Toxicology Research

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/toxicology

High-dose consumption of NaCl resulted severe degradation of lipoproteins associated with hyperlipidemia, hyperglycemia, and infertility via impairment of testicular spermatogenesis

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Running head: NaCl, lipoprotein, and sterility

Abstract

Although the effect of NaCl on serum lipid levels and hypertension has been well known, the detail mechanism on lipoprotein metabolism is still remained unclear. To study physiological effects of high salt consumption in lipoprotein metabolism, NaCl was treated to human cells and zebrafish. Wildtype zebrafish (10-week old) were fed 10% NaCl (wt/wt) in tetrabit diet with or without 4% cholesterol (wt/wt) for 21 weeks. Treatment with NaCl accelerated oxidation and glycation of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) as well as induced proteolytic degradation and aggregation. NaCl treatment also exacerbated phagocytosis of oxLDL into macrophage as well as cytotoxicity. Consumption of high salt diet (HSD, final 5% or 10% in diet, wt/wt) containing with or without 4% cholesterol for 21 weeks resulted remarkable elevation of serum cholesterol, triglyceride, glucose, and hepatic inflammation levels in zebrafish with significant weight loss. Fertility based on egg production was reduced by up to 45% in the HSD group. However, embryonic survivability after hatching was significantly lowered to less than 55%, whereas the control group showed 87% survival. HSD group showed abnormal testicular histology as well as spermatogenic defects, especially upon consumption of HCD. These results suggest that hyperlipidemia and high salt consumption have an additive effect on male fertility impairment. High salt consumption exacerbates hyperlipidemia, inflammation, spermatogenic defects, and infertility via modification of lipoproteins.

Key words; sodium chloride, lipoproteins, hyperlipidemia, infertility

Introduction

It is well known that high salt intake is directly associated with incidence of hypertension, metabolic syndrome, and cardiovascular disease [1-3]. An association between diabetes and hypertension has long been recognized, and metabolic syndrome is characterized by hyperinsulinemia, glucose intolerance, reduced levels of high-density lipoprotein-cholesterol (HDL-C), hypertriglyceridemia, and central obesity in association with hypertension.

Plasma HDL-C levels are correlated with reduced risk of cardiovascular disease (CVD) [4]. HDL (1.063<d<1.225) is a complex of apolipoprotein and lipids in plasma, and it exerts potent antioxidant and anti-inflammatory activities [5, 6]. However, beneficial HDL can be degenerated to dysfunctional HDL, which has pro-atherosclerotic and pro-inflammatory properties, via several modifications stimulated by aging stress, including oxidation [7], glycation [8], and enzymatic cleavage mediated by matrix metalloproteinase [9].

Modification of serum lipoproteins is the major process behind the initiation of metabolic syndrome, which is associated with mild inflammation and oxidative stress. Oxidation or glycation of LDL is directly linked with inflammatory cascades that trigger cardiovascular disease and diabetes [10]. Further, systemic oxidative stress in the testicular microenvironment affects spermatogenesis and sperm quality [11]. However, it is unknown whether or not high consumption of salt affects levels of plasma HDL and LDL cholesterol, which are important factors in such diseases.

Sodium intake is associated with elevated blood pressure as well as increased oxidative stress [12], resulting in arterial wall stiffness, stroke, and fibrosis of the heart and kidneys. Our research group reported that modification of lipoproteins by a sterilizer chemical causes

fibrosis of the heart aorta [13]. The results suggest that excess sodium can contribute to the modification of lipoproteins in serum via elevation of oxidative stress and inflammation.

Excess dietary salt is a major contributor to hypertension as well as a critical public health issue worldwide. Reduced sodium intake can be beneficial, especially for people with certain medical conditions such as high blood pressure, kidney disease, and heart problems [14]. Specifically, reduction of dietary salt intake by up to 3 g per day has been shown to reduce the incidence of coronary heart disease, stroke, and myocardial infarction across populations [15].

