Zebrafish larva as a reliable model for in vivo assessment of membrane remodeling involvement in the hepatotoxicity of chemical agents

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Title

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Short Title

Zebrafish larva for assessing membrane remodeling by hepatotoxicants

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Abstract

The easy-to-use *in vivo* model, zebrafish larva, is increasingly used to screen chemical-induced hepatotoxicity, with a good predictivity for various mechanisms of liver injury. However nothing is known about its applicability in exploring the mechanism called membrane remodeling, depicted as changes in membrane fluidity or lipid raft properties. The aim of this study was therefore to substantiate the zebrafish larva as a suitable *in vivo* model in this context. Ethanol was chosen as a prototype toxicant because it is largely described, both in hepatocyte cultures and in rodents, as capable of inducing a membrane remodeling leading to hepatocyte death and liver injury. Zebrafish larva model was demonstrated to be fully relevant since membrane remodeling was maintained even after a week-exposure without any adaptation as usually reported in rodents and hepatocyte cultures. It was also proven to exhibit a high sensitivity as it discriminated various levels of cytotoxicity depending on the extent of changes in membrane remodeling. In this context, its sensitivity appeared higher than that of WIF-B9 hepatic cells, which is suited for analyzing this kind of hepatotoxicity. Finally, the protection afforded by a membrane stabilizer, UDCA, or by a lipid raft disrupter, pravastatin, definitely validated zebrafish larva as a reliable model to quickly assess membrane remodeling involvement in chemical-induced hepatotoxicity. In conclusion, this model, compatible with a high throughput screening, might be adapted to seek hepatotoxicants *via* membrane remodeling, and also drugs targeting membrane features in order to propose new preventive or therapeutic strategies in chemical-induced liver diseases.

Short Abstract for Table of contents

The easy-to-use *in vivo* model, zebrafish larva, is increasingly used to screen chemical-induced hepatotoxicity. However nothing is known about its applicability in exploring the mechanism called membrane remodeling, depicted as changes in membrane fluidity or lipid raft properties. Its suitability and its sensitivity were demonstrated by the ability to maintain membrane remodeling after a week-ethanol exposure, and by alleviating or enhancing ethanol-induced hepatotoxicity after membrane manipulation with either a membrane stabilizer, lipid raft disrupter or stimulator of lipid raft clustering.

1. Introduction

In the last decade, an increasing body of evidence led to consider plasma membrane as a pivotal player in the first cell signaling events responsible for the early onset of chemical-induced tissue damages (Tekpli et al., 2013). Indeed, *in vitro* exposure to various xenobiotics has been reported to induce plasma membrane remodeling depicted as changes in membrane fluidity (Sergent et al., 2005; Tekpli et al., 2011) or physical and chemical alterations of lipid rafts (Tekpli et al., 2013). Lipid rafts are specialized membrane microstructures with highly packing and increased rigidity, stemming from their enrichment in sphingolipids, cholesterol and saturated fatty acids. These extremely dynamic microdomains are considered as membrane domains in a liquid ordered (Lo) phase among the bulk membrane in a liquid disordered (Ld) phase. They are able to cluster each other, building new signaling platforms thereby regulating intracellular pathways (Brown and London, 2000). Plasma membranes of the liver, which is the main organ for detoxification of exogenous compounds, are particularly targeted by chemical agents. Thus, numerous research efforts carried out *in vitro* in hepatic cells proved both 1) changes in membrane features and 2) their involvement in chemical-induced cytotoxicity (Table 1). However, *in vivo*, the complete demonstration of the involvement of membrane remodeling in chemical-induced liver injury was rarely performed (Table 2). Indeed, concerning membrane remodeling assessment, only very few papers showed lipid raft alteration *in vivo*. Most of them did not test compounds able to modify membrane fluidity or lipid raft structures before analysis of liver injury, thus excluding a direct demonstration of the membrane remodeling involvement in hepatotoxicity. In addition, the few rare articles that claimed the involvement of lipid rafts, used indirect ways, i.e. transgenic rodents knock out for proteins well-known to need recruitment to lipid rafts for their activation (Table 2). These difficulties might be explained by the fact that usual techniques in membrane remodeling assessment in rodents need large amount of biological samples or are time-consuming, thus limiting the screening of chemical toxicants. However, *in vivo* models are absolutely necessary because they are recognized to be more sensitive to hepatotoxicants (O'Brien *et al.*, 2006), and to integrate the complete physiology notably intercellular interactions that are particularly important in the liver.

This is the reason why we were interested in developing another model, the zebrafish larva (Danio rerio), which shares a large genomic homology with human (Howe *et al.*, 2013). Liver development in zebrafish is quite similar, but faster compared to those of higher

vertebrates like rodent or human; thus, a fully functional liver is obtained after 120 h post-fertilization (Goessling and Sadler, 2015). In addition, zebrafish larvae express a large number of phase I and phase II metabolizing enzymes with a strong analogy to human liver enzymes like cytochromes P450 3A4, 1A1, 2B6 (McGrath and Li, 2008; Alderton *et al.*, 2010), as well as the whole pathways needed to metabolize ethanol, including alcohol dehydrogenase and cytochrome P450 2E1 (Passeri *et al.*, 2009; Tsedensodnom *et al.*, 2013). Furthermore, zebrafish embryos and larvae remain transparent up to fourteen days, allowing quick and easy observation of internal organs, especially liver. They have a small size (1-4 mm) and are really easy to maintain, making them cost-effective for an *in vivo* study. Thus, this model has been successfully chosen to develop a predictive tool for assessing chemical-induced hepatotoxicity with similar effects to those obtained in rodents or human (Sukardi *et al.*, 2011; Hill *et al.*, 2012; Driessen *et al.*, 2013; He *et al.*, 2013; Vliegenthart *et al.*, 2014; Mesens *et al.*, 2015; Verstraelen *et al.*, 2016). In addition, it has been increasingly used to study mechanisms of liver injury following acute exposure to a classical hepatotoxicant, alcohol (Passeri *et al.*, 2009; Howarth *et al.*, 2011; Tsedensodnom *et al.*, 2013).

The aim of this study was therefore to substantiate the zebrafish larva as an *in vivo* model for investigating membrane remodeling in chemical-induced hepatotoxicity. To this end, ethanol was chosen as a prototype toxicant capable of inducing a membrane remodeling leading to hepatocyte death and liver injury. Indeed, as shown in tables 1 and 2, this chemical is one of the most studied in this context, by our team and others, both *in vitro* and *in vivo*. As we possess a good knowledge of membrane remodeling in the context of ethanol hepatotoxicity *in vitro* (Sergent *et al.*, 2005; Nourissat *et al.*, 2008; Aliche-Djoudi *et al.*, 2011, 2013), we decided to compare zebrafish larva responses with those obtained in an *in vitro* model of hepatic cells in order to fully validate the zebrafish model. Both structural disturbances of plasma membrane and their involvement in ethanol hepatotoxicity were determined *in vitro* and *in vivo*.

2. Materials and methods

2.1. *In vitro* and *in vivo* models

2.1.1. WIF-B9 hepatic cells

The WIF-B9 hybrid cells obtained by fusion of Fao rat hepatoma cells and WI 38 human fibroblasts were a generous gift from Doris Cassio (UMR Inserm S757, Université Paris-Sud,

Orsay, France). Cells were cultured in F-12 Ham medium with Coon's modification (Sigma-Aldrich, Saint Quentin Fallavier, France) containing 5 % fetal calf serum (Bio West, Nuaille, France), 0.22 g/L sodium bicarbonate, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.25 µg/mL amphotericin B (Sigma-Aldrich, Saint Quentin Fallavier, France), 2 mM glutamine and supplemented with HAT (10 µM Hypoxanthine, 40 nM Aminopterin, 1.6 µM Thymidine) (Gibco, Life Technologies, Courtaboeuf, France). They were incubated at 37°C in an atmosphere constituted of 5% CO_2 and 95% air. They were seeded at 12.5 x 10^3 cells/cm^2 and cultured 7 days in order to obtain approximately 80% confluence before drug exposure. Cultures were treated with 25 mM ethanol for 4 days. PUFAs were used at a final concentration of 10 µM. Cultures treated with PUFA vehicle (methanol 0.0065 %) were used as controls. Mediums and treatments were renewed daily. These treatments were briefly resumed in Table 3. For the experiments dealing with the involvement of membrane remodeling in oxidative stress and cell death, 25 µM UDCA (Calbiochem, Meudon, France), a membrane stabilizer, was added 1 hour before addition of ethanol.

2.1.2. Zebrafish larvae

Animals were handled, treated and killed in agreement with the European Union regulations concerning the use and protection of experimental animals (Directive 2010/63/EU). All protocols were approved by local ethic committee CREEA (Comité Rennais d'Ethique en matière d'Expérimentation Animale). Zebrafish fertilized embryos, collected following natural spawning, were obtained from the Structure Fédérative de Recherche Biosit (INRA LPGP, Rennes, France). Embryos and larvae were raised at 28°C according to standard procedures. Zebrafish larvae (sex unknown) were maintained on a 14:10 hour light:dark cycle at 28°C in embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) containing 0.416 mg/L methylene blue. From 4 days post-fertilization (dpf) until 12 days post-fertilization, larvae were fed daily with a standard diet (TetraMin®) during 1 hour before medium renewal. At 5 days post-fertilization, zebrafish larvae were treated by 43 mM ethanol (Prolabo, Paris, France) directly added to the incubation medium. Mediums and treatments were renewed each day until the end at 12 days post-fertilization. For PUFA experiments, 0.5 µM EPA and DHA (Sigma-Aldrich, Saint Quentin Fallavier, France) were added. Larvae treated with PUFA vehicle (methanol 0.000325%) were used as controls. These treatments were briefly resumed in Table 3. Some experiments were also realized with 10.75 or 21.5 mM ethanol in order to test the sensitivity of the model with regard to membrane remodeling and

toxicity. For the experiments dealing with the involvement of membrane remodeling in hepatotoxicity, 0.5 µM UDCA, a membrane stabilizer, and 0.5 µM pravastatin (PRAVA, Sigma-Aldrich, Saint Quentin Fallavier, France), a lipid raft disrupter which acts through inhibition of cholesterol synthesis, were added.

2.2. Toxicity evaluation

2.2.1. WIF-B9 cell death

WIF-B9 cells were tested for both apoptotic and necrotic cell death by fluorescence microscopic observation of cells stained with Hoechst 33342 (Life Technologies, Courtaboeuf, France) and propidium iodide (Sigma-Aldrich, Saint Quentin Fallavier, France). After each treatment, cells were stained with 10 µg/ml Hoechst 33342 and 10 µg/mL propidium iodide in the dark for 15 min at 37°C. Cells were then examined under fluorescence microscopy (Olympus BX60, France). Total population was always more than 400 cells. Cells with condensed and/or fragmented chromatin were counted as apoptotic and propidium iodide-stained cells were counted as necrotic cells.

2.2.2. Histological analysis of zebrafish larvae

After each treatment, larvae were washed in PBS and then fixed in 4 % paraformaldehyde in PBS at 4 °C before being embedded in paraffin according standard procedures. Then, 4 µm sections were stained with hematoxylin, eosin and safran red (HES) and imaged on Nanozoomer NDP from Hamamatsu Photonics K.K. (magnification x 400).

2.3. Oxidative stress measurement

Oxidative stress was determined by evaluation of lipid peroxidation.

2.3.1. Lipid peroxidation in WIF-B9 cells

Production of free malondialdehyde (MDA), a secondary end-product of degradation of oxidized polyunsaturated fatty acids, was measured in culture medium. Briefly, after each treatment, culture medium of 3 x 10^6 cells was collected and ultrafiltered through a 1000-Da membrane (Millipore, Saint-Quentin-les-Yvelines, France). Then, free MDA was measured by size exclusion chromatography with an ultraviolet detection at 267 nm as previously described (Morel *et al.*, 1990). The HPLC system [Agilent 1260 Infinity (Agilent, Nantes, France)] was equipped with a TSK-gel G1000 PW (7.5 mm x 30 cm) size exclusion column (Phenomenex, Le Pecq, France).

2.3.2. Lipid peroxidation in zebrafish larvae

Lipid peroxidation was estimated by the oxidation of the hydrophobic probe C11-Bodipy$^{581/591}$ (Molecular Probes, Life Technologies, Courtaboeuf, France) incorporated into membranes. Prior to the end of each treatment, 100 nM C11-Bodipy$^{581/591}$ were added to embryo medium. Larvae were then incubated at 28°C during 3 hours in the dark before euthanasia. Then, larvae were washed three times in PBS and mounted in 80% glycerol-PBS for observation with fluorescence microscope Ni-E (Nikon). Red fluorescence images of reduced probe were acquired with a TRITC filter whereas green fluorescence images of oxidized dye were acquired with a FITC filter (magnification x 100). Thus, a yellow fluorescence was indicative of lipid peroxidation in liver on the merge of both previously acquired images.

2.4. Determination of structural perturbation of plasma membrane

2.4.1. Membrane fluidity in WIF-B9 cells

Changes in membrane fluidity were evaluated by electron paramagnetic resonance (EPR) spectroscopy using paramagnetic reporter groups incorporated into membrane. Membranes were spin labeled by incubating cell suspensions at 37 °C for 15 min with 12-doxyl stearic acid (50 µg/ml). The EPR spectra of labeled samples were acquired at ambient temperature on a Bruker Elexsys EPR spectrometer operating at 3509 G center field, 20 mW microwave power, 9.86 GHz microwave frequency, 1.771 G modulation amplitude, and 100 kHz modulation frequency. The fluidity of the labeled membranes was quantified by calculating the order parameter S, which is inversely related to membrane fluidity.

2.4.2. Fluorescence staining of lipid rafts in WIF-B9 cells

Lipid rafts were stained by the binding of the green–fluorescent Alexa Fluor 488 conjugate of cholera toxin subunit B to the pentasaccharide chain of lipid raft ganglioside GM1 (Molecular Probes, Life Technologies, Courtaboeuf, France). Briefly, cells were seeded and treated on glass coverslips. At the end of each treatment, after three washes with PBS, adherent cells were fixed on coverslips with 4 % paraformaldehyde in PBS for 30 min at 4°C and washed three times with PBS. Then, cells were stained using a blocking solution containing 1 µg/mL cholera toxin conjugate. After washing, coverslips were finally mounted with PBS-glycerol-Dabco. Images were captured with a DMRXA Leica microscope (Leica Microsystem) and a

COHU high performance CCD camera, using MetaVue Research Imaging Software (Molecular Devices, LLC, Sunnyvale, CA).

2.4.3. Fluorescence staining of lipid rafts in zebrafish larvae

Plasma membrane lipid rafts in zebrafish liver were visualized by confocal fluorescence microscopy using the membrane order sensitive di-4-ANEPPDHQ dye (Molecular Probes, Life Technologies, Courtaboeuf, France). This dye displays a fluorescent spectral blue-shift from 620 nm when incorporated into membranes with a low lipid order (in a liquid-disordered phase, Ld) to 560 nm when inserted into membranes with a high lipid order (in a liquid-ordered phase, Lo). After acquisition using confocal fluorescence microscopy of both disordered- and ordered-phase fluorescence images, a new image, indicative of membrane lipid order, was obtained by calculating the generalized polarization (GP) value, a ratiometric measurement of fluorescence intensities for each pixel which is correlated to membrane lipid order (Owen *et al.*, 2012). As lipid rafts are membrane domains known to be highly packed and to exhibit an increased rigidity, they are characterized by a high lipid order; thus, lipid rafts were visualized by a yellow highlighting of plasma membrane regions with a high GP value in comparison to mean GP found in control liver. Briefly, after each treatment, larvae were washed in PBS and then fixed in 4 % paraformaldehyde in PBS at 4 °C. After three washes in PBS, larvae were stained with 5 µM di-4-ANEPPDHQ during 90 min. Then, larvae were washed twice in PBS and dipped in several baths at progressive glycerol concentrations. Thereafter, they were mounted in 80% glycerol-PBS for an observation with fluorescence confocal microscope LEICA DMI 6000 CS (Leica Microsystem). Under excitation at 488 nm with an argon ion laser, ordered membrane images were acquired with a PMT range of 500 to 580 nm whereas for disordered membrane images, PMT range was 620 to 750 nm (magnification x 400). Using Fiji imaging processing software (ImageJ, National Institutes of Health, USA) and the macro published by Owen et al. (Owen *et al.*, 2012), GP images were generated according to the following calculation: $GP = (I_{500-580} - I_{620-750})/ (I_{500-580} + I_{620-750})$. Plasma membrane regions with a high value of GP were highlighted in yellow and were considered as membrane regions with high lipid raft clustering.

2.4.4. Membrane fluidity in zebrafish larvae

Following fluorescence staining by the membrane order sensitive di-4-ANEPPDHQ (see above), areas in non-raft regions of liver plasma membrane were assessed to determine their

GP value which is inversely related to membrane fluidity. Using Fiji imaging processing software (ImageJ, National Institutes of Health, USA), three to five non-raft regions of liver plasma membranes per larva were selected and analyzed for their GP value, which were expressed as variations of the GP value found in control larvae.

2.5. Statistical analysis

All values were presented as means ± S.D from at least three independent experiments. Multiple comparisons among the groups were performed using One or Two Way Analysis of Variance followed by Bonferroni post-tests. All statistical analyses were performed using GraphPad Prism5 software (GraphPad Software, San Diego, CA, USA). Differences were considered significant when $p<0.05$.

3. Results

Based upon the fact that in rodents, ethanol-induced membrane remodeling and consequent liver injuries were studied after a long-term exposure to alcohol from 10 to 30 days (Oliva *et al.*, 1998; Yin *et al.*, 2001; Inokuchi *et al.*, 2011; Roh *et al.*, 2015), we decided to mimic this exposure time in zebrafish larvae by a 7-days-treatment with ethanol. Concerning the comparison with an *in vitro* model, our previous results obtained in primary rat hepatocytes could not be used due to the too short incubation time with ethanol (within a maximum of 5 hours). In order to work with a longer incubation time compatible with the cell survival, new experiments were carried out with WIF-B9 hepatic cells. These cells, which are highly differentiated and polarized, possess the advantages to contain the whole rat genome and a dozen of human chromosomes, to develop morphologic features close to primary rat and human hepatocytes (Decaens *et al.*, 1996), and to express both rat and human xenobiotic metabolism enzymes (Biagini *et al.*, 2006; McVicker *et al.*, 2006).

3.1. Zebrafish larvae as a suitable model to investigate changes in membrane features

First, we tested if, even after 4 days of treatment in WIF-B9 hepatic cells, ethanol was still able to induce membrane remodeling (Fig. 1) as demonstrated for shorter incubation times in primary rat hepatocytes. Thus, ethanol exposure led to an increase in membrane fluidity (Fig. 1A) without any adaptation of hepatocyte membranes. Other well-known fluidizing agents such as long chain polyunsaturated fatty acids (PUFAs) maintained this

property after 4 days of supplementation (Fig. 1A). It should be remembered the unique behavior of the liver compared to other organs, given that it is recognized as the only one to maintain such an increase in membrane fluidity during chronic intoxication with ethanol (Yamada and Lieber, 1984; Polokoff et al., 1985). In addition, as primary rat hepatocytes, WIF-B9 still exhibited an ethanol-induced lipid raft clustering (Fig. 1B). Consequently, WIF-B9 hepatic cells have proved to be ideally suited to compare the effect on membrane remodeling of an *in vitro* model with the zebrafish *in vivo* model. Concerning zebrafish, taking advantage of the transparency of zebrafish larvae, we used a technique allowing the measure of the membrane order directly in whole organism, which is inversely correlated to membrane fluidity (Owen *et al.*, 2012). As in WIF-B9 hepatic cells, ethanol or PUFAs induced an increase in membrane fluidity in non-raft regions of liver plasma membrane (Fig. 2A). It is noteworthy that, in zebrafish larvae but not in WIF-B9 cells, docosahexaenoic acid (DHA) induced a more pronounced increase in membrane fluidity than eicosapentaenoic acid (EPA). Besides, regarding the lipid rafts which were visualized in the liver after a quick staining of whole larvae with the fluorescent hydrophobic probe, di-4-ANEPPDHQ, ethanol and PUFAs induced alterations in the liver of zebrafish larvae similar to those observed in WIF-B9 cells , with no effect of DHA whereas ethanol or EPA induced raft clustering (Fig. 2B). Thus, zebrafish model seems perfectly suited to evaluate chemical-induced membrane remodeling even after a long-term exposure.

In order to definitely check whether zebrafish larva could be suitable as an *in vivo* model to investigate membrane remodeling, we decided to test the effect of ethanol in presence of DHA or EPA (Fig. 1B and 2B). Indeed, these n-3 PUFAs have been demonstrated by our team in primary rat hepatocytes (Aliche-Djoudi *et al.*, 2011, 2013) and by others in model membranes (Williams *et al.*, 2012; Shaikh *et al.*, 2014), in lymphocytes (Rockett *et al.*, 2012) or also *in vivo* in mice (Teague *et al.*, 2014), to have opposite effects on lipid raft properties. Thus, we previously showed that EPA increased ethanol-induced lipid raft clustering while DHA inhibited it. Interestingly, similar results were obtained *in vitro* with WIF-B9 cells confirming its ability to maintain treatment-induced membrane remodeling even after 4 days (Fig. 1B). Consequently, as n-3 PUFAs are well-recognized to modulate membrane remodeling, testing them in zebrafish larva seems us essential to definitely claim zebrafish larva as a relevant and sensitive model for assessing membrane remodeling. Firstly, we observed in zebrafish larvae that EPA enhanced the ethanol-induced lipid raft clustering (Fig. 2B). Thus, it proved the ability for this model to discriminate various types of chemical

and physical changes in membranes. Secondly, treatment with DHA led to an inhibition of ethanol-induced lipid raft clustering (Fig. 2B). These opposite effects between EPA and DHA, two compounds very close from a chemical point of view (EPA: C20:5(n-3); DHA: C22:6(n-3)), allowed us to confirm the sensitivity of the model regarding membrane remodeling.

3.2. Zebrafish larvae as a sensitive model to test the relationship between levels of changes in membrane features and the extent of toxicant-induced liver damages

As EPA enhanced the changes in plasma membrane features due to ethanol, we then decided to investigate if various levels of membrane remodeling could influence hepatotoxicity at various extents both in zebrafish larvae (Fig. 3) and in WIF-B9 hepatic cells (Fig. 4). For *in vivo* experiments with zebrafish larvae, cell death was estimated on liver histological sections. Exposure to ethanol or EPA alone increased the number of dead cells characterized by ballooning hepatocytes and hepatocyte dropouts (Fig. 3A and 3B). In addition, co-exposure to ethanol and EPA led to an increase of liver injuries compared to ethanol or EPA alone suggesting a good sensitivity of the zebrafish model (Fig. 3A). Furthermore, to thoroughly assess the sensitivity of zebrafish larva model, the impact of exposure to ethanol at various concentrations (from 10.75 to 43 mM) was tested on both membrane remodeling and liver damages (Fig. 3C and 3D). Zebrafish larva model was able to discriminate the effect on membrane remodeling according to the toxicant dose as an elevation of lipid raft clustering was detected in a dose-dependent manner (Fig. 3C). Interestingly, this increase in membrane remodeling was associated with a gradual rise in liver injuries demonstrated by the elevation of cell death on histological sections (Fig. 3D). This correlation between membrane remodeling and hepatotoxicity as a function of the toxicant dose clearly confirmed the high sensitivity of zebrafish larva model.

To compare with an *in vitro* model, cell death was also estimated in WIF-B9 cells by the evaluation of apoptosis and necrosis (Fig. 4A and 4B). Treatment by ethanol but not by EPA alone induced both cell death types. However, EPA was still able to significantly increase ethanol-induced apoptosis (Fig. 4A), with no significant effect regarding necrosis (Fig. 4B).

Finally, one mechanism well-known to participate to ethanol-induced liver damages, namely oxidative stress, was evidenced by lipid peroxidation evaluation. To this end, the C11-Bodipy$^{581/591}$ fluorochrome was incorporated into living larvae and the appearance of a yellow fluorescence testified for lipid peroxidation (Fig. 5A). Ethanol or EPA alone led to an

elevation of lipid peroxidation in liver. Once again, the zebrafish model was able to detect an EPA increase in ethanol-induced lipid peroxidation (Fig. 5A). The *in vitro* model exhibited an elevation of lipid peroxidation for ethanol but not for EPA alone (Fig. 5B). Yet, as in zebrafish larvae, the *in vitro* model could display the ability of EPA to rise oxidative stress levels in presence of ethanol (Fig. 5B).

3.3. Zebrafish larvae as a reliable model to prove the membrane remodeling involvement in hepatotoxicity

In order to definitely validate the effectiveness and applicability of the zebrafish larva model as a powerful tool to obtain evidence about membrane remodeling involvement in hepatic injury, liver damages were evaluated in presence of a well-recognized membrane stabilizer, ursodeoxycholic acid (UDCA) (Güldütuna *et al.*, 1993) or a lipid raft disrupter, pravastatin (Wei *et al.*, 2013). In the liver of zebrafish larvae, UDCA partially reduced ethanol- or EPA-induced injuries evaluated by the number of ballooning hepatocytes and cells dropout on histological sections (Fig. 6), thus suggesting the sensitivity of zebrafish larva to membrane fluidity changes even after a long-term exposure to toxicant. In WIF-B9 hepatic cells, the pre-treatment with UDCA inhibited ethanol-induced cell death (Fig. 7A and 7B) and oxidative stress (Fig. 7C). Pravastatin, known to disrupt lipid rafts by inhibiting HMG-CoA-reductase activity (Wei *et al.*, 2013), an enzyme required for cholesterol biosynthesis, also protected zebrafish liver against both ethanol- and EPA-induced injury, as well as the increase in cytotoxicity after co-exposure (Fig. 6). In addition, pravastatin inhibited ethanol- and/or EPA-induced lipid raft clustering, as expected (Fig. S1). Thus, zebrafish larva appeared as a reliable model since toxicant-induced lipid raft clustering could be demonstrated to be fully involved in liver damages. It should be noted that the *in vitro* model also indicated the involvement of lipid rafts in hepatotoxicity since cholesterol oxidase, another lipid raft disrupter which produces cholestenone, thereby breaking the cholesterol bond to sphingolipid (Rouquette-Jazdanian *et al.*, 2006; Castro *et al.*, 2009), inhibited apoptosis due to ethanol and EPA in WIF-B9 hepatocytes (Fig. S2).

Besides the hindsight about the effects of UDCA and pravastatin on membrane remodeling, the choice of these compounds for the experiments in zebrafish was also justified by their regular use in pharmacotherapy of human liver diseases (Poupon, 2012; Nseir and Mahamid, 2013; Xiang *et al.*, 2013; Herrick *et al.*, 2014). The above results allowed us to consider the zebrafish larva not only as a tool for assessing membrane remodeling but also as

a screening model of potential drug candidate for "membrane therapy". In this context, as DHA, a long chain polyunsaturated fatty acid found in fatty fishes, was shown to protect from ethanol-induced lipid raft clustering (Aliche-Djoudi *et al.*, 2013), we decided to test it on the liver injury due to ethanol in order to also evaluate zebrafish larva as a model for assessing beneficial diet or bioactive compounds from food. As predicted by its effect on membranes, DHA totally reduced ethanol-induced liver damages (Fig. 8A) and lipid peroxidation (Fig. 8B) in zebrafish larvae. In WIF-B9 hepatocytes, DHA treatment completely inhibited apoptosis but only partially reduced necrosis (Fig. 9A and 9B) and lipid peroxidation (Fig. 9C) due to ethanol.

4. Discussion

As no *in vitro* cell model can mimic the combination of interaction between the various cell types of the whole liver, zebrafish larvae constitute an attractive *in vivo* model to study the toxicant-induced membrane remodeling and its impact on cytotoxicity. Indeed, zebrafish larvae exhibit advantages equivalent to those of cell cultures such as small size, easy maintenance, convenient breeding and high proliferation; in addition, they provide a whole-organism approach without the very costly and time-consuming procedures related to *in vivo* rodent experiments. Thus, zebrafish larva model has already been proved to be suitable for assessing chemical toxicant-induced hepatotoxicity (Hill *et al.*, 2012; Driessen *et al.*, 2013; He *et al.*, 2013; Vliegenthart *et al.*, 2014; Goessling and Sadler, 2015; Verstraelen *et al.*, 2016); it shows a good predictivity for various mechanisms of liver injury such as steatosis, cholestasis or oxidative stress (Mesens *et al.*, 2015), but nothing is known about its interest in exploring another mechanism, the membrane remodeling, depicted as changes in membrane fluidity or in physical and biochemical properties of lipid rafts. Thus, we demonstrated for the first time the full relevance of zebrafish larvae for this kind of research since membrane remodeling was maintained even after a week-exposure to ethanol or n-3 PUFAs. This point is worth stressing since the liver is recognized as the only tissue that does not adapt to the fluidizing effect of ethanol during chronic exposure of rats (Yamada and Lieber, 1984) or hepatocyte cultures (Polokoff *et al.*, 1985). In addition, zebrafish larvae, unlike rodent models, allowed us to easily demonstrate both the ability of ethanol to induce membrane remodeling and its involvement in hepatotoxicity of ethanol. Interestingly, zebrafish larva model exhibited a high sensitivity since it was able to discriminate various levels of

cytotoxicity depending on the extent of membrane remodeling as shown by the experiments performed with both ethanol and EPA or with ethanol alone at various concentrations. In addition, its sensitivity would be higher than that of WIF-B9 hepatocyte cultures. First, membrane remodeling was almost similar in both models whatever the test condition, except for DHA supplementation. Thus, in zebrafish larvae, DHA led to a more pronounced fluidizing effect than EPA whereas in hepatocyte cultures, both n-3 PUFAs have the same behavior. Yet, by referring to the acyl chain length and unsaturation degree, DHA should logically increase membrane fluidity more than EPA, which is consistent with the higher sensitivity of zebrafish larvae. Secondly, a higher sensitivity for zebrafish larvae also appeared when looking at results concerning liver injury. Thus, zebrafish larvae were successful in exhibiting a hepatotoxicity and oxidative stress due to EPA alone related to a lipid raft clustering whereas the *in vitro* model did not demonstrate any increase in cell death or lipid peroxidation despite such a clustering. In the same context, in hepatocyte cultures, DHA alone only partially reduced ethanol-induced necrosis while it totally inhibited lipid raft clustering, whereas in zebrafish larvae, this PUFA almost completely protected from ethanol-induced cell death. The lower sensitivity of hepatocyte cultures for assessing the membrane remodeling was in accordance with results obtained by other teams that compared zebrafish larvae with hepatocyte cultures. Indeed, sensitivity of zebrafish larvae towards liver injury due to well recognized hepatotoxicants (86% of true-positive; i.e. 32 out of 37 tested compounds were correctly identified as hepatotoxicant) was better than that of *in vitro* models (HepG2 hepatocytes or primary human hepatocytes, with sensitivity of 67% and 60%, respectively) (Jones *et al.*, 2009; Hill *et al.*, 2012).

When considering the involvement of membrane remodeling in liver injury, the treatment of zebrafish larvae by a membrane stabilizer (UDCA) or a lipid raft disrupter (pravastatin) provided a protection towards ethanol and/or EPA-induced liver injury as in WIF-B9 hepatocytes, thus leading us to claim the applicability of the zebrafish model to evaluate this mechanism of liver injury. However, once again, the sensitivity of zebrafish larvae appeared better than that of hepatocyte cultures. Indeed, the effect of UDCA or pravastatin was partial in zebrafish larvae whereas the protection was total in hepatocyte cultures except for the condition EPA+ethanol, for which it was also partial. It should be pointed out that UDCA and pravastatin which allow manipulating membrane features, were essentially chosen for this study due to their regular use in pharmacotherapy of human liver diseases, thus ensuring their safety (Poupon, 2012; Nseir and Mahamid, 2013; Xiang *et al.*,

2013; Herrick *et al.*, 2014). Our aim was also to demonstrate zebrafish larvae as an appropriate model to screen potential drug candidates for "membrane therapy". Results obtained with zebrafish larva model were closer to those obtained *in vivo* in rodents or in human, that were not fully convincing about the protection provided by UDCA in monotherapy (Haedrich and Dufour, 2011; Liechti and Dufour, 2012; Ratziu, 2012; Ali *et al.*, 2015) or statins (Eslami *et al.*, 2013; Pastori *et al.*, 2015; Tziomalos *et al.*, 2015) against steatohepatitis. Taken altogether, our data indicated that zebrafish larva is a reliable model for 1) revealing plasma membrane remodeling as a mechanism thereby toxicants induce liver injury, and 2) discovering drugs such as UDCA and statins, or nutrients such as DHA, which impact on membrane fluidity or lipid rafts; this could allow proposing new preventive or therapeutic strategies for chemical-induced liver diseases. In addition, this model is compatible with a high throughput screening. Indeed, zebrafish larvae can live for several days in a single well of a 96 multiwell plate which consumes small amounts of compounds to test. In addition, the transparency of zebrafish for several days post-fertilization enables *in vivo* observation of internal organs including the liver and therefore, various tools for automating the imaging in zebrafish larvae are currently under investigation (Chang *et al.*, 2012; Pulak, 2016). Notably, through the use of a quick and easy staining of zebrafish larvae with the fluoroprobe di-4-ANEPPDHQ, visualization of plasma membrane lipid order in whole zebrafish larva offers a unique possibility to study membrane remodeling directly *in vivo.* Finally, studies about zebrafish transcriptome exhibited some hepatotoxicity-associated genes such as *fabp10a* that could be used instead of the challenging and time consuming histopathology (Driessen *et al.*, 2013; Mesens *et al.*, 2015; Verstraelen *et al.*, 2016).

In conclusion, taking advantage of *in vitro* testing methodologies, zebrafish larva model allows exploring membrane remodeling in the liver of a whole animal exposed to hepatotoxicants. Our data strongly support the use of this model as a powerful tool to rapidly screen *in vivo* chemicals for their capacity to induce a membrane remodeling leading to liver injury, before to be fully confirmed in rodent models. Therefore, as proposed by Sukardi *et al*., zebrafish larva model could be considered as a bridge between *in vitro* cell-based models and *in vivo* mammalian models (Sukardi *et al.*, 2011). In addition, the other important perspective of this promising model is the screening of nutritional or pharmacological candidates that can alleviate this kind of hepatotoxicity. As explained above, in the near future, the research development of new methods for a high throughput screening will be a

pivotal milestone to definitely conclude on the high potential of this model for hepatotoxicity research in the context of membrane remodeling.

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Table 1. *In vitro* assessment of membrane remodeling involvement in chemical-induced hepatotoxicity.

Chemicals	Models	Results		References
		Membrane remodeling assessment	Membrane remodeling involvement in cytotoxicity	
Anandamide	HSC-T6 rat liver stellate cells	↑ lipid raft clustering	↑ cell death ↑ cytokines expression	Yang *et al.*, 2010
Benzo(a)pyrene	F258 rat hepatic epithelial cells	↑ membrane fluidity	↑ apoptosis	Gorria *et al.*, 2006
Benzo(a)pyrene	F258 rat hepatic epithelial cells	↑ lipid raft disruption NHE1 protein relocation outside lipid rafts	↑ apoptosis ↑ reactive oxygen species production	Huc *et al.*, 2007; Tekpli *et al.*, 2010, 2012
Doxorubicin	Hepa1–6 murine liver cancer cells		↑ cell death	Mohammad *et al.*, 2015
Ethanol	Primary rat hepatocytes	↑ membrane fluidity ↑ lipid raft clustering Recruitment of PLCγ1 to lipid rafts	↑ oxidative stress ↑ apoptosis	Sergent *et al.*, 2005; Nourissat *et al.*, 2008
Ethanol	RAW264.7 mouse macrophages Primary mouse macrophages (Kupffer like macrophages)	Recruitment and activation of TLR4 into lipid rafts	↑ cytokines expression	Fernandez-Lizarbe *et al.*, 2008
Ethanol / Benzo(a)pyrene mixture	Primary rat hepatocytes	↑ membrane fluidity Alterations of lipid rafts Recruitment of PLCγ1 to lipid rafts	↓ cell viability ↓ ATP content ↑ oxidative stress	Collin *et al.*, 2014
Lovastatin	HepG2 human hepatocarcinoma cells		↓ glucose uptake	Nowis *et al.*, 2014
Ochratoxin A	HepG2 human hepatocarcinoma cells	Differential plasma membrane protein expression	↓ cell viability ↑ reactive oxygen species production	Zhang *et al.*, 2016
Tacrine	Primary rat hepatocytes	↑ membrane fluidity	↑ necrosis	Galisteo *et al.*, 2000

Table 2. *In vivo* assessment of membrane remodeling and of its potential involvement in chemical-induced hepatotoxicity.

Chemicals	Models	Results		References
		Membrane remodeling assessment	Membrane remodeling involvement in liver injury	
Acetaminophen, Carbon tetrachloride, D-galactosamine, Thioacetamide	Ceramide synthase 2 knock-out mice	Mislocalization of connexin 32 in lipid raft	↑ necrosis	Park *et al.*, 2013
Bromodichloromethane	High-fat diet-fed TLR4 knock-out mice	Recruitment of TLR4 into lipid rafts	↑ inflammation	Das *et al.*, 2015
Cadmium	Druckrey rats	↑ membrane fluidity		Yadav and Khandelwal, 2006
Carbon tetrachloride	Sprague-Dawley rats	↓ membrane fluidity		Aranda *et al.*, 2010
Cyclosporin A	Sprague-Dawley rats	↓ membrane fluidity		Yasumiba *et al.*, 2001
Diethylnitrosamine	Mice	↑ membrane fluidity		Gupta *et al.*, 2013
Ethanol	Sprague-Dawley rats	↑ membrane fluidity	↑ steatosis and oxidative stress	Oliva *et al.*, 1998
Ethanol	CD14 knock-out mice		↑ inflammation and necrosis	Yin *et al.*, 2001
Ethanol	TLR4 chimeric mice		↑ steatosis, inflammation and necrosis	Inokuchi *et al.*, 2011
Ethanol	TLR2 or TLR9 knock-out mice		↑ apoptosis, ↑ necrosis and inflammation	Roh *et al.*, 2015
Halothane	Louvain rats	↑ membrane fluidity		Thalhammer *et al.*, 1993
Manganese	Wistar rats	↓ membrane fluidity		Huang *et al.*, 2012
PCB126	Chick embryos	↑ membrane fluidity		Katynski *et al.*, 2004
Selenium	Sprague-Dawley rats	↓ membrane fluidity		Pan *et al.*, 2012
2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)	Sprague-Dawley rats	↓ membrane fluidity		Alsharif *et al.*, 1990; Stohs *et al.*, 1990

Table 3. *In vitro* and *in vivo* exposure conditions.

Models		**WIF-B9 hepatic cells**	**Zebrafish larvae**
Compounds	Ethanol	25 mM	43 mM
	DHA	10 µM	0.5 µM
	EPA	10 µM	0.5 µM
Exposure time		4 days (daily renewal)	7 days (daily renewal)

FIGURES LEGENDS

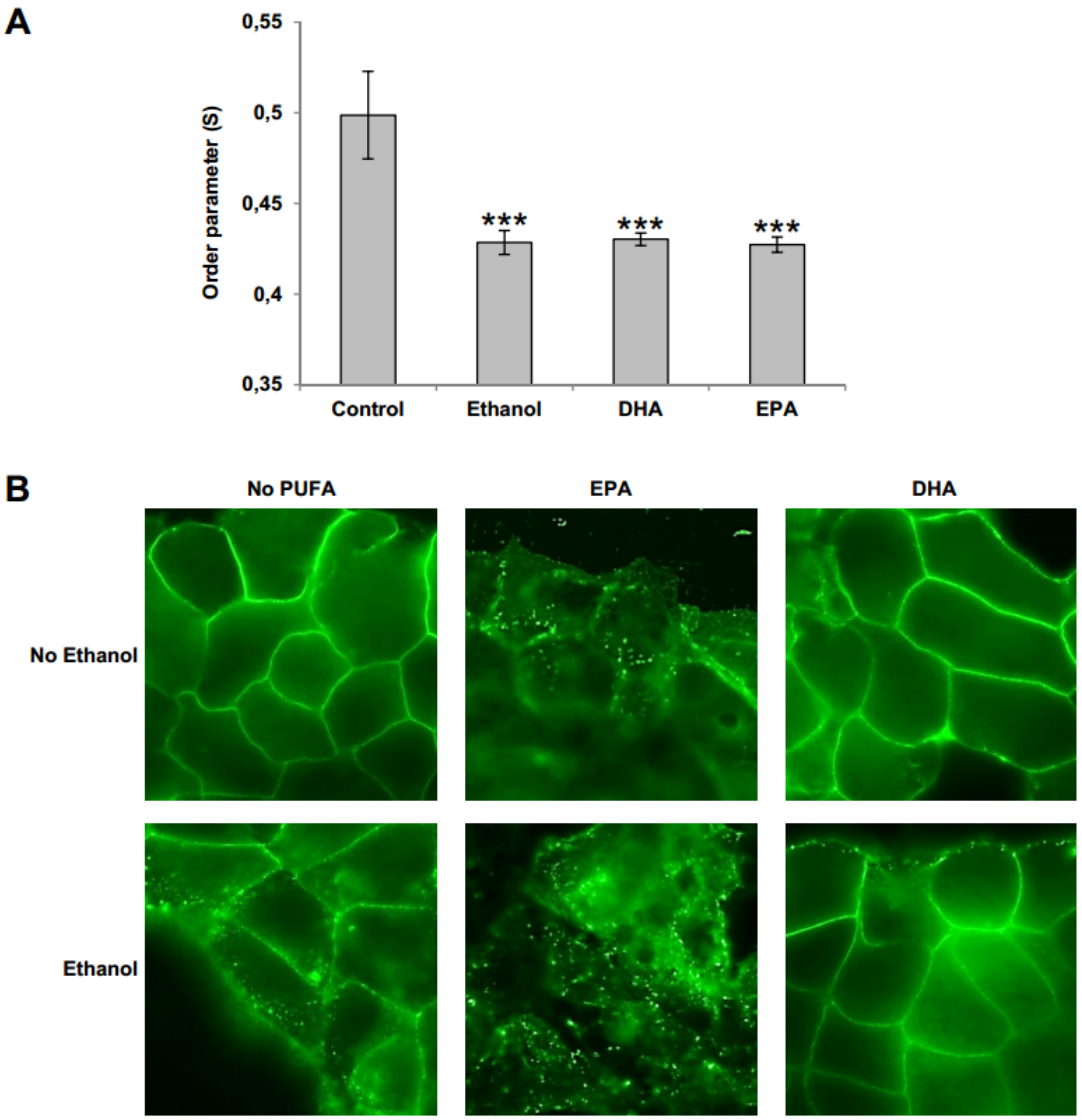

Figure 1: Plasma membrane feature changes in WIF-B9 hepatic cells were maintained even after four days of treatment. (A) Membrane fluidity in WIF-B9 hepatic cells was monitored by electron paramagnetic resonance analysis of 12-doxyl stearic acid embedded in membranes. This analysis resulted in the determination of the order parameter S, which is inversely related to membrane fluidity. (B) Membrane lipid rafts in WIF-B9 cells were visualized by fluorescence microscopy with cholera toxin subunit B conjugated with Alexa Fluor 488, which binds the raft-associated glycosphingolipid GM1 (magnification x 400). In control cells, the green fluorescence throughout the whole plasma membrane indicated

uniform GM1 membrane distribution whereas ethanol treatment led to the appearance of a fluorescence punctuation indicative of lipid raft clustering. Pictures are representative of at least 3 experiments. Values are the mean ± SD of three independent experiments. Treatment *versus* no treatment: * p<0.05; ** p<0.01; *** p<0.001.

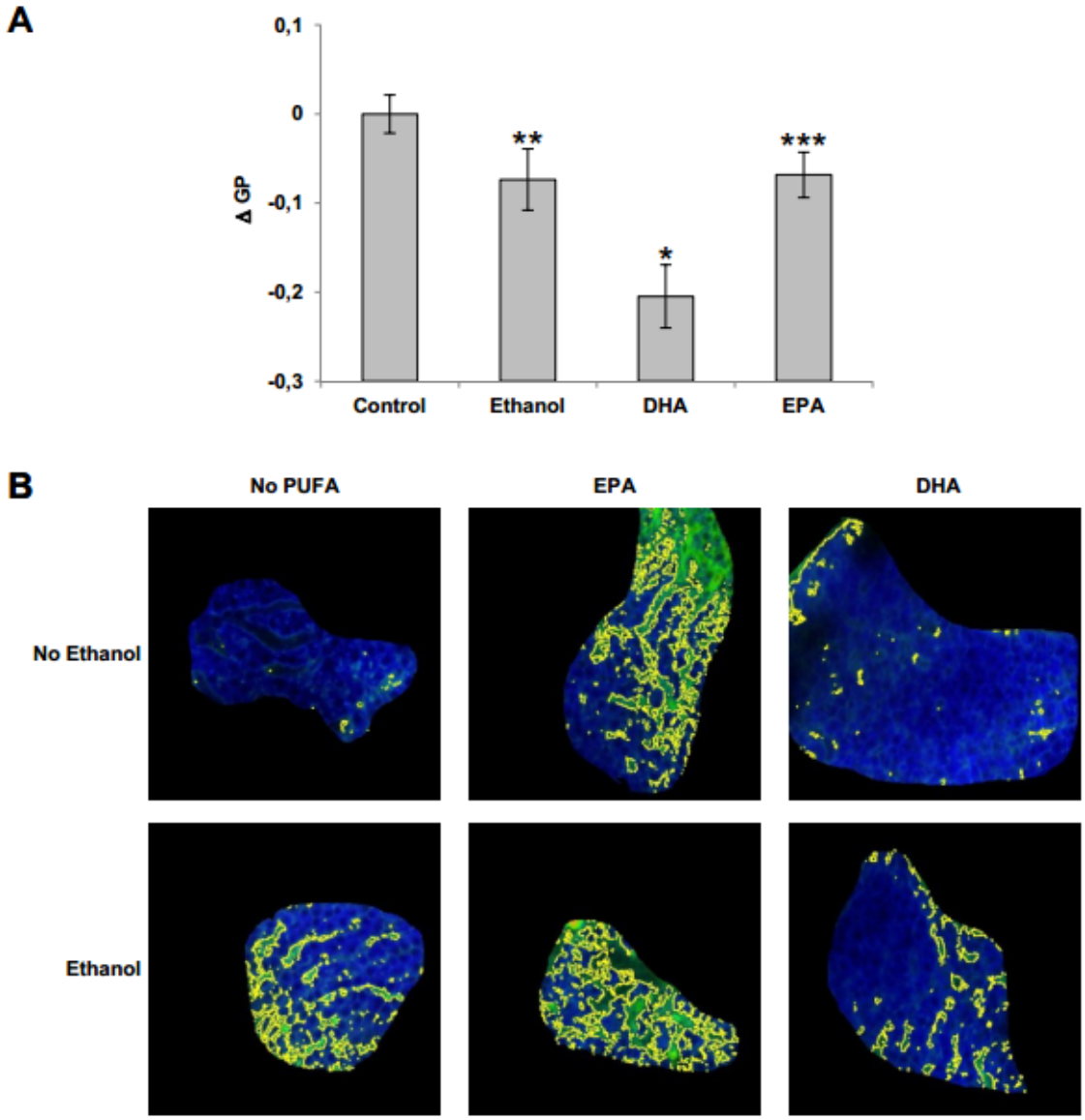

Figure 2: Suitability of zebrafish larvae to investigate plasma membrane remodeling shown by membrane feature changes comparable to those obtained for WIF-B9 cell model. Membrane remodeling was assessing in zebrafish larva by confocal fluorescence microscopy after staining with di-4-ANEPPDHQ, a fluorescent probe sensitive to membrane order. (A) Membrane fluidity in non-raft regions of zebrafish liver plasma membrane were evaluated by the calculation of the GP parameter, inversely related to membrane fluidity.

Changes in GP values in non-raft regions were expressed as the difference between individual larva GP value and the mean of GP found in control larvae (ΔGP). (B) Membrane lipid rafts in livers were visualized on images representing GP value (magnification x 400). Membrane regions with high degree of membrane order, the biophysical hallmark of lipid rafts, appeared in yellow. Pictures are representative of at least 3 larvae. Values are the mean ± SD at least 3 larvae. Treatment *versus* no treatment: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

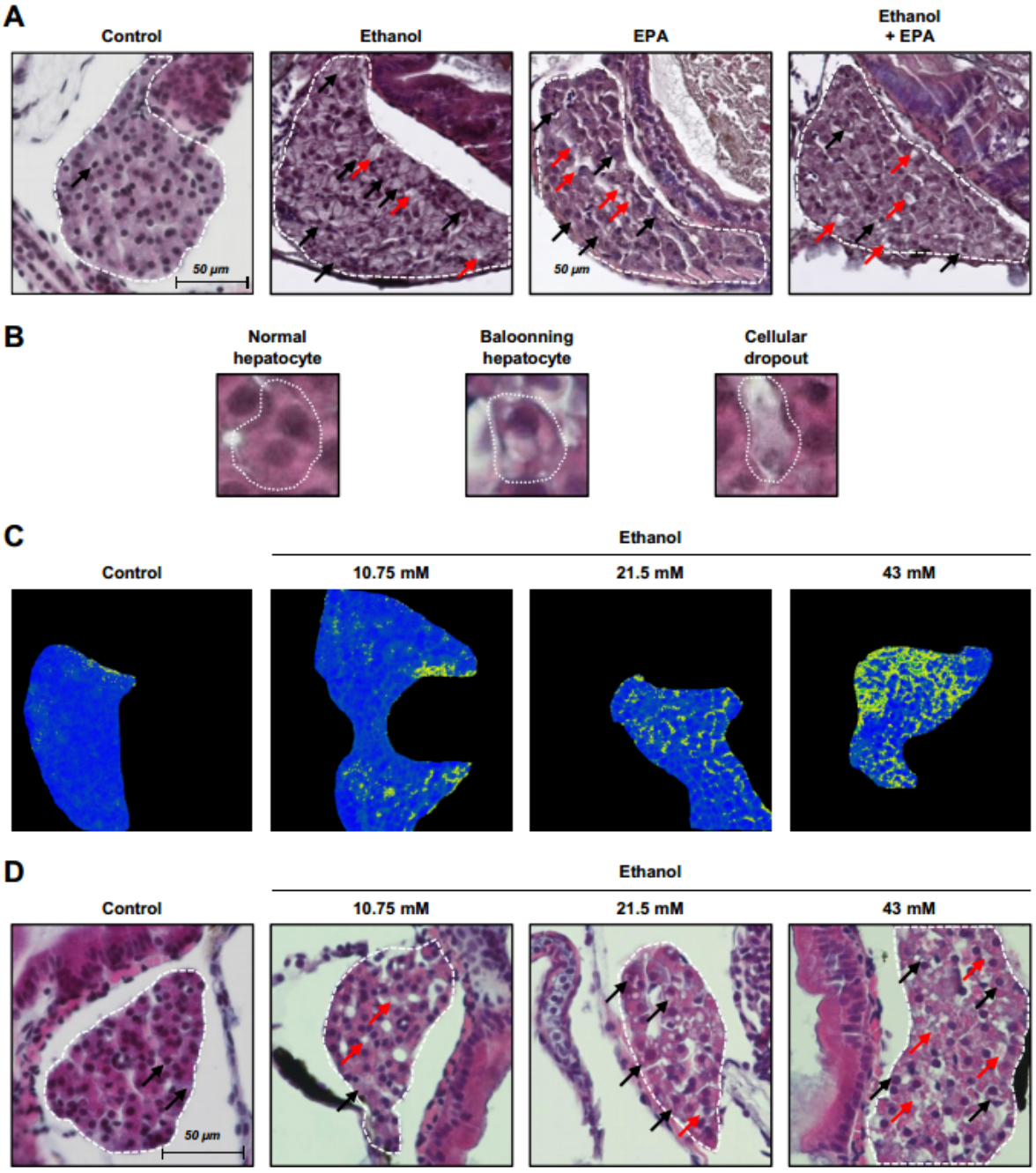

Figure 3: Assessment of zebrafish larvae sensitivity evidenced by the influence of various membrane remodeling levels on liver damage severity. (A, D) Cell death was observed on zebrafish liver section after HES staining (magnification x 400). White dotted line outlines liver. Red arrows indicate hepatocyte dropouts, and black arrows show ballooning hepatocytes. (B) Numerical magnifications of images of histological preparations of liver sections show respectively, surrounded in white dotted lines, a normal hepatocyte, a ballooning hepatocyte with vacuolization and a cellular dropout. (C) Membrane lipid rafts in livers were visualized by confocal fluorescence microscopy after staining with di-4-ANEPPDHQ (magnification x 400) Membrane regions with high degree of membrane order, the biophysical hallmark of lipid rafts, appeared in yellow. Zebrafish larvae were exposed daily to ethanol at 0, 10.75, 21.5 or 43 mM from 5 dpf to 12 dpf (C, D). Pictures are representative of at least 3 larvae.

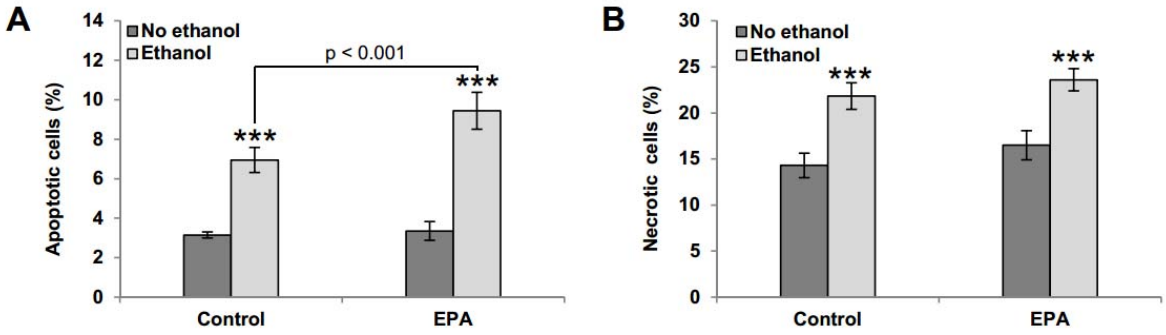

Figure 4: Influence of the extent of membrane remodeling on cell death in WIF-B9 cell model. (A) Apoptotic and (B) necrotic cell death were determined by microscopic counting after WIF-B9 hepatocyte staining with Hoechst 33258 and propidium iodide. Values are the mean ± SD of three independent experiments. Treatment *versus* no treatment: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

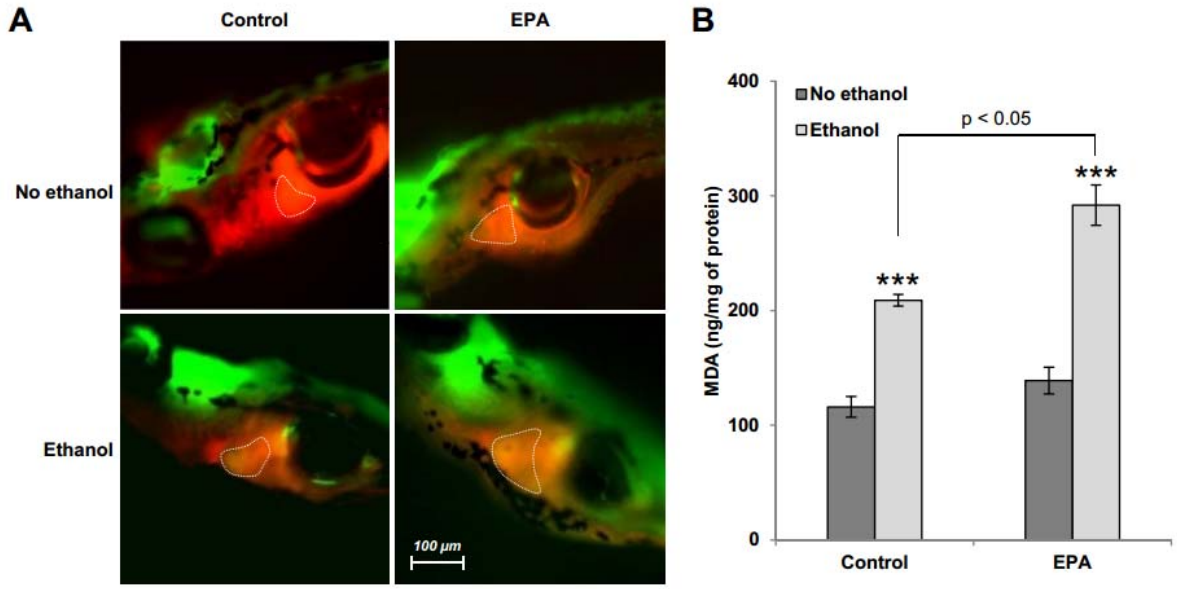

Figure 5: Comparison of zebrafish larva and WIF-B9 cell models for the study of the correlation of membrane remodeling with oxidative stress. (A) Oxidative stress in zebrafish liver was visualized by fluorescence microscopy after staining of living zebrafish with C11-Bodipy$^{581/591}$, a hydrophobic fluorescent probe; a yellow fluorescence was indicative of lipid peroxidation in the liver (magnification x 100). White dotted line outlines the liver. (B) Malondialdehyde (MDA), a lipid peroxidation end-product, was measured by HPLC-UV as a marker of oxidative stress in WIF-B9 cells. Pictures are representative of at least 3 larvae. Values are the mean ± SD of three independent experiments. Treatment *versus* no treatment: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

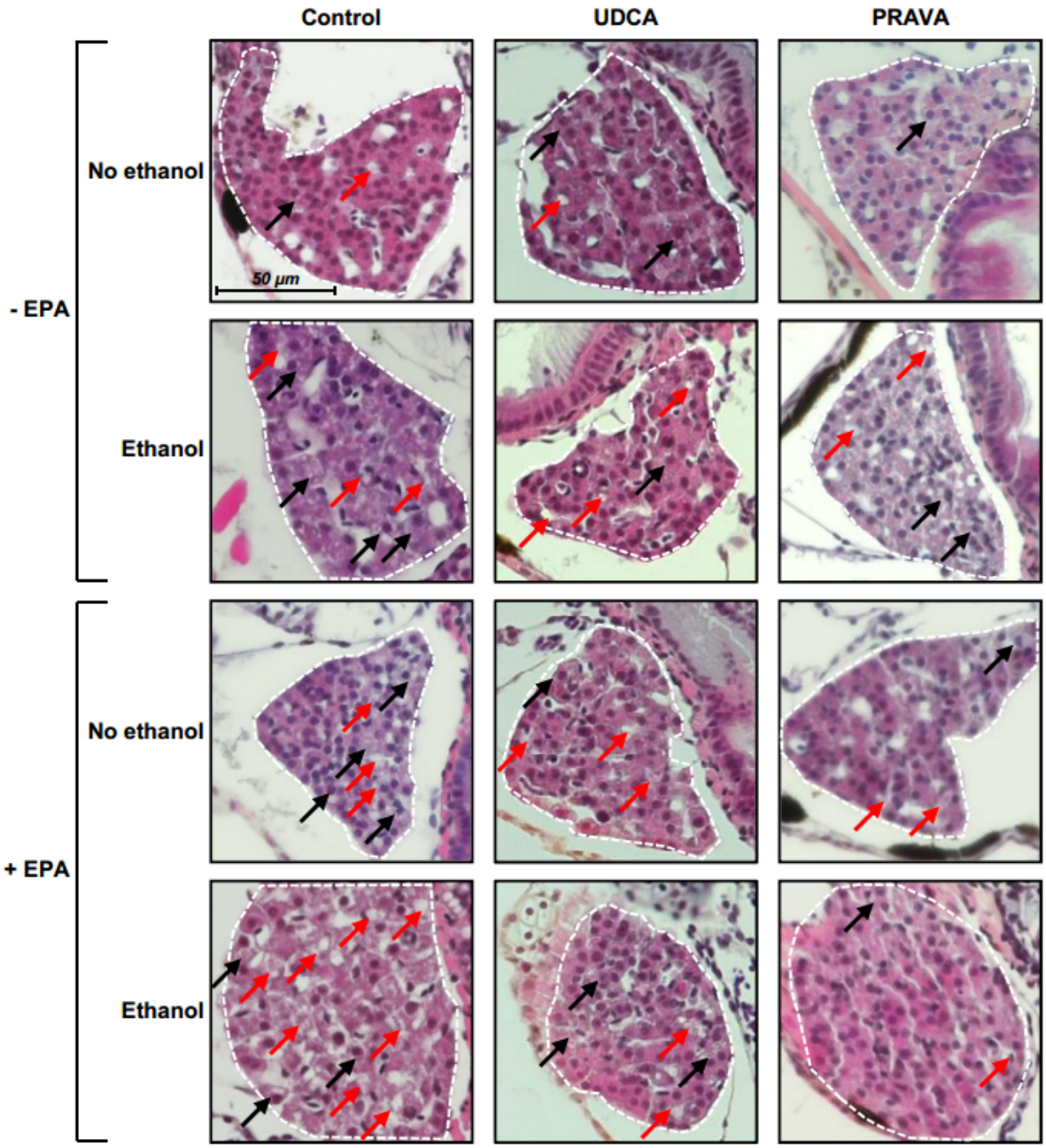

Figure 6: Zebrafish larvae as an applicable model to prove the involvement of membrane remodeling in hepatotoxicity induced by ethanol and EPA. Cell death was observed on zebrafish liver section after HES staining (magnification x 400). White dotted line outlines the liver. Red arrows indicate hepatocyte dropouts and black arrows show ballooning hepatocytes. Some larvae were co-exposed to 0.5 µM UDCA, a membrane stabilizing agent, or to 0.5 µM Pravastatin (PRAVA), a lipid raft disrupter. Pictures are representative of at least 3 larvae.

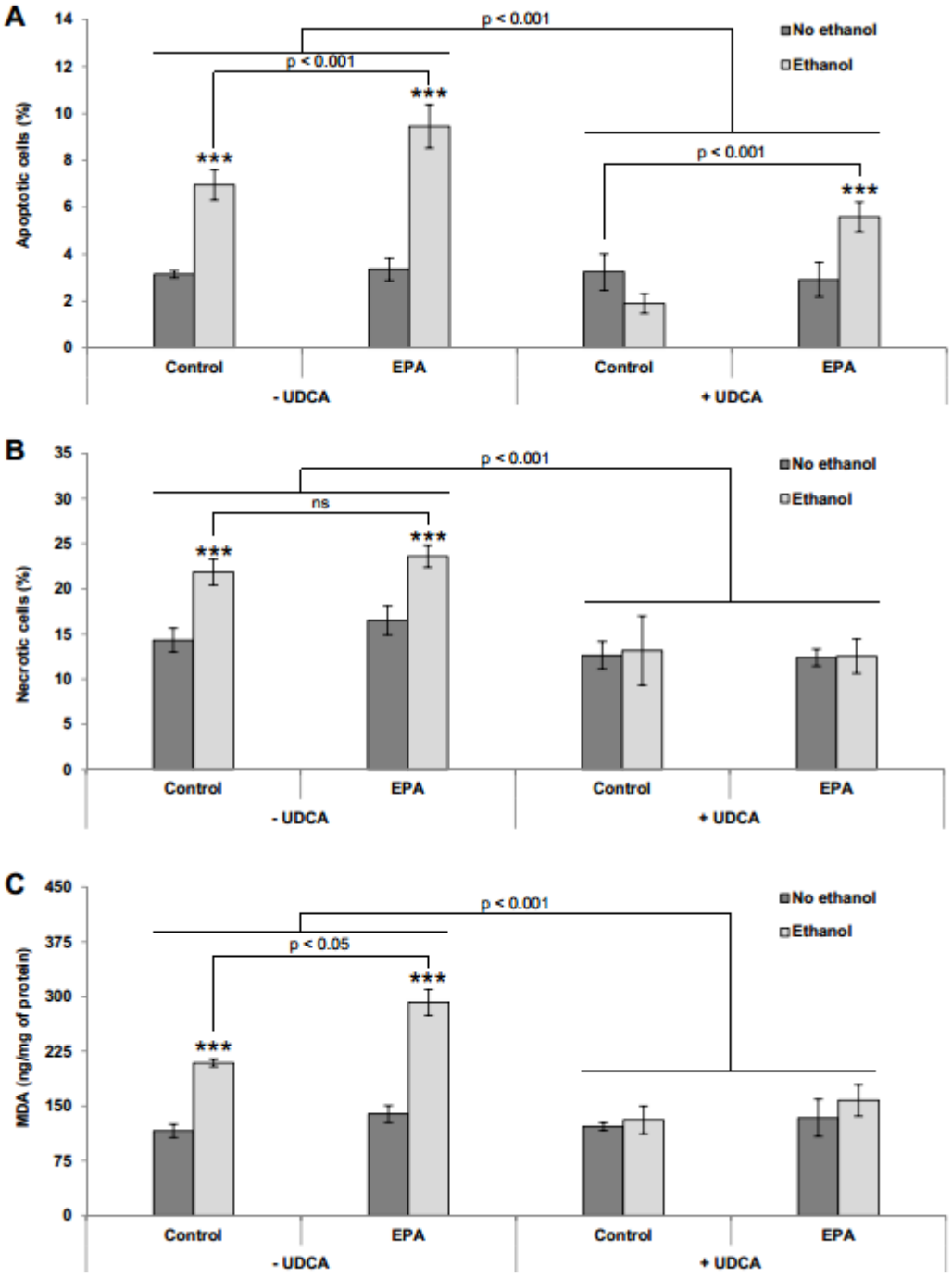

Figure 7: Involvement of membrane remodeling in cytotoxicity induced by ethanol and EPA in WIF-B9 hepatocytes. (A) Apoptotic and (B) necrotic cell death was determined by microscopic counting after WIF-B9 hepatocytes staining with Hoechst 33258 and propidium iodide. (C) Malondialdehyde (MDA), a lipid peroxidation end-product, was measured by HPLC-UV as a marker of oxidative stress in WIF-B9 cells. Some cultures were pre-treated for

one hour with 25 µM UDCA, a membrane stabilizing agent. Values are the mean ± SD of three independent experiments. Treatment *versus* no treatment: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

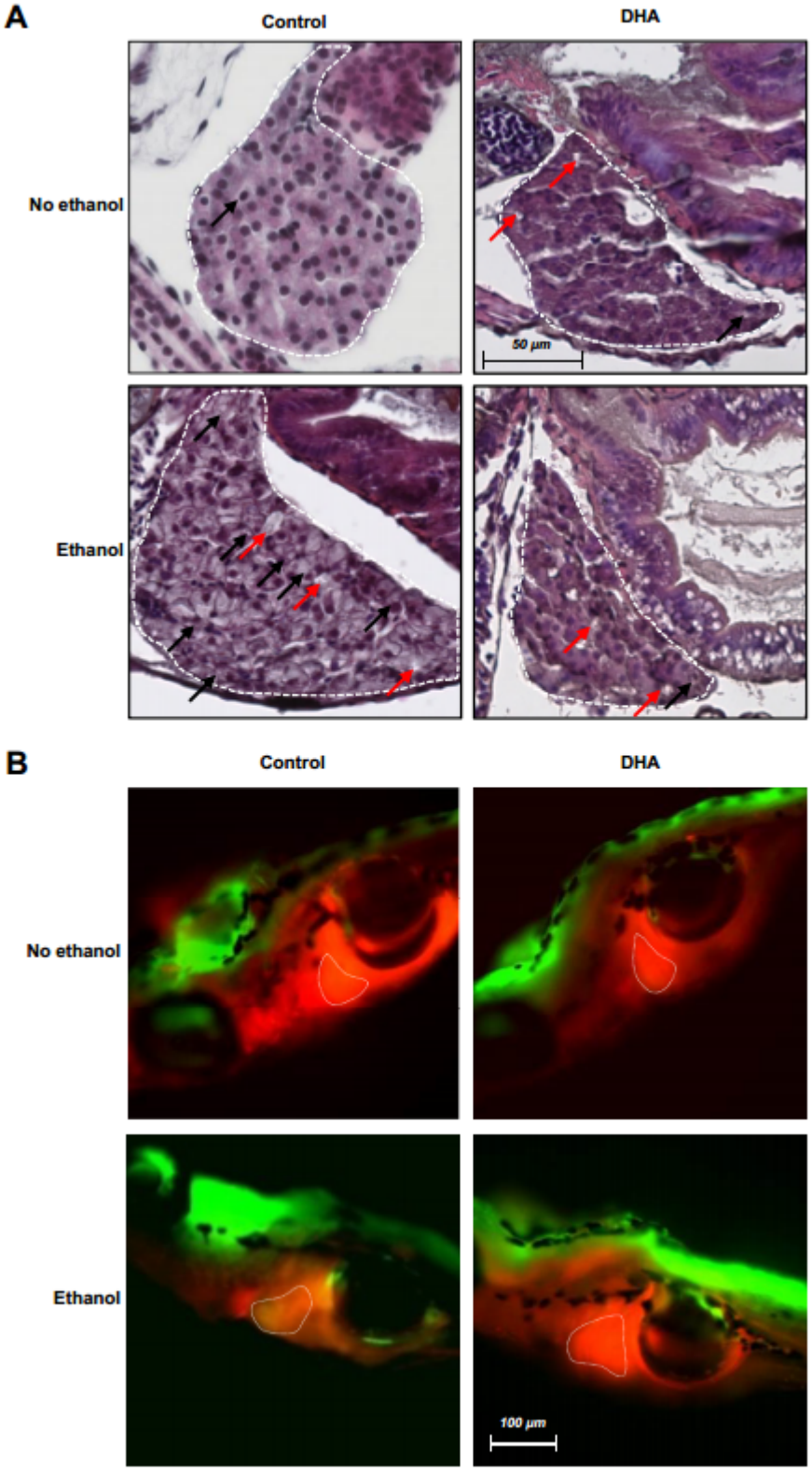

Figure 8: Zebrafish larvae as an interesting model to find beneficial compounds from food (DHA) against hepatotoxicity due to membrane remodeling. (A) Cell death was observed on zebrafish liver section after HES staining (magnification x 400). White dotted line outlines liver. Red arrows indicate hepatocyte dropouts, and black arrows show ballooning hepatocytes. (B) Oxidative stress in zebrafish liver was visualized by fluorescence microscopy after staining of living zebrafish with C11-Bodipy$^{581/591}$, a hydrophobic fluorescent probe; a yellow fluorescence was indicative of lipid peroxidation in the liver (magnification x 100). Pictures are representative of at least 3 larvae.

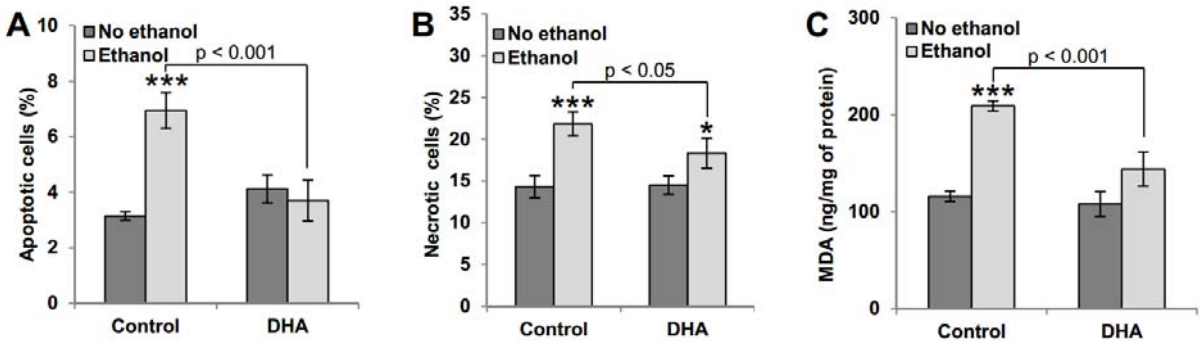

Figure 9: Protective effect of DHA against ethanol-induced membrane remodeling and toxicity in WIF-B9 hepatic cells. (A) Apoptotic and (B) necrotic cell death were determined by microscopic counting after WIF-B9 hepatocyte staining with Hoechst 33258 and propidium iodide. (D) Malondialdehyde (MDA), a lipid peroxidation end-product, was measured by HPLC-UV as a marker of oxidative stress in WIF-B9 cells. Values are the mean ± SD of three independent experiments. Treatment *versus* no treatment: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.