Evaluation of cytotoxicity, genotoxicity and embryotoxicity of insecticide propoxur using flounder gill (FG) cells and zebrafish embryos

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A B S T R A C T
Cytotoxicity, genotoxicity and embryotoxicity of carbamate insecticide propoxur were evaluated using flounder gill (FG) cells and zebrafish embryos. The cytotoxicity of propoxur in FG cells was analyzed by MTT, neutral red uptake (NRU), lactate dehydrogenase (LDH) release and Hoechst 33342 and propidium iodide double staining, and acute cytotoxic effects were observed in a concentration-dependent manner. The 24 h-LC50 values of 89.96 ± 1.04, 103.4 ± 1.14 and 86.59 ± 1.13 μg/ml propoxur were obtained by MTT, NRU and LDH assays, respectively. The lethal effects were induced in FG cells mainly through necrosis but not apoptosis as evidenced by double fluorescence staining. Comet assay showed weak genotoxic effects and statistically significant DNA damages were recorded in the cells exposed to highest tested concentration of 75 μg/ml propoxur (p < 0.05). Propoxur exerted obvious acute toxic effects on the survival, spontaneous movement, hatching and heart rate, and development (yolk and pericardial sac edema) of zebrafish embryos in both time- and concentration-dependent manner only at ≥ 100 μg/ml. The corresponding 24 h-, 48 h- and 96 h-LC50 values of propoxur in zebrafish embryos were 166.4 ± 1.06, 146.3 ± 1.07 and 134.8 ± 1.06 μg/ml, respectively. The above data obtained suggest a low acute toxicity of propoxur to the in vitro cultured FG cells and zebrafish embryos.

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1. Introduction

Propoxur (2-isopropoxyphenyl methylcarbamate) is a N-methylcarbamate insecticide and acaricide. It was introduced to the market in 1959 and has been widely used against turf, forestry, and household pests and fleas. It is also used in pest control for other domestic animals, mosquitoes, bugs, ants, gypsy moths, and other agricultural pests (Tomlin, 1994). The main toxic mechanism of action for propoxur in target species is to inhibit the activity of acetylcholinesterase (AChE), resulting in the disruption of normal nervous system function causing a rapid “knockdown” effect and possible death (Baron, 1991; WHO, 2005). In addition, propoxur has also been shown to be toxic to non-target species like honeybees, birds and even mammals including humans, though its toxicity varies according to the species (CDPR, 1997; FAO, 2006; Lakota et al., 1981). Propoxur is classified to be moderately toxic (Toxicity Category II) for oral exposure and slightly toxic (Toxicity Category III) via the dermal and inhalation routes of exposure by the U. S. Environmental Protection Agency (EPA). It is also classified as a Group B2 probable human carcinogen (USEPA, 1997). Propoxur may remain in the environment for weeks to several months, longer than most carbamates. It is also likely to be moderately persistent and mobile in soils, having characteristics which could produce leaching to groundwater (USEPA, 1997) and may impose adverse effects on human health.

Despite the obsolete and restrictive use of propoxur in some regions, the widespread application of propoxur have been found increasing particularly in the developing countries, propoxur has attracted increasing concerns on the safety of aquatic organisms as this pesticide eventually ends up into the aquatic environment. Fish are often used for monitoring toxicity in the aquatic environments. Accumulating evidences showed that propoxur is moderately to slightly toxic to freshwater fish (FAO, 2006). Lakota et al. (1981) determined the behavioral changes and median lethal concentration (LC50, 7.34 μg/ml propoxur) of five month-old common carp (Cyprinus carpio) fries after exposure to propoxur. Srivastava and Singh (1982) reported the 96 h-LC50 value of 6.50 μg/ml propoxur for adult female Indian catfish (Heteropneustes fossilis) and the acute toxicity of propoxur on carbohydrate metabolism. The reported 96 h-LC50 values of propoxur in rainbow trout (Salmo gairdneri), fathead minnow (Pimephales promelas), bluegill (Lepomis macrochirus) and goldfish (Carassius auratus) were 8.13, 25.12, 4.78 and 36.2 μg/ml, respectively (Vittozzi and De Angelis, 1991; Wang...
et al., 2009), Hanson et al. (2007) examined the toxic effects of propoxur on the growth and reproduction, as shown by gonadosomatic indices, of three freshwater fish species of Nile tilapia (Oreochromis niloticus), Bagrid catfish (Chrysichthys nigrofasciatus) and African catfish (Clarias gariepinus). Recently, sublethal toxicity and in vivo genotoxicity of propoxur in the common carp (C. carpio) were evaluated by hematological/biochemical biomarkers and histopathological examination and indicated the genotoxic potential of this insecticide (Gul et al., 2012). However, all the above-mentioned studies have focused on the toxicity of propoxur on juvenile and adult fish species, and the embryotoxicity of propoxur on fish is lacking.

Fish embryos and larvae are generally the most sensitive stages in the life cycle of teleosts (Laale and Lerner, 1981; Lele and Krone, 1996) and they are ideal for determining the toxic responses to environmental pollutants. Thus fish embryo toxicity (FET) tests have been developed to evaluate the developmental toxicity of toxicants in fish, and most of these tests were carried out in zebrafish embryo because of its transparency and easy maintenance. The transparent nature of eggs and embryos allows the visualization of morphological and structural abnormalities in the whole body following exposure to chemicals (Brannen et al., 2010; Fraysse et al., 2006; OECD, 2006). Up to date, studies on the developmental toxicity of carbamate pesticides in zebrafish embryos are scarce. Lin et al. (2007) reported the toxic effects of carbachlor, a kind of carbamate pesticide, on zebrafish embryos including the viability, malformations in the tail region, pericardial edema, red blood cell accumulation and bradycardia. Schock et al. (2012) also observed the defects in heart formation, decreased heart rate and developmental delay/defect in cardiac looping in zebrafish embryos after exposure to carbachlor. The toxic effects of another carbamate insecticide aldicarb and its metabolite aldicarb-sulfoxide to zebrafish embryos demonstrated sublethal effect, with the significant increase of heart rate at lower concentrations up to 1 μM and decrease at test concentrations above 30 μM, only for aldicarb-sulfoxide on the organismic level (Kuster and Altenburger, 2007). Thus investigating the embryotoxicity of propoxur in zebrafish will markedly contribute to the safety assessment of propoxur for aquatic organisms.

In vitro cell cultures, as an alternative of whole animals, provide us a useful tool to examine the cytotoxic and genotoxic effects of chemicals and environmental pollutants in a rapid and cost-effective way (Bols et al., 2005). Although negative carcinogenic and teratogenic toxic effects were observed in live rats and rabbits (FAO, 2006), cytotoxic and inconsistent genotoxic effects of propoxur have not yet been reported in cultured mammalian cells. Wang et al. (1998) found that the propoxur has less cytotoxic effect than its N-nitroso derivative in the hamster lung fibroblast (V79) cells and primary rat tracheal epithelial (KTE) cells. However, no mutagenic effects of propoxur were recorded on either type of the above-mentioned cells. Maran et al. (2010) also reported the growth inhibitory effect of propoxur in Chinese Hamster Ovary cell line (CHO-K1). But Ündeger and Basaran (2005) reported that the propoxur significantly induced DNA damage in human peripheral lymphocytes at all concentrations tested. However, information regarding the cytotoxicity and genotoxicity of propoxur to fish cell line is unknown.

Up to now, various in vitro cultured fish cells have been used for the safety assessment of chemicals and environmental pollutants in fish (Babcich and Borenfreund, 1991; Castaño et al., 2003; Segner, 1998). The continuous marine flatfish cell line of flounder gill (FG), maintained in our laboratory since its establishment by Tong et al. (1997), has already been successfully used to evaluate the cytotoxicity and genotoxicity as well as the corresponding toxic mechanism of action of an array of environmental pollutants (Guo and Zhang, 2002; Li and Zhang, 2001; Na et al., 2009; Xiao et al., 2011, 2007; Yang et al., 2010; Yin et al., 2007). However, the toxic effects of propoxur have not yet been evaluated in FG cells.

The main purpose of the present study was therefore to examine the toxic effects of propoxur in the in vitro cultured FG cells and zebrafish embryos with a view to record its cytotoxicity, genotoxicity and embryotoxicity. In specific, in vitro studies were performed using FG cell line to determine the cytotoxic effects of propoxur by MTT reduction, neutral red (NR) uptake, lactate dehydrogenase (LDH) release, and Hoechst 33342 and propidium iodide (PI) double staining assays; genotoxic effect was evaluated by comet assay. The embryotoxicity test dealt with the assessment of propoxur in developing zebrafish embryos by observing diverse general morphological endpoints.

2. Material and methods

2.1. Chemicals

Propoxur (2-isopropoxyphenyl methylcarbamate, 99.8% pure), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), neutral red (NR), dimethyl sulfoxide (DMSO), low melting point agarose (LMPA), 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), ethylene diamine tetraacetic acid (EDTA), trypsin and tris base were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade. Plastic cell culture flasks (25 cm²) and 24- and 96-well culture plates were from Corning Incorporation (NY, USA). A 100 μg/ml stock solution of propoxur was prepared in DMSO and stored at 4 °C before being used. Working solutions were prepared by dilution of the stock solution into culture media immediately before use. The final concentration of DMSO in the working solutions was always <0.5% (v/v).

2.2. Cell line

The continuous flounder gill (FG) cell line, derived from the gill tissues of flounder (Paralichthys olivaceus) and maintained in this laboratory since 1993 (Tong et al., 1997), was used for cytotoxicity and genotoxicity assays. Briefly, the cells were cultured at 20 °C in minimal essential medium (MEM; Gibco BRL, New York), supplemented with 10% bovine calf serum (BCS; Hyclone, USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin, buffered to pH 7.4 in plastic cell culture flasks at 20 °C.

2.3. Zebrafish and eggs

Adult zebrafish (Danio rerio) were purchased locally from a fish dealer and acclimatized and kept in five glass aquaria of 10 l each filled with matured water. They were maintained at 26 ± 1 °C under a 14/10 h (light/dark) photoperiod cycle (Westerfield, 2000). Fish were fed twice daily with live nematodes and a part of the water was exchanged every day. In the evening, male and female fish (2:1) were placed in a spawning box. Spawning was triggered once the light was turned on the next morning and the fertilized eggs were collected and examined under a stereomicroscope.

2.4. MTT assay

MTT assay, described by Borenfreund et al. (1988), is based on the reduction of soluble yellow MTT tetrazolium salt to a blue insoluble MTT formazan product by mitochondrial succinic dehydrogenase in cells. MTT assay was carried out according to the method described previously (Yang et al., 2010). Briefly, FG cells were exposed to fresh MEM medium containing 0 (control), 0.5, 1, 10, 25, 50, 75, 100, 150, and 200 μg/ml propoxur, respectively.
for 24 h, then incubated with 20 μl aliquots of 5 mg/ml MTT in phosphate-buffered saline (PBS) per well (96-well plates) for 4 h. After removal of MTT solution by gentle aspiration and PBS rinse, the formazan crystals formed upon reduction of MTT were dissolved by adding 150 μl DMSO per well and the absorbance was measured by spectrophotometer (Tecan GENios, Austria) at 490 nm. The 24 h-IC$_{50}$ (50% inhibitory concentration after a 24 h exposure period of the test agent) value was determined by logistic nonlinear regression analysis (dose–response-inhibition, log (inhibitor) vs. response-variable slope) using the GraphPad Prism 5 software.

Further validation of the cytotoxicity of propoxur was done by a time-dependent MTT assay using 75 μg/ml propoxur, which is about 80% of 24 h-IC$_{50}$ value of concentration-dependent MTT assay. Briefly, FG cells were exposed to 75 μg/ml propoxur in MEM and sampled at 3, 6, 9, 12, and 24 h, respectively, and subjected to MTT assay.

All concentrations were tested in triplicate and the mean absorbance at each concentration was calculated and expressed as the percentage of absorbance of treated cells against the control.