Regarding the effects serum lipids on sterility, it has been reported that a high level of serum cholesterol is associated with male infertility in animals and humans [16-18]. Reduced semen quality, sperm count, motility, and morphology have all been observed in hypercholesterolemic rats, and histopathological abnormalities in the testis are associated with a high cholesterol diet (HCD) [19]. Moreover, in a clinical study, Ramírez-Torres et al. reported that high incidences of hyperlipidemia (65%) and hypertension (26%) in a group of infertile men in which 80% of patients were < 40-years-old [20].

Until now, the mechanism of action of HCD on impaired testicular function remains to be investigated. In the current study, we compared the effects of high salt consumption with or without HCD on serum lipid profiles, embryonic toxicity, and reproduction ability, especially in males, in order to examine the association between high salt intake and oxidative stress, dyslipidemia, and sterility.

For this, in the current study, we examined the modification of serum lipoproteins and lipid metabolism upon high salt intake *in vitro* using serum lipoprotein fractions as well as *in vivo* using zebrafish over the course of 21 weeks. Changes in serum lipid profiles, fertility,

embryonic survivability, and testicular histology were also examined in the zebrafish model.

Materials and methods

Materials

Sodium chloride (NaCl, #7647-14-5) was purchased from Merck KGaA (Darmstadt, Germany), and cholesterol (#C8667) was obtained from Sigma (St Loius, MO).

Purification of lipoproteins and apoA-I

Human plasma was isolated by low-speed centrifugation from healthy human males who fasted for at least 16 hours before donating blood voluntarily. Very low-density lipoprotein (VLDL, d<1.019 g/mL), low-density lipoprotein (LDL, 1.019<d<1.063), high-density lipoprotein (HDL_2, 1.063<d<1.125), and HDL_3 (1.125<d<1.225) were isolated via sequential ultracentrifugation, and the density was appropriately adjusted by addition of NaCl and NaBr in accordance with standard protocols [21]. Samples of each lipoprotein fraction were centrifuged for 22 hours at 10^{o}C and 100,000 g using a Himac CP-90α (Hitachi, Tokyo, Japan) at the Instrumental Analysis Center at Yeungnam University. After centrifugation, each lipoprotein sample was extensively dialyzed against Tris-buffered saline (TBS; 10 mM Tris-HCl, 5 mM EDTA, and 140 mM NaCl [pH 7.4]) for 24 hours in order to remove NaBr [22].

Human apoA-I was purified from HDL by ultracentrifugation and column chromatography according to a previously described method [23]. Protein purity of at least 95% was confirmed by SDS-PAGE. Protein concentration was determined according to Lowry-Markwell protein assay, modified for lipoproteins as in our previous report [24], using bovine serum albumin as a standard.

Oxidation and glycation of HDL and apoA-I in the presence of NaCl

Human HDL_3 (1 mg/mL) was oxidized by $CuSO_4$ treatment (final concentration, 10 μM) at 37°C for up to 72 hours in the presence of NaCl. Oxidized HDL_3 was aliquoted and analyzed by electrophoresis to determine antioxidant ability.

Human HDL_3 was incubated with NaCl (final 0.8, 5, or 10%) and fructose (5 mM) for the designated time up to 72 hours at 37°C in the presence of 5% CO_2. After incubation, each HDL_3 fraction was characterized by electrophoresis and fluorescence spectroscopic analysis in order to confirm the extent of glycation.

To mimic consumption of fast food, which contains high contents of both fructose and NaCl, we tested the extent of protein fructosylation in HDL in the presence of NaCl. In the preliminary test, the extent of fructosylation increased with increasing NaCl content.

Cupric ion-mediated LDL oxidation

To compare the degree of cupric ion-mediated LDL oxidation, 300 μg of LDL was incubated with 5 μM $CuSO_4$ for up to 2 hours in the presence of NaCl (final 0.8, 5, or 10%). After the reaction, the amount of oxidized product in the mixture was quantified based on the conjugated diene level [25] as well as the thiobarbituric acid reactive substance (TBARS) method [26] using malondialdehyde as a standard. To confirm the spectroscopic data, oxidized LDL was subjected to agarose gel electrophoresis, as previously described [27].

LDL phagocytosis assay

THP-1 cells, a human monocytic cell line, were obtained from the American Type Culture Collection (ATCC, #TIB-202^{TM}, Manassas, VA, USA) and maintained in RPMI-1640 medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum until needed. Cells that had undergone no more than 20 passages were incubated in medium containing phorbol 12-myristate 13-acetate (PMA, 150 nM) in 24-well plates for 24 hours at 37^{o}C in a humidified incubator (5% CO_2, 95% air) in order to induce differentiation into macrophages. Differentiated and adherent macrophages were then rinsed with warm PBS and incubated with 400 μL of fresh RPMI-1640 medium containing 1% FBS, either 50 μL of LDL (1 mg of protein/mL in PBS) or oxLDL in the presence of NaCl (final 0.8, 5, or 10%), and 50 μL of PBS for 48 hours at 37^{o}C in a humidified incubator. After incubation, cells were washed with PBS three times and then fixed in 4% paraformaldehyde for 10 minutes. Next, fixed cells were stained with oil-red O staining solution (0.67%) and then washed with distilled water. THP-1 macrophage-derived foam cells were then observed and photographed using a Nikon Eclipse TE2000 microscope (Tokyo, Japan) at 600x magnification.

Supplementation of NaCl to zebrafish

Maintenance and procedures involving zebrafish were approved by the Committee of Animal Care and Use of Yeungnam University (Gyeongsan, Korea). Zebrafish, larvae, and embryos were maintained in 6-well plates at 28^{o}C in a system cage maintained under a 14:10 hr light:dark cycle. Wild-type zebrafish and their embryos were maintained according to

standard protocols [28].

High salt diet (final 10%, wt/wt) was prepared by addition of NaCl to Tetrabit (Tetrabit Gmbh D49304; 47.5% crude protein, 6.5% crude fat, 2.0% crude fiber, and 10.5% crude ash containing vitamin A [29770 IU/kg], vitamin D3 [1860 IU/kg], vitamin E [200 mg/kg], and vitamin C [137 mg/kg]; Melle, Germany). After redissolving NaCl and Tetrabit, the mixture was lyophilized again to prevent loss during feeding. High cholesterol diet (HCD) consisting of 4% cholesterol was prepared by soaking Tetrabit in a diethyl ether solution of cholesterol (Sigma # C-3045), as previously described [29, 30].

As designated in Table 1, blood (2 μL) was drawn from the hearts of adult fish after feeding for 21 weeks, combined with 5 μL of PBS-EDTA, and then collected into EDTA-treated tubes (final 1 mM). Serum total cholesterol (TC), HDL-cholesterol, and triglyceride (TG) levels were determined using a commercial assay kit (cholesterol, T-CHO; Wako Pure Chemical, Osaka, Japan; triglycerides, Cleantech TS-S, Wako Pure Chemical). Concentrations of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were measured using a commercially available assay kit (Asan Pharmaceutical, Hwaseong, Korea).

Embryo production and embryonic toxicity

Embryo survivability was measured after each group had mated, as previously described [31, 32]. One male from each group was placed together with one female in a 1 L mating cage in according to our previous report [33]. Embryonic morphology was observed at 48 hours post-fertilization, whereas imaging of reactive oxygen species (ROS) was carried out according to our previous report [33]. After treatment with HCD and HSD, the elevated

ROS and inflammatory responses were imaged using dihydroethidium (DHE, cat # 37291; BioChemika), as previously described [34]. The embryonic image was obtained by fluorescent observation (Ex=588 nm and Em=605 nm) using a Nikon Eclipse TE2000 microscope (Tokyo, Japan). Quantification of the fluorescent area in the embryo was carried out via computer-assisted morphometry using Image Proplus software (version 4.5.1.22, Media Cybernetics, Bethesda, MD).

Histologic analysis

Testes were collected from zebrafish fed a high salt diet (10% NaCl) and/or HCD (x%) for 24 weeks. Tissues were fixed in Bouin solution for 2 days and then dehydrated in 30% sucrose solution for 2 days. Frozen testes in OCT cryo-embedding compound were freshly sectioned to a thickness of 6 μm and stained using standard H&E staining protocol. H&E staining was also performed on formalin-fixed, paraffin-embedded 3-μm sections after deparaffinization, as previously described [35]. Testicular abnormalities were examined by measuring spermatogenesis, the size of seminiferous tubules, as well as the size of the interstitial space in the testis slides from at least three fishes per group. Spermatogenic defects were examined by measuring the area of each spermatogenic cell, as previously defined by previously [36].

In order to compare antioxidant ability, aliquots of hepatic tissue (50 mg of liver in 0.5 mL of PBS) from each group were homogenized for 3 min (150 rpm) in an ice bath using a tissue homogenizer (Euro-ST; Eurostar, IKA-WERKE, Staufen, Germany). After brief centrifugation (10,000g) and protein determination using Bradford reagent, equally diluted supernatants (100 μg of protein in 0.05 mL) were used for determination of oxidative species

using the thiobarbituric acid reactive species (TBARS) method [26]. In the homogenate aliquot, ferric ion reduction ability was compared according to our recent report [30].

Statistical analysis

All data are expressed as the mean±SD from at least three independent experiments with duplicate samples. Comparisons between results were made via analysis of variance (ANOVA), and differences were sought by Student′s *t*-test using the SPSS program (version 14.0; SPSS, Inc., Chicago, IL, USA) for the *in vitro* experiment. For the *in vivo* test, the data were evaluated via one-way ANOVA using the SPSS program, and differences between the means were assessed using Duncan′s multiple-range test (Table 1). Statistical significance was defined as $p<0.05$.

Results

Oxidation of LDL and HDL by cupric ion and NaCl

In the absence of cupric ion, NaCl treatment resulted in slower electromobility of the LDL fraction in a dose-dependent manner (lanes 1-3, Fig. 1A). In the presence of cupric ion, LDL moved faster due to oxidation (lane 4, Fig. 1). However, NaCl treatment induced slower electromobility of LDL in a dose-dependent manner (lane 6, Fig. 1A). Therefore, excess sodium uptake can alter the electromobility of LDL. Oxidation increased the negative charge and degradation of LDL, as shown in Fig. 1B. Especially, co-treatment of NaCl and cupric ion (final 10 μM) caused severe proteolytic degradation of LDL (lanes 4-6, Fig. 1B). Measurement of conjugated diene levels showed that co-treatment of NaCl and cupric ion accelerated oxidation compared to cupric ion treatment alone. After 60 minutes, NaCl and cupric ion co-treatment increased A_{234} by 1.4-fold compared to cupric ion treatment alone, as shown in Fig. 1C.

Although cupric ion-mediated oxidation occurred in the HDL fraction (lanes 4-6, Fig. 2), co-treatment with NaCl also reduced the electromobility of HDL in the native state (Fig. 2A, 2C). Proteolytic degradation of HDL also occurred upon co-treatment of cupric ion and NaCl for 4 hours, as determined by SDS-PAGE (lanes 4-6, Fig. 2B and 2D). Specifically, the major bands of HDL, apoA-I (28 kDa), and apo-E (34 kDa) almost disappeared and multimerized at the top of the gel (lanes 4-6, Fig. 2D). Further, co-treatment of NaCl and cupric ion reduced the electromobility of both HDL_3 and apoA-I (Fig. 2C, 2D).

Acceleration of lipoprotein glycation by NaCl

As shown in Fig. 3, fructosylation of LDL and HDL was accelerated by increasing NaCl treatment. Further, glycation of lipoproteins as evidenced by yellowish fluorescence was elevated in a time- and dose-dependent manner with increasing NaCl content. After 24 hours, both the fructose- and 10% NaCl-treated HDL fractions showed 1.5- and 7.4-fold increased yellowish fluorescence, respectively, compared to fructose-treated HDL and HDL alone (Fig. 3A). Agarose gel electrophoresis after 48 hours revealed that NaCl-treated HDL showed slower electromobility and proteolytic degradation, as shown in Fig. 3C.

Lipoproteins were incubated for 72 hours in the presence of both fructose and NaCl underwent severe protein degradation in a time-dependent manner, as shown in Fig. 4. HDL_3 fraction showed severe proteolysis and tetramer multimerization after 24 hours with increased NaCl treatment (lane 5, panel B). After 72 hours, co-treatment of NaCl and fructose resulted in disappearance of the apoA-I band as well as protein aggregation at the top of the gel (panel C, Fig. 4). Furthermore, LDL degradation was more accelerated upon co-treatment of fructose and NaCl. The apo-B band almost disappeared after 24 hours and completely disappeared after 72 hours.

NaCl exacerbates phagocytosis of oxLDL by macrophages and cytotoxicity

As shown in Fig. 5, increasing NaCl treatment accelerated uptake of oxLDL into macrophages, as evidenced by oil-red O staining. Specifically, oil-red intensity was elevated 10-fold by 5% compared to 0.8% NaCl treatment (physiological salt content). However, 10% NaCl treated resulted in severe cytotoxicity due to increased osmolarity.

High salt intake causes hyperlipidemia in zebrafish despite weight loss

After 21 weeks of NaCl treatment (final 10%, wt/wt) with or without cholesterol supplementation (final 4%, wt/wt), zebrafish showed a remarkable body weight loss of 16% compared to the control group (Table 1). On the other hand, zebrafish fed a high cholesterol diet (HCD) with NaCl showed elevated serum total cholesterol (TC) and triglyceride (TG) levels. In the normal diet (ND) group, NaCl diet increased serum TC levels by 1.5-fold, whereas serum TG levels were unchanged. Zebrafish fed HCD with NaCl showed 1.2- and 1.6-fold increases in serum TC and serum TG levels.

Likewise, serum glucose levels in both the ND and HCD groups increased by 1.4-fold upon NaCl supplementation. The serum GOT level also increased by 1.4-fold in the HCD group fed 10% NaCl. These results suggest that serum TC, TG, glucose, and hepatic inflammatory levels were elevated upon NaCl consumption despite body weight loss, especially combined with HCD.

NaCl consumption reduces embryo production and survivability

Fertility based on egg production was reduced by up to 45% in both the ND and HCD groups following 15 weeks of NaCl consumption (Fig. 6A), and there was no difference between the ND and HCD groups.

Further, embryonic survivability was monitored 4 days after fertilization (Fig. 6B). After hatching, embryos from the HCD group showed lower survivability (81%) than those from the ND group (91%). Whereas the ND group fed NaCl showed similar embryonic survivability (81%), the HCD group fed NaCl showed reduced survivability (53%). After 48 hours, the HCD group showed reduced developmental speed as well as increased production of ROS (Fig. 6C). The HCD group fed NaCl showed the highest DHE staining intensity,

whereas ND and HCD groups fed NaCl showed 1.6-fold ROS elevation based on fluorescence intensity (Fig. 6D). This result suggests that NaCl consumption caused impairment of embryonic production and survival.

Salt intake reduces male fertility

To determine the effects of salt intake on male fertility, we examined spermatogenesis and testicular abnormalities. Histological examination of testes from zebrafish fed a ND showed seminiferous tubules with full cell populations adherent to the basal membrane with few gaps between them and the interstitium (Fig. 7A). However, zebrafish fed high salt displayed irregularly outlined seminiferous tubules with disarranged cellular layers as well as a broken lamina basal membrane, indicating reduced spermatogenesis. Morphometric results of spermatogenesis in zebrafish fed a high salt diet revealed that spermatids and sperm (mature forms) were smaller in area compared to the ND group. The average areas of spermatids and sperm in seminiferous tubules from the ND, ND+NaCl, HCD, and HCD + NaCl groups were 58.1%, 49.1%, 35.8%, and 25.9% of the total area (ND vs. HCD and ND+NaCl vs. HCD+NaCl, $p<0.05$), respectively, thereby indicating spermatogenic defects. High fat diet also induced abnormal testicular histological patterns. The average size of seminiferous tubules significantly decreased compared to the control group (219 vs. 439 arbitrary units, $p<0.05$). Strikingly, high salt intake induced degeneration of the seminiferous tubules with empty spaces in the inter- and intra-seminiferous tubules (arrow, Fig. 7B). Areas of these empty spaces significantly increased in both the high salt and HCD groups, as shown in the graph (Fig. 7C). Due to the fragile testicular interstitium, tissue tearing occurred during H&E staining in the cryosection slides for tissues treated with 10% NaCl. We also performed

paraffin sectioning to confirm our results. As shown in Fig. 8, the interstitial space of the group treated with NaCl and HCD was larger. Spermatogonia (SG), spermatocytes (SC), and chromatin of spermatocytes looked uniform with punctuate condensations. Spermatid (ST, residual bodies were condensed and small) and spermatozoa (SZ) were marked in seminiferous tubules (Fig. 8A). Based on DHE staining, ROS production was 1.4- and 1.9-fold higher in the ND and HCD groups fed NaCl, respectively (Fig. 8B).

From the DAPI staining, live cell number decreased by 35% and 75% by NaCl feeding under ND and HCD conditions, respectively (Fig. 8B). ROS production was elevated 1.5- and 1.9-fold by NaCl consumption under ND and HCD (Fig. 8B). These results suggest that testis damage was more prevalent in the HCD group via cell death and ROS production.

We also analyzed ovarian tissue. As shown in Suppl. Fig. 1, there was no notable damage observed in the histological comparison using H&E staining or ROS production using DHE staining between the control and HSD regardless of ND and HCD. There was also no increase of inflammatory cell infiltration in ovarian tissue in the HSD group using H&E staining or ROS production using DHE staining.

Salt intake caused hepatic inflammation

In liver sections obtained from zebrafish fed NaCl, the ND (photo b) and HCD groups (photo d) showed more severe infiltration of inflammatory cells compared to the control (photos a and c), as determined by H&E staining (Fig. 9). Fatty liver changes were more severe in the HCD group (photos g and h) than in the ND group (photos e and f), as determined by oil-red O staining. The highest level of ROS production was detected in the

HCD plus NaCl group (photo i), whereas the ND plus NaCl group (photo j) showed the second highest ROS production (Fig. 9B). Interestingly, although hepatic fatty liver changes were more severe in the HCD control (photo g) than in the ND plus NaCl group (photo f), the ND plus NaCl group (photo j) showed higher ROS production (Fig. 9B). This result suggests that consumption of NaCl alone is more dangerous than HCD alone in regarding hepatic inflammation.

DAPI staining in hepatic tissue revealed that more nuclei number were detected in ND plus NaCl group (photo n in Fig. 9A) and HCD plus NaCl group (photo p in Fig. 9A) than ND (photo m) and HCD (photo o) alone, respectively, due to infiltration of inflammatory cell. Under ND and HCD, NaCl consumption caused 3.5-fold and 2.5-fold higher infiltration of inflammatory cell.

In addition, as shown in Suppl. Fig. 2A, ferric ion reduction ability (FRA) of the homogenate was significantly reduced in the HCD+NaCl group by 13%, whereas the HCD group showed a 24% increase. In the ND and ND+NaCl groups, FRA ability increased by 33% and 27%, respectively, suggesting that co-consumption of NaCl and HCD caused the most severe loss of antioxidant ability. As shown in Suppl. