed position, length was measured by following the curvature of the notochord (Osano et al., 2002).

The overall study design consisted of three distinct phases. During the first phase, ritodrine (2–60 mg/L) was administered within the scope of five repeat experiments to 5100 embryos (1700 control and 3400 assay embryos) obtained from five females. During the second phase, nifedipine (0.0001–1.5 μg/L) was administered within the scope of five repeat experiments to 2100 embryos (700 control and 1400 assay embryos) obtained from five females. During the third phase, a 5 mg/L ritodrine solution was mixed with nifedipine solutions with concentrations ranging between 0.1 μg/L and 1.5 μg/L, and the resulting ritodrine + nifedipine solution was administered within the scope of five repeat experiments to 1800 embryos (600 control and 1200 assay embryos) obtained from five females. All repeated experiments were performed on petri dishes, and with twenty embryos per petri. Each one of the abovementioned concentrations were tested on two separate dishes.

Analytical methods

Data were analyzed by using the SPSS 19.0 package program. Probit analysis was used to measure the effects of ritodrine and nifedipine on the *Xenopus laevis* embryos. The probabilities that the embryos were normal, abnormal, or dead were given in absolute numbers and percentages for each concentration. Dunnett's multiple comparison procedure was used to calculate the values for the non-observed adverse effect concentration (NOAEC) and the lowest observed adverse effect concentration (LOAEC). Minimum concentration to inhibit growth (MCIG) was calculated by performing comparisons of length at different concentrations with the aid of the analysis of variation (ANOVA). p values greater than 0.05 were considered as being statistically significant.

The Coefficients of Variability (CV) is a dimensionless number defined as the standard deviation of a set of measurements divided by the mean of the set. The % Coefficients of Variability (CV) for each sample is calculated by finding the standard deviation of results 1 and 2, dividing that by the duplicate mean, and multiplying by 100. The inter-assay CV is an expression of plate-to-plate consistency that is calculated from the mean values for the high and low controls on each plate. The intra-assay CV reported in these studies is an average value calculated from the individual CVs for all of the duplicates, even if the total number of samples requires the use of multiple assay plates (Salimetrics, 2014).

Results

Incidence of normal, abnormal and dead *Xenopus laevis* embryos

Figure 1 shows the mean ratios of dead and abnormal embryos for the control group and the ritodrine-administered groups. The mean ratios for dead, abnormal and normal embryos within the control group were 0.60%, 1.61% and 97.80%, respectively. It was thus determined the control group satisfied the 95% criterion described by the ASTM (2004). The mean ratios for dead, abnormal and normal embryos were 2.50%, 1% and 96.50%, respectively, in the group administered with 2 mg/L ritodrine; and 100%, 0% and 0%, respectively, in the group administered with 60 mg/L ritodrine.

Figure 2 shows the mean ratios of dead and abnormal embryos for the control group and the nifedipine-administered groups. The mean ratios for dead, abnormal and normal embryos within the control group were 0.4%, 1.6% and 98%, respectively. The mean ratios for dead, abnormal and normal embryos were 2.50%, 1% and 96.50%, respectively, in the group receiving 0.0001 μg/L nifedipine; 21.5%, 78.5% and 0%, respectively, in the group receiving 0.01 μg/L nifedipine; and 100%, 0% and 0%, respectively, in the group receiving 1.5 μg/L nifedipine.

Figure 3 shows the ratios of dead and abnormal embryos for the control group and the groups administered with 5 mg/L ritodrine together with varying concentrations (μg/L) of nifedipine. The mean ratios for dead, abnormal and normal embryos within the control group were 98.17%, 1.83% and 0.0%, respectively. The mean ratios for dead, abnormal and normal embryos were 0%, 96% and 4%, respectively, in the 5 mg/L ritodrine + 0.1 μg/L nifedipine group; 0%, 79% and 21%, respectively, in the 5 mg/L ritodrine + 0.8 μg/L nifedipine group; and 0%, 0% and 100%, respectively, in the 5 mg/L ritodrine + 1.5 μg/L nifedipine group.

Lethal (LC50) and effective concentration (EC50) values

Table 1 provides the lethal (LC_{50}) and effective (EC_{50}) concentration values identified during this study. According to Probit analyses, the LC_{50} values for ritodrine and nifedipine were 28.571 mg/L and 0.606 μg/L, respectively, while the LC_{50} value for nifedipine in the 5 mg/L ritodrine + nifedipine combination was 1.050 μg/L. The EC_{50} values for nifedipine and 5 mg/L ritodrine + nifedipine were 0.006 μg/L and 0.868 μg/L, respectively, while the EC_{50} value of ritodrine could not be determined. The teratogenicity index (TI) value (LC_{50}/EC_{50}) of ritodrine could also not be calculated due to the inability to determine the EC_{50} value for ritodrine. On the other hand, the TI value of nifedipine was 101, and the TI value of the ritodrine + nifedipine combination group was determined as 1.5 (Table 1).

NOAEC and LOAEC values

For ritodrine, the NOAEC value was determined as 2 mg/L, while the LOAEC value was determined as 4 mg/L. NOAEC

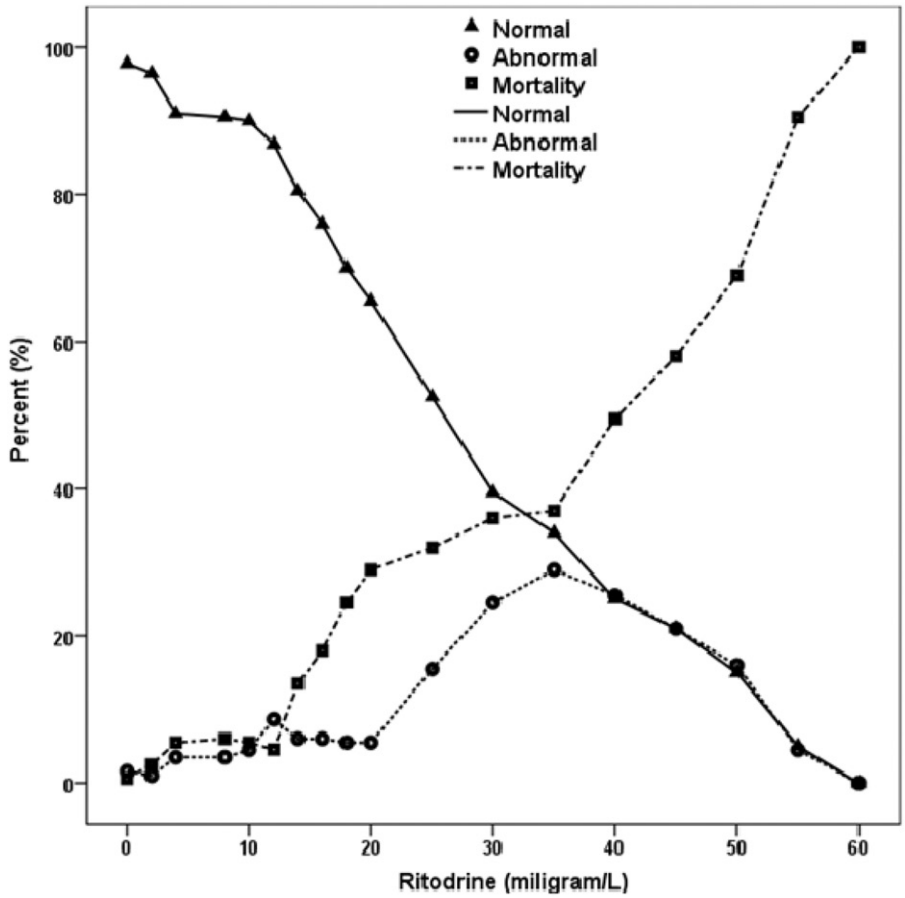

Figure 1. The mean ratios of normal, abnormal and dead embryos in the control group and the ritodrine-administered groups based on the FETAX test.

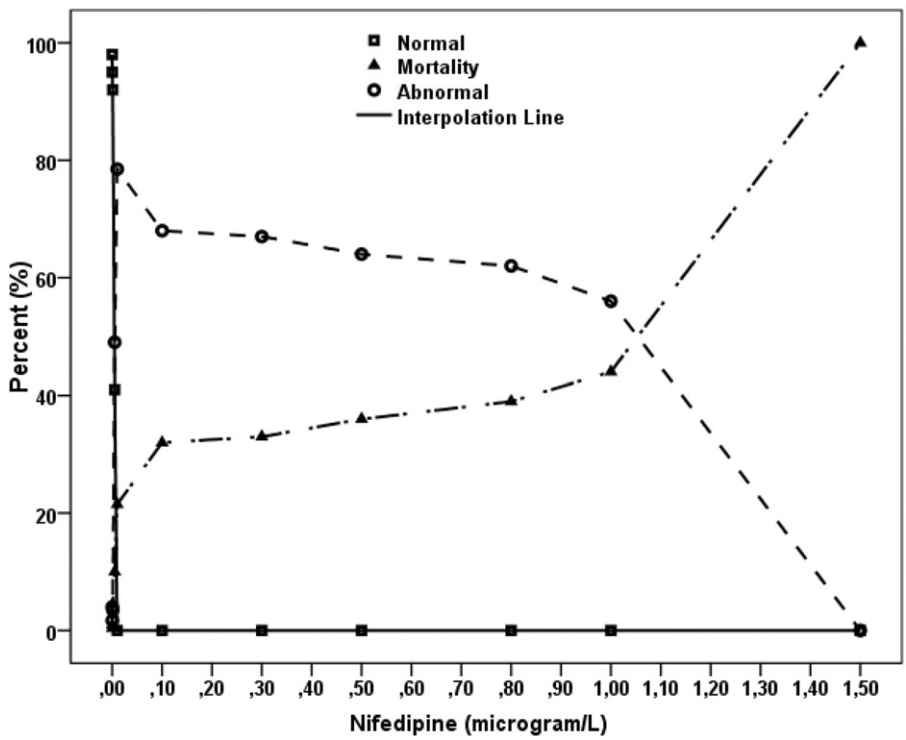

Figure 2. The mean ratios of normal, abnormal and dead embryos in the control group and the nifedipine-administered group based on the FETAX test.

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could not be determined for the nifedipine and the ritodrine + nifedipine groups, while the LOAEC values of these groups were 0.0001 µg/L and 0.1 µg/L, respectively (Table 2).

MCIG values

Table 3 shows the lengths (mm) of the *Xenopus laevis* embryos in the control and administered groups. The total

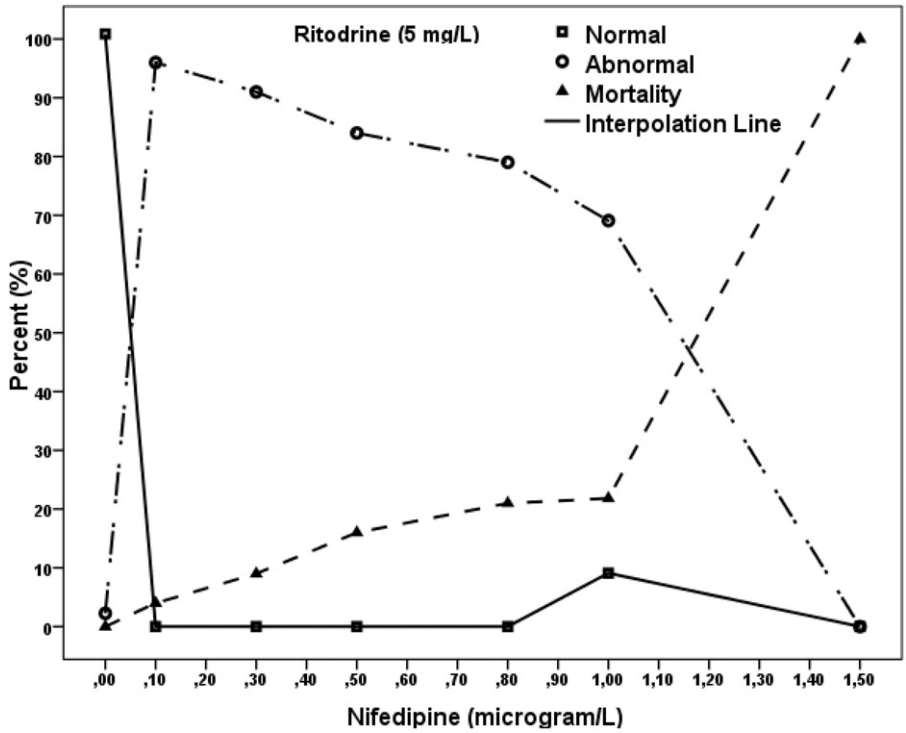

Figure 3. The mean ratios of normal, abnormal and dead embryos in the control group and the ritodrine + nifedipine-administered groups based on the FETAX test.

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Table 1. The LC50 (Lethal concentration) and EC50 (effective concentration) and TI (Teratogenicity Index) of the ritodrine and nifedipine-administered groups based on the FETAX test.

Probit analyzes of drugs	LC_{50}	EC_{50}	TI
Ritodrine (mg/L)	28.571 (27.774–29.399)	c.f	c.f
Nifedipine (μg/L)	0.606 (0.548–0.673)	0.006 (0.004–0.010)	101
Ritodrine (mg/L) + Nifedipine (μg/L)	1.050 (0.986–1.126)	0.868 (0.768–0.968)	1.5

*95% Confidence Limits Mean For Limits *100 Conc was presented in parenthesis as lower and upper value; c.f: couldn't found

Table 2. NOAEC and LOAEC values of the ritodrine, nifedipine, and ritodrine + nifedipine-administered groups based on the FETAX test.

Probit analyzes of drugs (Estimated concentrations)	NOAEC	LOAEC
Ritodrine (mg/L)	2	4
Nifedipine (μg/L)	c.f	0.0001
Ritodrine (mg/L) + Nifedipine (μg/L)	c.f	0.1

NOAEC, LOAEC are expressed as mg/L in ritodrine and as μg/L in nifedipine ($p < 0.05$)

Table 3. Lengths and MCIG values of the ritodrine, nifedipine, and ritodrine + nifedipine-administered groups based on the FETAX test.

Control (9.261mm ± 0.043)	MCIG value	Length (±SE) (mm)
Ritodrine	12 mg/L	9.11 mm ± 0.059
Nifedipine	0.0001 μg/L	8.52 mm ± 0.060
Ritodrine + Nifedipine	5 mg/L + 0.1 μg/L	6.950 mm ± 0.190

mean length of the control embryos (N: 1700) was 9.26 ± 0.043 mm, while the MCIG values for the ritodrine, nifedipine and ritodrine + nifedipine groups were 12 mg/L (mean length: 9.11 ± 0.057 mm) (Figure 4), 0.0001 μg/L (mean length: 8.52 ± 0.06 mm) (Figure 5), and 5 mg/L + 0.1 μg/L (mean length: 6.950 ± 0.190 mm) (Figure 6), respectively ($P < 0.05$). The MCIG values indicated that nifedipine and the ritodrine + nifedipine combination had a greater detrimental effect on the length of the *Xenopus laevis* embryos than ritodrine alone.

Coefficients of Variability (CV)

Table 4 shows the CV of the *Xenopus* embryos in the control and administered groups. In order to express the precision, or repeatability of test results, CV assay was used. While Inter-assay % CVs of less than 15 are generally acceptable, Intra-assay % CVs should be less than 10. In our study; intra and inter assay results were 12,11.5 and 9.1%; 3, 2.9, 2.07%, respectively. According to this knowledge CV values ritodrine, nifedipine and the ritodrine + nifedipine combination group's results were repeatable.

Observed anomalies

Various abnormalities were caused by nifedipine administration. Gut malrotation, eye malformations (e.g. microphthalmia), body malformations (e.g. kinked and somite abnormalities), and dermal blister or bullae were all observed at ratios less than 1% with nifedipine concentrations ranging from 0.0001 μg/L to 1.5 μg/L. Even at relatively low concentrations of nifedipine (0.0001 μg/L) (Figure 7), different types of abnormalities were observed in *Xenopus laevis* embryos in comparison to the control group (Figure 8).

The inter-tissue areas of embryos administered with ritodrine, nifedipine and ritodrine + nifedipine were swollen with fluids, and widespread edema was observed across their extremities, bodies and heads. The abnormalities appeared to precede the swellings and oedema; this suggested that the swellings and oedema were the consequence rather than the

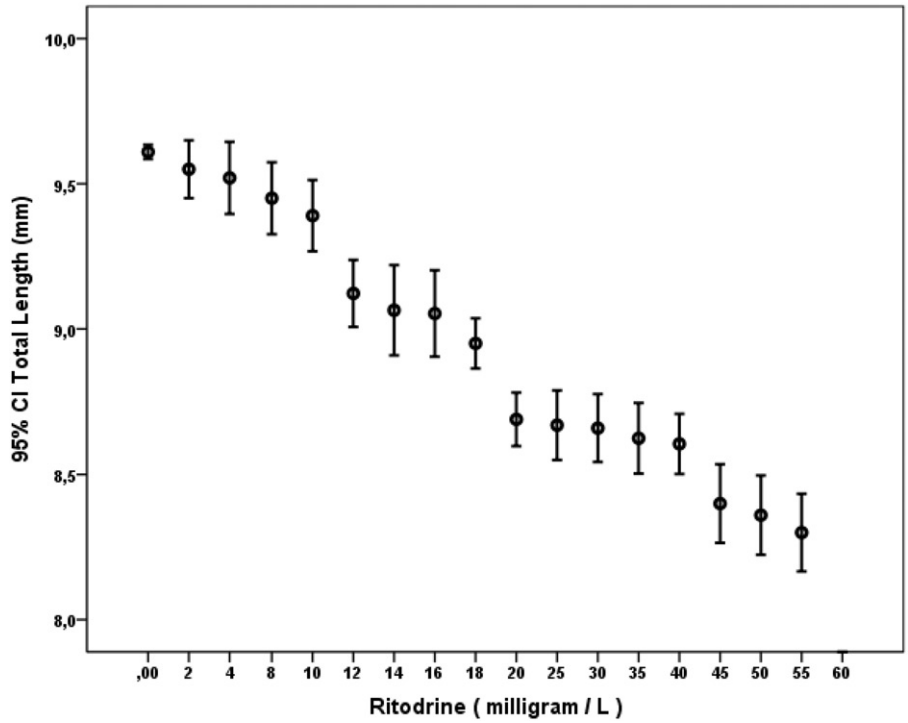

Figure 4. Total lengths (mm) of *Xenopus laevis* embryos administered with ritodrine. 95% CI error bars are shown.

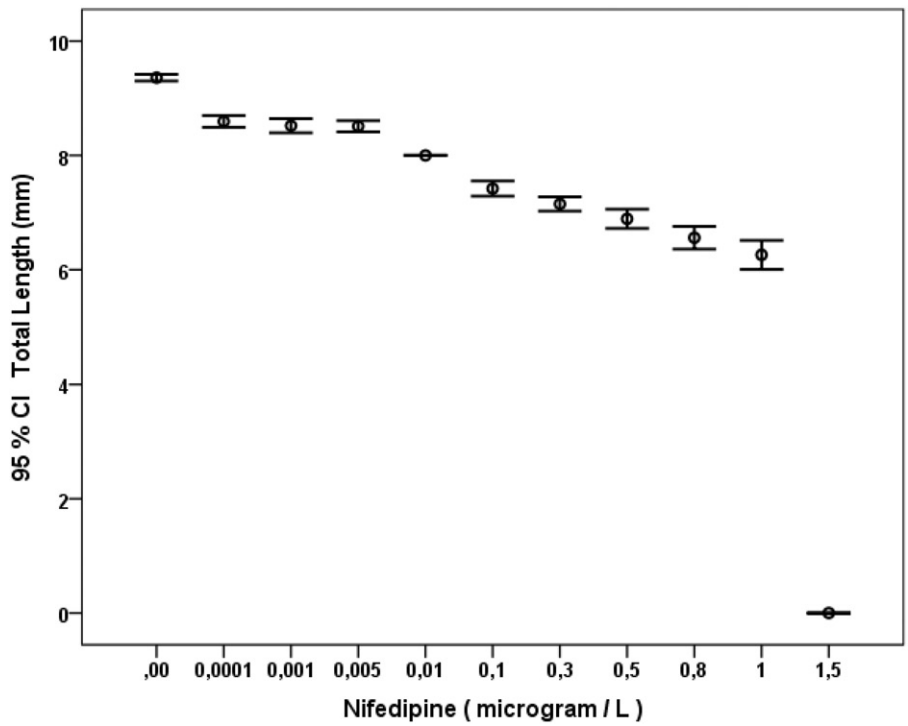

Figure 5. Total lengths (mm) of *Xenopus laevis* embryos administered with nifedipine. 95% CI error bars are shown.

cause of the abnormalities. There were cases, however, in which edema further contributed to damage and displacement in the visceral organs, thus acting at the same time as the cause of other abnormalities. Various types of microcephaly were also observed in the embryos, and some even displayed alteration in the proper orientation of their eyes (e.g. medial emplacement rather than lateral). Certain embryos also demonstrated cyclopia perfecta (Boga et al., 2012), a condition in which the mouth is absent or malformed. Furthermore, nifedipine led to the formation of kinked tails in the embryos by causing cysts to develop between the axial skeletal structures and the skin. Two or more kinks were also observed in certain embryos; however, such kink-related abnormalities were, ultimately, not fatal for the *Xenopus laevis* bred within the context of this experiment. All embryos with kinked tails were also identified as having malformed notochords, suggesting that the kink-related abnormalities might have initially stemmed from the notochord and then spread to the other tissues (Boga et al., 2000). In certain cases, nifedipine resulted in the reduction of both posterior structures (anteriorization) and anterior structures (posteriorization) (Boga et al., 2000). Two-headed *Xenopus laevis* tadpoles

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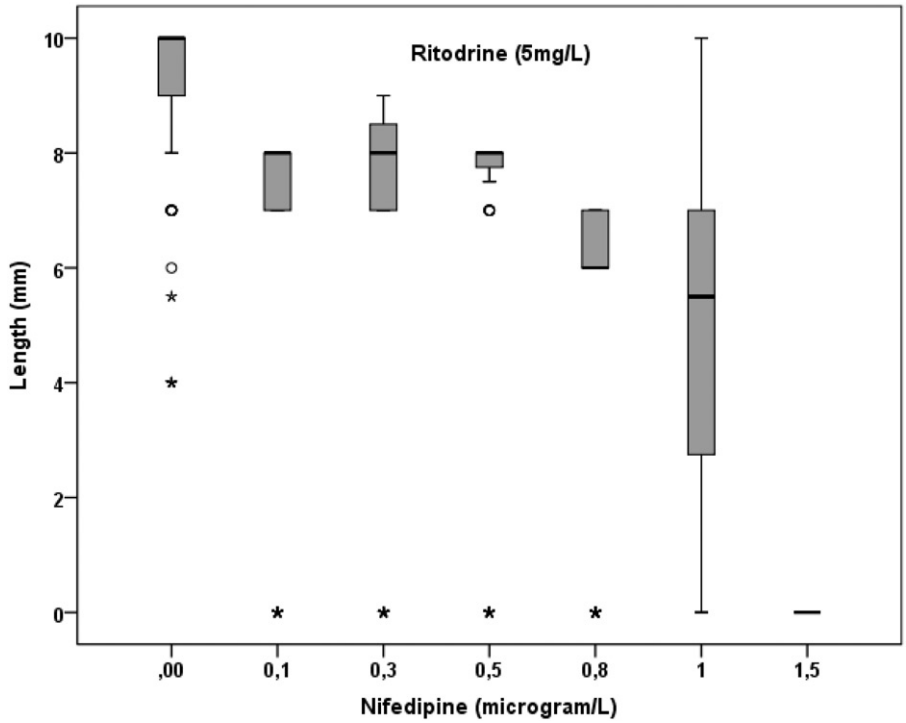

Figure 6. Total lengths (mm) of *Xenopus laevis* embryos administered with ritodrine + nifedipine.

Table 4. Coefficients of Variability of Ritodrine, Nifedipine and Ritodrine + Nifedipine combination group groups.

	Intra-assay CV (%)	Inter-assay CV (%)
Ritodrine (mg/L)	12	3
Nifedipine (μg/L)	11.5	2.9
Ritodrine (mg/L) + Nifedipine (μg/L)	9.1	2.07

were observed among nifedipine-administered embryos. The abnormal presence of RNA encoding the signaling molecule plakoglobin can lead to atypical signaling, which in turn can give rise to two-headed tadpoles (Klymkowsky, 2011).

Data from other animal studies on the abnormalities induced by ritodrine and nifedipine in embryos is limited. For this reason, it was not possible to perform comparisons between the dose-dependent increase in ritodrine- and nifedipine-induced abnormalities observed in our study and the abnormalities observed in other animal studies (Boga et al., 2012).

Figure 9 shows that embryos in the ritodrine-administered groups had relatively light abnormalities, including somite anomalies. A comparison of ritodrine and nifedipine doses revealed that abnormalities began to appear at concentrations of 2 mg/L for ritodrine and 0.0001 μg/L for nifedipine. The difference between these two concentrations is greater than 2×10^7.

Discussion

Due to scarcity as well as the contradictory nature of the data on the toxicity and teratogenicity of ritodrine and nifedipine in animals and humans, these two drugs were investigated in this study by using the FETAX test. To the best of our knowledge, our study represents the first prospective study comparing the toxicity and teratogenicity of ritodrine and nifedipine by using FETAX.

Clinical studies have previously demonstrated that beta-adrenergic drugs can easily pass through the placenta and induce similar side effects in both the mother and the embryo (Pryde et al., 2001). In our embryo tests, only mild adverse effects were observed. In addition, we could not determine TI values for the ritodrine-administered embryos in our study. While the LC_{50} value for ritodrine was 28.571 mg/L, its EC_{50} value could not be determined for reason that the abnormality ratio never reached 50% in this arm of the study.

In one another study, no differences were identified with respect to growth and development between infants exposed to ritodrine while in the uterus and the controls (Laros et al., 1991). This observation is in agreement with results of our study, which also showed that the difference between the ritodrine-administered group and the control embryos with respect to growth and developmental stages values were not statistically significant.

Nandita et al. (2007) suggested that, in comparison to ritodrine, nifedipine was more effective in arresting preterm labor, and also associated with less side effects and better tolerability. However, our results indicated that ritodrine is associated with less side effects than nifedipine.

Studies on human pregnancy have demonstrated that calcium channel blockers result in no noticeable teratogenicity towards human embryos (Magee et al., 1996; Sorensen et al., 1998). However, the result of our FETAX test indicated TI value (LC_{50}/EC_{50}) of 101 for nifedipine. According to the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the American Society for Testing and Materials (ASTM), a TI value >3.0 is actually indicative

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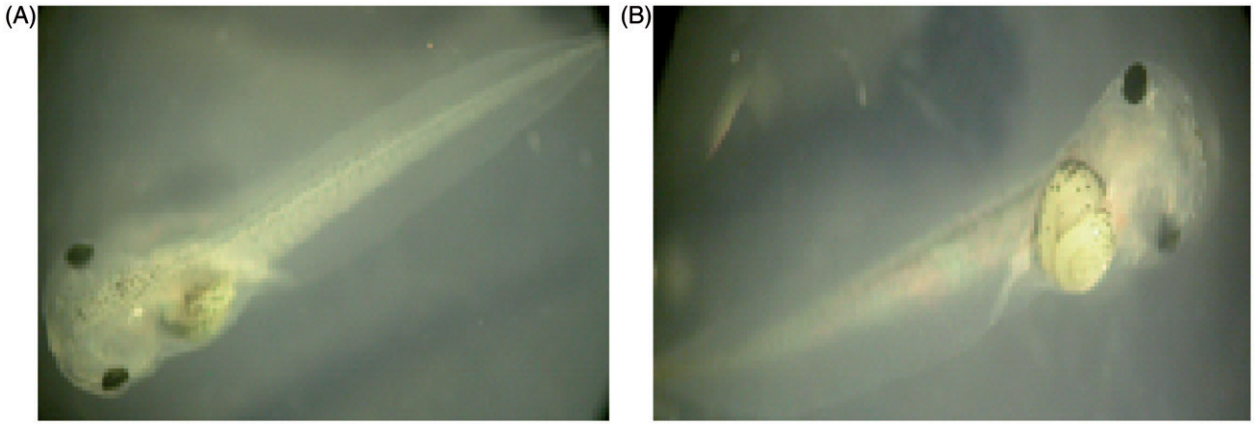

Figure 7. Five-day-old *Xenopus* larvae. (A) Dorsal view of a control larvae and (B) Ventral view of a control larvae.

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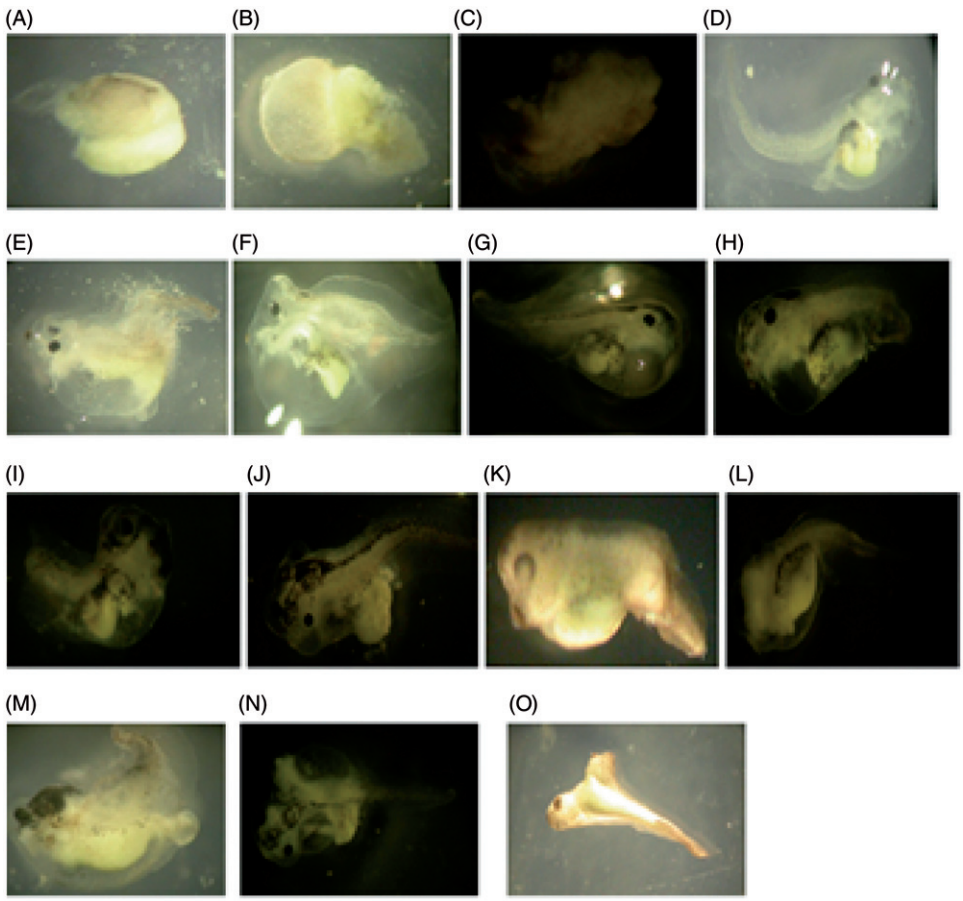

Figure 8. Five-day-old *Xenopus* larvae. (A), (B) Anteriorization (C) Posteriorization (A–O) larvae administered with 0.0001–1.5 µg/L nifedipine. (D–N) Pluri-malformed larva affected by generalized edema, abnormal gut coiling, microphthalmia, kinked tail, microphthalmia, tail flexure (I–J) Cyclopia (N–O) Two-headed tadpole.

strong teratogenicity. Therefore, our study results demonstrated that nifedipine has a strong teratogenic effect for *Xenopus laevis* embryos. Its use should hence be avoided in contexts that could result in embryo exposure.

In addition, our study results also indicated that the combination of ritodrine + nifedipine may have protected *Xenopus* embryos from the side effects of nifedipine used separately.

Our test results and calculated TI values showed that ritodrine is non-teratogenic, while nifedipine was highly teratogenic. However, the combination of nifedipine + ritodrine was only weakly teratogenic. These results were promising, and an appropriate and tolerable combination dose for these two drugs might be identified in future clinical studies. In pharmacology, combination of drugs was preferred because it allows to reduce the doses and thus also side effects

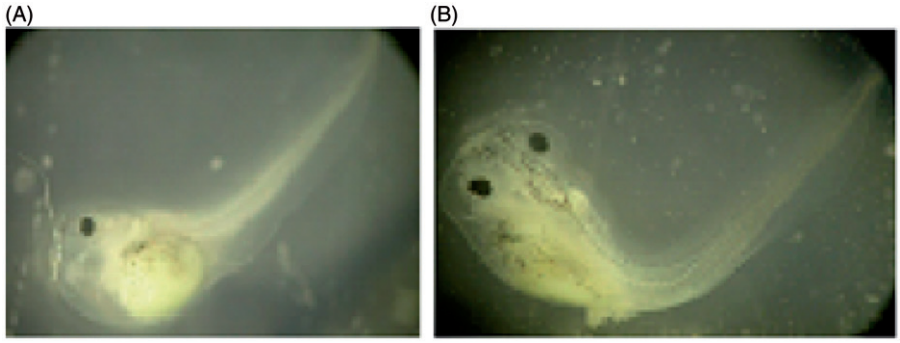

Figure 9. Five-day-old *Xenopus* larvae. (A), (B) malformed larva affected by light edema and kinked tail (larvae exposed to 2–55 mg/L ritodrine).

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of each drug used. In the present experiment, combination dose of nifedipine and ritodrine were the same with those of used alone. So we can not suggest that low teratogenic effect of nifedipine was due to the combination. It could be possible that once nifedipine and ritodrine are in the same solution, a chemical reaction may occur and at the end of this chemical reaction nifedipine may be ineffective as a drug. And also the teratogenic effect seen in the present study in larvae could belong to the ritodrine alone.

Gallagher et al.'s studies were undertaken to evaluate the efficacy of the beta 2-adrenergic agonist ritodrine and the calcium channel blocker nifedipine, alone and in combination, for inhibition of contraction in isolated rat uterine strips. Because the mechanisms of action of these two agents are different. Ritodrine uses G protein and second Messenger pathway (Bernal, 2007) and nifedipine uses RhoA/Rho kinase pathway (Webb, 2003). It is reasonable to suggest that they be used in combination to control premature labor. The results of Gallagher's study suggest they may provide inhibition of contraction superior to either agent alone, and could therefore provide more effective inhibition and possibly reduce untoward side effects common (Gallagher & Gautieri, 1992). This knowledge supported our results.

It was observed that ritodrine did not inhibit embryo growth; on the other hand, nifedipine inhibited embryo growth even at low initial doses. The minimum concentration to inhibit growth (MCIG) value for ritodrine at 12 mg/L was 9.11 ± 0.0594 mm, while the MCIG value for the control group was 9.261 ± 0.043 mm. The MCIG value for nifedipine was 8.52 ± 0.06 mm at 0.0001 µg/L and $6.950 \text{ mm} \pm 0.190$ at 5 mg/L ritodrine + 0.1 µg/L nifedipine. Furthermore, in the combined application of nifedipine + ritodrine, the inhibition of growth started at concentrations about 10^3 times higher than the concentrations observed in the nifedipine arm of the study.

The NOAEC and LOAEC values for ritodrine were 2 and 4 mg/L, respectively. Although a NOAEC value could not be determined for nifedipine, its LOAEC value was calculated as 0.0001 µg/L. For the nifedipine + ritodrine combination, the LOAEC value was 0.1 µg/L, while the NOAEC value could not be determined. There were no NOAEC values for nifedipine, since each dose of nifedipine caused significant abnormalities in the embryos. As a result, significant differences were observed between the LOAEC values of these two drugs. Nifedipine's negative effects on *Xenopus laevis* embryos were significantly more pronounced than effects of ritodrine. Among the nifedipine-administered embryos, 96.5% showed abnormalities at hour 96 of incubation (96 hours for *Xenopus laevis* embryos correspond to the first trimester of a human embryo in terms of development). With nifedipine, abnormalities began to be observed at a concentration of 0.0001 µg/L. The abnormalities were of a rather severe nature. On the other hand, the ritodrine-administered embryos demonstrated only mild anomalies.

In conclusion, our study results have shown that ritodrine is more effective and less teratogenic than nifedipine for *Xenopus laevis* embryos. Further comparative studies must be conducted with nifedipine and ritodrine not only to elucidate their therapeutic effects, but also to gain a better understanding of their teratogenic potential. In addition, the differences in the therapeutic and teratogenic effects of these drugs between different species should also be considered and evaluated within the context of another study.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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