2.5. Neutral red (NR) uptake assay

NR uptake assay was conducted following Borenfreund and Puerner (1985). It measures the inhibition of cell growth, which is based on the absorbance of the vital dye NR by living cells. In brief, after 24 h exposure of FG cells to 0, 0.5, 1, 10, 25, 50, 75, 100, 150, and 200 μg/ml propoxur, the MEM in each well was removed and replaced with 200 μl MEM containing 50 μg/ml NR. After in situ incubation for 3 h at 37 °C, the cells were rinsed with warm PBS followed by de-staining with 200 μl glacial acetic acid:ethanol:water (1:50:49) solution. The cells were then let to stand for 10 min at room temperature with gentle agitation on a microplate shaker, followed by measurement of absorbance from each well at 540 nm with a microplate reader, and then the 24 h-IC$_{50}$ value was calculated as previously described.

2.6. LDH release assay

LDH release assay examines the changes of membrane permeability in the exposed cells, by detecting the LDH leakage from the cytosol of damaged or lysed cells into the supernatant (Korzenniowski and Callewaert, 1983). Following exposure of FG cells to the varied concentrations (0, 1, 50, 75, 100 and 150 μg/ml) of propoxur for 24 h, the medium was collected and centrifuged at 250 × g for 10 min and the cell-free supernatant was obtained for LDH activity assay using a commercial LDH detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. Briefly, NADH and pyruvate (0.1%, w/v) were added into the samples and incubated at 37 °C for 15 min followed by further incubation with 25 μl 2, 4-dinitrophenyhydrazine for 15 min. The reaction was stopped by the addition of 250 μl 0.4 mol/l NaOH and the absorbance was measured at 450 nm in the microplate reader. The spontaneous LDH release was also examined by analyzing the LDH activity in the cell-free supernatants from the control wells. And the maximal LDH release was determined by analyzing the LDH activity in the supernatants from the control wells after the cells were lysed by ultrasonic vibrations followed by centrifugation. The LDH release percentage for each sample was then calculated according to the following equation:

LDH leakage (%) = (Experimental release OD – Spontaneous release OD)/maximal release OD × 100.

Further validation of the results was done by a time-dependent LDH release assay using 75 μg/ml propoxur. Briefly, following exposure of FG cells to 75 μg/ml of propoxur in MEM, the exposed cells were sampled at 3, 6, 9, 12, and 24 h, respectively, and subjected to LDH release assay as described above.

2.7. Hoechst 33342 and propidium iodide (PI) double staining assay

The discrimination of normal, apoptotic and necrotic cells was carried out by double fluorescence staining of the nuclei of FG cells after exposure to varied concentrations of propoxur for 24 h. The ‘all-cell’ dye of Hoechst 33342 is membrane permeable and can stain all the nucleated cells blue (excitation filter: 346 nm, emission filter: 460 nm), that is, both live and dead cells were detected with blue nuclei. Nevertheless, Hoechst 33342 tends to stain the condensed chromatin in apoptotic cells more brightly than that in live cells, allowing for the discrimination of apoptotic cells from live cells. However, the ‘dead-cell’ dye of PI is not membrane permeable and can only stain the dead cells red (excitation filter: 488 nm, emission filter: 615 nm). Therefore, light blue and light red nuclei were found in the live cells, bright blue and light red nuclei in the apoptotic cells, and light blue and bright red in the dead cells, respectively, under the fluorescence microscope (Carl Zeiss).

Briefly, cells were seeded into 24-well plates and exposed to different concentrations (0, 0.5, 1, 10, 50, 100, 150 μg/ml) of propoxur for 24 h followed by washing the cells twice with PBS. Hoechst 33342/PI double staining kit (Shanghai Majorbio Bio-Pharm Technology Co. Ltd., Shanghai, China) was used to stain the exposed cells according to the manufacturer’s instructions. For this, fresh 400 μl staining solution containing 5 μg/ml each of Hoechst 33342 and PI was added to each well and incubated for 15 min at room temperature in darkness followed by washing with PBS. Finally, the cells of blue and red fluorescence were separately examined under fluorescence microscope.

2.8. DNA fragmentation assay

DNA fragmentation assay was performed to determine whether the cell damage was associated with apoptosis or necrosis at genome level by propoxur using DNA ladder technique and visualized by electrophoresis in agarose gels. Cell culture flasks were each seeded with 5 × 10$^5$ FG cells and cultured at 20 °C for 24 h. When the cells reached desired density, 0, 50, 100, and 150 μg/ml of propoxur were added to each flask and the cells were incubated for 24 h followed by centrifugation at 1600 × g for five minutes to harvest the FG cells. Then DNA was extracted from the exposed cells using DNA extraction kit (CoWin Biotech Co., Ltd., Beijing, China) and 10 μl of each DNA sample was loaded on 2% agarose gel containing ethidium bromide and the DNA bands were visualized with a UV-illuminated camera (Peiqing, China).

2.9. Comet assay

Comet assay, also called single-cell gel electrophoresis, allows detecting DNA breakages induced by genotoxic agents and has been modified variously to suit different types of cell assays (Collins, 2004). In this study, comet assay was performed as described by Singh et al. (1988). Briefly, cell culture flasks were each seeded with 5 × 10$^5$ FG cells and cultured at 20 °C for 24 h. The FG cells were then exposed to 0 (negative control), 1, 10, 25, 50, and 75 μg/ml propoxur for 24 h, respectively. FG cells exposed to 5 μg/ml bleomycin were used as a positive control. The cells were harvested by trypan blue exclusion test. In order to avoid false positive response of the toxicant due to cytotoxicity, cell viabilities exceeding 75% were further processed for the evaluation (Henderson et al., 1998).
The comet assay was carried out according to the method described previously (Yang et al., 2010). Two slides per specimen were prepared and 50 cells per slide (total 100 cells per concentration) were scored randomly and analyzed visually according to the relative intensity of the fluorescence in the tail that classified the comets from grades 0–3 (from undamaged, 0 to maximally damaged, 3) using fluorescence microscope (Carl Zeiss) equipped with appropriate filters. The total scores for the sample gel were between 0 and 300 arbitrary unit (au). All the experiments were performed in triplicate. The same observer (MR Pandey) analyzed all the slides throughout the study to minimize inter-scoring variability.

For the time-dependent genotoxicity test, 75 μg/ml of propoxur was chosen. Briefly, cell culture flasks were each seeded with 5 x 10^6 FG cells and incubated at 20 °C for 24 h. After removal of media, the FG cells were exposed to 75 μg/ml of test medium and the exposed cells were sampled at 3, 6, 12, 24, 48, 72, and 96 h, respectively, and subjected to comet assay.

2.10. Embryotoxicity assay

The assay was based on the OECD draft guideline on Fish Embryo Toxicity (FET) test (OECD, 2006) and was carried out as described by Fraysse et al. (2006). The test started with newly fertilized eggs exposed to different concentrations of 0, 25, 50, 100, 200, 400 and 800 μg/ml of propoxur prepared by dilution with standard E 3 ("egg water": 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂ and 0.33 mM MgSO₄, pH (7.0 ± 1.0)) (Brand et al., 2002), and run for 5 days. The eggs were cultured in 24-well microplates at 28.5 °C with one egg per well in 2 ml E₃ solution. Twelve of normally fertilized eggs at 2- to 4-cell stage were selected for each concentration (84 eggs per experiment) and the experiment was repeated four times. Embryos and larvae were observed daily under a stereomicroscope connected to a camera device (Olympus SZX12, Japan) at specific time points (4, 8, 12, 24, 48–60, 72–84, and 96 h). During the period of 48–60 h and 72–84 h records were made every 2 h and 4 h, respectively for hatching rate calculation. Heart beat rates and spontaneous movements were recorded by visual inspection for 20 s each (Fraysse et al., 2006; Lin et al., 2007). In the embryo phase, the parameters evaluated were egg coagulation, malformations, eye development, body pigmentation, otolith formation, spontaneous movement, hatching rate, heart beat rate and mortality.

2.11. Data analysis and statistics

All the experiments were conducted at least in triplicate. Statistical analyses were performed using the GraphPad Prism 5 (GraphPad software, Inc., San Diego, USA) and SPSS 16.0 (SPSS Inc., Chicago, Il.). The statistical tools used for the comparison between groups depend on the type of data. Data were presented as the mean ± SEM. The data were tested for homogeneity and normality. If these assumptions were met, one-way analysis of variance (ANOVA) was performed to detect the significant differences between the groups for normally distributed data set, otherwise, the non-parametric test viz. the Kolmogorov–Smirnov (KS) normality test and χ² tests were performed in order to evaluate the non-normal distribution of group data to assess the homogeneity between the tested concentrations. If significant results were found, the post hoc range tests and pairwise multiple comparisons were made using the Tukey HSD test (depending on the nature of the test, parametric or non-parametric, respectively) to verify differences between the tested concentrations and control. Significance level was set at p < 0.05.

3. Results

3.1. In vitro cytotoxic effects of propoxur in FG cells

3.1.1. Toxic effects on the growth of FG cells

Propoxur treatment was seen to inhibit the proliferation of FG cells in a concentration-dependent manner as evidence from Fig. 1A, which indicates a decrease in the viability of FG cells with an increase in propoxur concentration. Significant toxic effects were observed at concentrations of >75 μg/ml propoxur. The 24 h-I₅₀ values for propoxur in FG cells determined by MTT and NRU assays were 89.96 ± 1.04 and 103.4 ± 1.14 μg/ml, respectively. A time-dependent cytotoxic effect was also observed in the FG cells after exposed to 75 μg/ml propoxur for 3–24 h as determined by MTT assay (Fig. 1B). And statistically significant (p < 0.05) toxic effect in the time-course toxic test was obtained only after 24 h exposure.

3.1.2. Toxic effects on the membrane integrity of FG cells

The toxic effects of propoxur on the membrane integrity of FG cells were examined by LDH release assay. As shown in Fig. 2A, the cells displayed increasingly significant LDH leakage after 24 h exposure to 75 μg/ml propoxur and above. The obtained 24 h-I₅₀ value for LDH release assay was 86.59 ± 1.13 μg/ml propoxur, indicating a good agreement with the 24 h-I₅₀ value obtained by MTT assay. Similar to MTT assay, a time-dependent cytotoxic effect on the membrane integrity of the FG cells after exposed to 75 μg/ml propoxur for 3–24 h was also observed (Fig. 2B). And statistically significant (p < 0.05) toxic effect in the time-course toxic test was obtained only after 24 h exposure.

The above data by LDH release assay were further confirmed by examining the morphological changes of the exposed FG cells using inverted microscope (Nikon Eclipse, TS100, Japan). The normal morphology of FG cells is epitheloid and the cells are well spread (Tong et al., 1997). Obvious morphological changes were observed in the FG cells after exposed to 100 μg/ml propoxur and above for 24 h (Fig. 3), inferring that the release of LDH had happened before observable morphological changes occurred. With further increase of the concentration of propoxur, the treated cells started to shrink and distort into irregular shape, and eventually detached from the substrate surface and lysed.

3.1.3. Propoxur induced necrosis rather than apoptosis in the FG cells

In order to investigate the toxic mechanism of action of propoxur, live, necrotic and apoptotic FG cells after 24 h exposure to varied concentrations of propoxur were discriminated by Hoechst 33342 and PI double fluorescence staining. As shown in Fig. 4 and 5, based on the observations of the morphology and intensity of blue and red fluorescence of the nuclei of FG cells, the toxicity of propoxur was seen manifested in terms of necrosis and in a concentration-dependant way rather than apoptosis since apoptotic cells were noticed sparsely. Significant cell damage was observed at the concentration of 100 μg/ml propoxur and above.

3.2. In vitro genotoxic effects of propoxur in FG cells

3.2.1. No obvious DNA laddering was induced by the propoxur tested in the FG cells

DNA laddering is considered as a specific biochemical marker for the apoptotic cells. As shown in Fig. 6, only a single band was obtained in the treated FG cells for all the exposure concentrations of propoxur, which was the characteristic of intact genomic DNA. Thus, no obvious DNA laddering was induced by the propoxur tested in the FG cells.
Fig. 1. (A) Concentration-dependent in vitro cytotoxicity of propoxur to FG cells after 24 h exposure as determined by MTT and NRU assay. (B) Time-course cytotoxicity of propoxur at 75 μg/ml to FG cells within 24 h exposure period as determined by MTT assay. The percentage absorbance was calculated in comparison to untreated cells taken as 100%. Data are reported as means from three independent experiments and the values are expressed as mean ± SEM. *p < 0.05.

Fig. 2. (A) Concentration-dependent in vitro cytotoxicity of propoxur to FG cells as determined by LDH release assay. (B) Time-course cytotoxicity of propoxur at 75 μg/ml to FG cells within 24 h exposure period as determined by LDH release assay. The relative percentage of LDH release was calculated in comparison to untreated cells. Data are reported as means from three independent experiments and the values are expressed as mean ± SEM. *p < 0.05.

Fig. 3. Morphological changes of FG cells after exposed to varied concentrations of propoxur for 24 h. Panels A through D correspond to 0 (control), 100, 150, and 200 μg/ml propoxur, respectively.
3.2.2 Weak genotoxic response of the FG cells to propoxur by comet assay

The comet assay in the present study found that weak DNA damage occurred in the FG cells exposed to 10–75 μg/ml of propoxur. The scorings of treated cells based on the degree of damage according to comet appearance are shown in Table 1 and Fig. 7A–B. The maximal and acceptable concentration, at which necrosis did not interfere with the performance of comet assay (Henderson et al., 1998), was 75 μg/ml of propoxur. Despite the low incidence of DNA damage, the corresponding comet assay scores (au) of propoxur at different concentrations ranged from 14.33 ± 0.33 to 71.67 ± 14.31 (Table 1).

The DNA damage scores and the results demonstrated that the damage grades were not significantly different from the control (p > 0.05) up to 50 μg/ml propoxur and a little concentration response relationship was evident. Though not statistically significant up to 50 μg/ml, the cells were affected the most at a concentration of 75 μg/ml propoxur.

The results of genotoxic response for the time-dependent exposure to 75 μg/ml propoxur, based on the concentration that

![Fig. 4. Representative fluorescent micrographs showing the morphology and intensity of blue and red fluorescence of live, necrotic and apoptotic cells after 24 h exposure to varied concentrations of propoxur with Hoechst 33342 and PI double staining. The microscopic images in panels A and B depict control, C and D, E and F, and G and H for cells treated with propoxur at 50, 100 and 150 μg/ml, respectively. Panels A, C, E and G are stained with Hoechst 33342 whereas, cells in panels B, D, F and H are stained with PI. Live cell nuclei are seen in light blue and light red, dead cell nuclei are in light blue and bright red, and apoptotic cell nuclei are in bright blue and light red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image-url)
produced similar cytotoxicity, from 3 to 96 h in FG cells are shown in Table 2 and Fig. 7C. It is clear from the results that the DNA damage increased with the increase of the exposure period to propoxur. Significant DNA damage was seen from 24 to 96 h of exposure as compared with the control. The corresponding comet assay scores (au) at different exposure time ranged from 13 ± 3 to 91 ± 6.35 (Table 2).

3.3. Embryotoxicity of propoxur to zebrafish

A series of exposure studies were conducted on developing zebrafish embryos to determine the developmental effects and endpoints associated to propoxur exposure. The control group displayed normal embryonic development (Kimmel et al., 1995). Embryotoxicity of propoxur including malformations, mortality and alteration of function and other findings are as followed.

As shown in Fig. 8A–C, slowdown of epiboly was first evident at 12 hours post fertilization (hpf), at which time about 95.83 ± 2.41% of the control embryos were found in the stage of 90% epiboly whereas only 70.83 ± 5.38% and 2.08 ± 2.08% embryos were found reaching at the same stage for 100 and 200 µg/ml of propoxur exposure, respectively. The remaining exposed embryos were observed at the stage of about 50%–85% epiboly instead. In contrast, no obvious slowdown of epiboly was observed in the embryos exposed to 25 and 50 µg/ml propoxur at 12 hpf in comparison with the control. Morphological malformations of the exposed embryos were found after 12 hpf in comparison to control and mostly in the form of yolk sac edema first monitored at 24 hpf (Fig. 8 D–F), followed by pericardial sac edema from 48 hpf (Fig. 8G–L). And the number of the malformed embryos increased with concentration and in a time-dependent manner.

At 24 hpf, the total percentage effects for 100 and 200 µg/ml propoxur exposure were 30.34 ± 4.70% and 95.83 ± 4.17%, respectively for yolk sac edema. At 48 hpf, the edema percentages for the yolk and pericardial sacs of exposed embryos were similar and values of 35.83 ± 2.50% and 95.00 ± 5.00% were obtained for 100 and 200 µg/ml propoxur exposure, respectively. But at 96 hpf, the respective percentage effects for 100 and 200 µg/ml propoxur exposure were 24.17 ± 5.67% and 100% (for yolk sac), and 29.44 ± 5.91% and 100% (for pericardial sac). The alterations in body shape were also observed due to morphological malformations of developing embryos at the concentrations of 100 and 200 µg/ml propoxur.

As shown in Fig. 8A, on the average, embryos exposed to lower concentrations (i.e. ≤50 µg/ml) showed low rates of malformation (≤18.75 ± 0.02% up to 96 hpf) and mortality (≤10.42 ± 0.04% up to 48 hpf and ≤14.58 ± 0.04% at 96 hpf). However, the percentage of malformation and mortality increased as the concentration of propoxur administered increased, with malformation of ≤27.08 ± 0.04% and mortality of ≤27.08 ± 0.08% of the total embryos at 100 µg/ml up to 96 hpf. As the mortality increased at 200 µg/ml, the malformation of 27.08 ± 0.07% at 24 hpf.

Table 1
Concentration-dependent DNA damage as assessed by visual scoring of comet assays in the FG cells after 24 h exposure to different concentrations of propoxur. Comet scores are given in arbitrary units (au). The values are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>DNA damage score (AU)</th>
<th>No. of cells in each damage grade</th>
<th>Viability (%)</th>
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<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>11.33 ± 2.67</td>
<td>89.33 ± 2.33</td>
<td>10 ± 2</td>
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<tr>
<td>50</td>
<td>41.67 ± 13.17</td>
<td>71.67 ± 8.88</td>
<td>19.33 ± 6.01</td>
</tr>
<tr>
<td>75</td>
<td>71.67 ± 14.31</td>
<td>54.67 ± 10.17</td>
<td>27 ± 6.08</td>
</tr>
</tbody>
</table>

*p < 0.05.
18.75 ± 0.05% at 48 hpf, and 10.42 ± 0.02% of the total embryos at 96 hpf were observed.

As shown in Fig. 9B, zebrafish embryos exposed to increasing concentrations of propoxur for 24, 48 and 96 hpf demonstrated elevated mortality rates in obvious concentration-dependent manner, but in low time-dependent manner. The lowest observed effect concentration (LOEC) of propoxur was 100 μg/ml (p < 0.05) with 24 h-, 48 h- and 96 h-LC50 (50% lethal concentration after the exposure of test agent at certain time period) values of 166.4 ± 1.06 μg/ml, 146.3 ± 1.07 μg/ml and 134.8 ± 1.06 μg/ml, respectively.

Cumulative hatching rates from 48 to 96 hpf are shown in Fig. 9C. The statistically significant effect on hatching at 72 hpf was observed at 200 μg/ml of propoxur. However, we observed the significant effect for both concentrations (100 and 200 μg/ml) at 96 hpf when compared with the control (p < 0.05). But in low time-dependent manner. The lowest observed effect concentration (LOEC) of propoxur was 100 μg/ml (p < 0.05), however, no significant difference was observed at ≤50 μg/ml propoxur (p > 0.05).

Effects of different concentrations of propoxur on spontaneous movement of embryos were recorded from the movements of each embryo observed for about 20 s (Fig. 10A). Resulting effects were discrete and the distribution was not symmetric over the mean. About 25 ± 4.81% and 37.50 ± 4.17% of the embryos showed a frequency of movement of 2 and 3 per 20 s in control groups. But at 25 μg/ml propoxur exposure, about 37.50 ± 5.38% and 25 ± 4.81% of the embryos showed 2 and 3 movement frequency. However, at 50 μg/ml, 1 and 2 times movement frequency recorded were 18.75 ± 3.99% and 27.08 ± 2.08%, respectively and 0, 1, and 2 times movements at 100 μg/ml were 37.50 ± 7.22%, 20.83 ± 2.41% and 25 ± 3.40%, respectively. But at 200 μg/ml, the maximum of about 87.50 ± 7.98% of the embryos showed no spontaneous movement. The results show that the frequency of movement decreased with increasing concentrations of propoxur. As the obtained data are not suitable for parametric law, the comparison between the movement counts was done with Kolmogorov–Smirnov (KS) and χ² tests for the non-normal distribution of group data. KS test shows that there is no uniform distribution between the spontaneous movements among all tested concentration groups (p < 0.05). χ² test also indicates the statistical difference between the number of spontaneous movements (p > 0.12).

The results show that the frequency of movement decreased with increasing concentrations of propoxur. As the obtained data are not suitable for parametric law, the comparison between the movement counts was done with Kolmogorov–Smirnov (KS) and χ² tests for the non-normal distribution of group data. KS test shows that there is no uniform distribution between the spontaneous movements among all tested concentration groups (p < 0.05). χ² test also indicates the statistical difference between the number of spontaneous movements (p > 0.12).

Mean of heart rates of the embryos at 48, 72 and 96 hpf following exposure to propoxur at different concentrations (n = 48 for each concentration) are given in Fig. 10B. The mean heart beats ranged from 48.58 ± 0.70 to 24.38 ± 4.23 beats/20 s at 48 hpf; 51.57 ± 0.46 to 25.70 ± 4.89 beats/20 s at 72 hpf; and 53.59 ± 0.75 to 25.75 ± 4.33 beats/20 s at 96 hpf for control and 200 μg/ml, respectively.

Significant decline in heart rates were observed at propoxur concentrations of 100 and 200 μg/ml in comparison to control at 48, 72 and 96 hpf (p < 0.05). Moreover, we also observed that the heart rates of embryos showing yolk sac edema and pericardial edema were feeble and irregular and reduced to as low as 16 beats/20 s at 48, 72 and 96 hpf for the higher propoxur concentrations of 100 and 200 μg/ml. Heart rates were affected in 35.83 ± 2.50% and 95.00 ± 5.00% of embryos showing yolk and edema were feeble and irregular and reduced to as low as 16 beats/20 s at 48, 72 and 96 hpf for the higher propoxur concentrations of 100 and 200 μg/ml. Heart rates were affected in 35.83 ± 2.50% and 95.00 ± 5.00% of embryos showing yolk

Table 2
Detection of time-course DNA damage in FG cells on exposure to 75 μg/ml of propoxur. Comet scores are given in au. The values are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>DNA damage score (AU)</th>
<th>No. of cells in each damage grade</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.67 ± 2.33</td>
<td>90.33 ± 1.86 6.67 ± 1.45 3 ± 0.58 0</td>
<td>97.68</td>
</tr>
<tr>
<td>3</td>
<td>13 ± 3</td>
<td>89.67 ± 2.33 7.67 ± 1.67 2.67 ± 0.67 0</td>
<td>96.68</td>
</tr>
<tr>
<td>5</td>
<td>20 ± 0.58</td>
<td>80.67 ± 0.33 18.67 ± 0.88 0.67 ± 0.67 0</td>
<td>95.60</td>
</tr>
<tr>
<td>12</td>
<td>38.67 ± 7.54</td>
<td>69.67 ± 6.67 23.67 ± 5.90 5 ± 2.52 1.67 ± 0.88 89.02</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>64.67 ± 11.20</td>
<td>55 ± 6.93 29.67 ± 6.36 11 ± 2.52 4.33 ± 1.86 82.03</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>69.33 ± 11.32</td>
<td>56.33 ± 4.26 25 ± 1.53 11.67 ± 1.45 7 ± 2.89 80.92</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>79.33 ± 7.06</td>
<td>54.33 ± 3.28 23 ± 4.62 11.67 ± 0.33 11 ± 3.06 79.08</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>91 ± 6.35</td>
<td>46.67 ± 3.48 27.33 ± 1.20 14.33 ± 2.40 11.67 ± 0.33 77.30</td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.05.
pericardial sac edema at 48 hpf; 31.66 ± 1.76% and 100% at 72 hpf, and 29.44 ± 5.91% and 100% at 96 hpf for 100 and 200 µg/ml propoxur.

No abnormality was observed in other developmental endpoints, such as tail detachment, otolith formation, somite formation, eye development and body pigmentation in the treated embryos (Table 4).

4. Discussion

The present study was designed to assess the cytotoxic, genotoxic and embryotoxic effects of propoxur using in vitro cultured continuous fish cell line of FG and zebrafish embryos with concerns on the possible adverse effects of this pesticide along with their toxic potential.

The cytotoxicity of propoxur in in vitro cultured mammalian cells has been examined. The cytotoxicity of propoxur to mammalian V79 and RTE cells were evaluated and their LC50 values were estimated as 43 µg/ml and 288 µg/ml, respectively (Wang et al., 1998). Maran et al. (2010) determined the 24 h-IC50 values of propoxur in CHO-K1 cells as 96.0 ± 4.1 µg/ml and 112.2 ± 9.3 µg/ml for MTT and NRU assays, respectively. Similar cytotoxicity data were obtained in the present study with the 24 h-IC50 values of 89.96 ± 1.04 µg/ml, 103.4 ± 1.14 µg/ml and 86.59 ± 1.13 µg/ml propoxur in FG cells for MTT, NRU and LDH leakage assays, respectively. It seems that LDH leakage and MTT assays are more sensitive than NRU assay in the cytotoxicity detection of propoxur. Both the different mechanisms of toxicity detection for these assays and the toxic mechanism of action of propoxur in FG cells may account for the above-mentioned different sensitivity. LDH leakage assay detects the released LDH enzymes from the dead cells upon loss of their membrane integrity by toxicants. However, MTT assay is designed to detect the perturbation of the mitochondrial function of live cells by toxicants, whereas NRU assay is for the loss of lysosomal activity of live cells. But the NR accrual and retention is also dependent on an intact plasma membrane and adequate energy metabolism in addition to a functional lysosome (Bols et al., 2005). Schmuck and Mihail (2004) had showed a moderate toxic effect of propoxur on mitochondrial inner membrane potential of primary cells from brain tissues of fetal rats, and Maran et al. (2010) also suggested the involvement of the...
Fig. 9. Embryotoxicity of varied concentrations of propoxur on zebrafish eggs exposed for 24, 48 and 96 hpf. Panel (A) summarizes the total percentages of normal, malformed and dead embryos after 24, 48 and 96 hpf exposure (n = 48 for each concentration). Panel (B) demonstrates the obvious concentration-dependent and low time-dependent lethal toxicity of propoxur to zebrafish embryos. Panel (C) shows the cumulative hatching rates of the exposed embryos observed from 48 to 96 hpf. The values are expressed as mean ± SEM. (n = 48 for each concentration). *p < 0.05.

Table 3
Median hatching time (HT50) of zebrafish embryos on exposure to propoxur. The values are expressed as mean ± SEM. (n = 48 for each concentration).

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Control 25 µg/ml</th>
<th>50 µg/ml</th>
<th>100 µg/ml</th>
<th>200 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT50 (h)</td>
<td>55.14 ± 1.06</td>
<td>55.27 ± 1.06</td>
<td>55.27 ± 1.04</td>
<td>59.19 ± 1.02</td>
</tr>
</tbody>
</table>

**"-"** Indicates less and/or no hatched embryos.

* p < 0.05.
mitochondrial pathway of propoxur-induced cell death in CHO-K1 cells. Thus, the interruption of mitochondrial function and injury of membrane integrity by propoxur may to some degree result in a relatively lower sensitivity of NRU assay.

Taken together, propoxur imposed low acute cytotoxicity on FG cells, and the order of sensitivity for these three assays, based on their 24 h-IC_{50} values obtained, was LDH > MTT > NRU. In addition, the obtained cytotoxicity by propoxur in FG cells was closely correlated in all these assays, independent of the cytotoxic endpoints employed, which corroborated the view of Ekwall (1995) that most cell lines have a similar response to toxicants when toxicity is measured with various endpoints relating to basal functions and structures.
Morphological alterations are the primary indications of cytotoxicity and its underlying mechanisms. Despite very limited histopathology study on propoxur exposure, the gill tissues of carp fry were found to be affected by the exposure of propoxur causing alterations in the structure of gill tissues (Gul et al., 2012; Lakota et al., 1978; Meyers and Hendricks, 1982). Here, FG cells also demonstrated obvious morphological alterations like shrinkage, detachment and eventual lysis leading to the membrane breakage when exposed to 100 μg/ml propoxur and above for 24 h. Of interest, the significant LDH leakage was observed in the FG cells after 24 h exposure to 75 μg/ml propoxur and above, indicating that the LDH leakage had already happened before the obvious morphological changes occurred in the exposed cells.

It was also found in the present study that propoxur at the concentrations up to 100 μg/ml failed to induce apoptosis at significant levels in the exposed FG cells. Furthermore, no apparent DNA laddering was detected by agarose gel electrophoresis analysis and the results of the Hoechst 33342 and PI double staining assay also showed the necrotic appearance (nuclei in light blue and bright red) rather than apoptosis (nuclei in bright blue and light red), suggesting that the main pathway involved in the cytotoxicity by propoxur was not apoptosis. Thus, the overall cytotoxic response observed indicates that the propoxur cause significant cell death by necrosis.

Both positive and negative genotoxic effects have been reported for carbamates using different testing systems. For propoxur, positive genotoxic results were found in only 3 studies out of 40 in vitro and in vivo genotoxicity investigations as summarized by the Working Groups of WHO/FAO (1989). Since then, few articles have been published which suggest that this substance has a weak genotoxic effect (Siroki et al., 2001). Propoxur was found to be non-mutagenic whereas its nitroso derivative, nitroso-propoxur, was strongly mutagenic in Salmonella typhimurium, Escherichia coli and Saccharomyces cerevisiae (Blevins et al., 1977a; Seiter, 1977; Siebert and Eisenbrand, 1974). Furthermore, propoxur and its nitroso derivative, nitroso-propoxur, were also found genotoxic and increased the frequencies of sister-chromatid exchanges and micronuclei in human lymphocytes in vitro (Gonzalez Ced et al., 1990). However, it was found non-mutagenic to V79 and RTE cells, and not genotoxic to human skin cells and mouse bone marrow (Blevins et al., 1977b; Vasudev and Krishnamurthy, 1994; Wang et al., 1998). Significant DNA damage was observed at 50, 100 and 200 μg/ml of propoxur using comet assay in human lymphocytes (Undeger and Basaran, 2005). Comet assay is considered as a predictor of genotoxicity and possibly carcinogenic activity of chemicals in animal and human cells (Undeger and Basaran, 2005). But there are limited and inconsistent data in the literature about the genotoxic effects of propoxur in the comet assay and little is known about the genotoxic effects of propoxur on fish cells. In the present study, comet assay was found useful and sensitive for the genotoxicity detection of propoxur in FG cells, showing a weak genotoxic response in both concentration and time-dependent manner instead.

The toxic effects of propoxur in terms of LC50 values have been examined in several fish species (Gul et al., 2012; Hanso et al., 2007; Vitozzi and De Angelis, 1991; Wang et al., 2009). But the data regarding the embryotoxicity of propoxur to fish is lacking. In the present study, we observed significant toxic effects of propoxur on developing zebrafish embryos, as evidence of the slowdown of epiboly, edema of yolk and pericardial sac area, decrease of spontaneous movement, heart beat rate and hatching rates. As summarized in Table 4, the slowdown of epiboly of the exposed zebrafish embryos at 12 hpf was the earliest disrupted developmental process and was obvious only up to 100 and 200 μg/ml of propoxur exposure. Furthermore, the exposed embryos with delayed or unfinished epiboly at 12 hpf later ended in either malformation or mortality.

Spontaneous movements in the developing embryos indicate the uncontrolled action potential of the motoneurons (Frayss et al., 2006). And the joint development of muscular and motoneural systems are responsible for raising the frequency of spontaneous movements of the developing embryos (Kimmel et al., 1995; Myers et al., 1997). In the present study, at 24 hpf, a concentration-dependent decrease in the frequency of spontaneous movement was revealed in the zebrafish embryos after exposed to varied concentrations of propoxur, indicating an adverse effect of propoxur on the development of fish motoneural systems. Moreover, zebrafish has been reported to lack the butyrylcholinesterase (BChE) gene or activity (Bertrand et al., 2001). Only AChE is responsible for both acetylcholinohiole and butyrylcholinohiole hydrolysis in this species. Therefore, any adverse effects due to the presence of a cholinesterase inhibitor should only be caused by a disruption of AChE activity (Lin et al., 2007). The AChE inhibitory ability of propoxur may account for the toxic effects on the motoneural system as well as the heart rates in the developing zebrafish embryos (Behra et al., 2002; Lin et al., 2007; WHO, 2005).

Besides lowering of heart rates from 100 μg/ml, edemas of the yolk and pericardial sac were the obvious malformations in the exposed zebrafish embryos along with some other body shape deformity. Such type of teratogenic effects including malformations were also exhibited in zebrafish and medaka when exposed to other types of carbamate compounds (Lin et al., 2007; Schock et al., 2012; Solomon and Weis, 1979), but the effects can be toxicant- and concentration-specific. The observed delayed and retarded hatching of zebrafish embryos after exposed to relatively higher concentrations of propoxur might be due to the disturbance of the hatching enzyme and induced hypoxia by propoxur. The proteolytic haching enzyme, secreted from the gland cells of the hatching embryo, has the role of digesting the chorion during the normal hatching process of teleost embryos. Moreover, the interference of hatching-enzyme activity by osmotic disturbances, increased consumption of oxygen by embryos/larvae, and behavioral deficits resulting in weakened spontaneous muscular movement are other factors that can result in the hatching delay as described earlier in other literatures (David and Pancharata, 2009; Haendel et al., 2004; Strmac et al., 2002). But it is difficult to pinpoint the actual causes at present. And this type of hatching delay was also observed earlier in another carbamate insecticide, carbaryl (Lin et al., 2007; Todd and Van Leeuwen, 2002).

In summary, the current study demonstrated that the exposure of FG cells to propoxur for 24 h could induce low acute cytotoxic and weak genotoxic effects in a concentration-dependent manner. FG cells also reacted differently to the different cytotoxicity assays, confirming the suitability of this cell line in the screening of cytotoxic and genotoxic effects of this type of pesticide. Using zebrafish embryo as a model, our research also provided more information on the toxic effects of propoxur on the early embryo development of fish. The exposed zebrafish embryos exhibited a series of toxic effects, including mortality, slowdown of epiboly, decreased spontaneous movement, yolk and pericardial sac edemas, lowering of heart rates and hatching rates as well as delayed hatching (at 100 μg/ml). In addition, the concentration responsive endpoints analyzed in all the tests in FG cells indicate that the significant toxic effects at all the concentrations tested were observed from 75 μg/ml propoxur. However, we observed significant toxic effects in the developing zebrafish embryos at 100 and 200 μg/ml of propoxur except the effect on cumulative hatching rate at 72 hpf, in which we found significant only at the concentration of 200 μg/ml. Fish embryos, different from in vitro cultured FG cells, have protective chorions. This difference may to some degree account for the relatively higher concentration of propoxur in embryotoxicity examination than cytotoxicity tests. Lawrence et al. (2009) reported the highest level of propoxur in river up to 0.97 μg/l, a
level much lower than the LOEC (>100 μg/mL) for zebrafish embryos obtained in this study, thus relieving our safety concerns of propoxur to fish population in this case.

Conflict of interest

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

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2013.11.010.

References


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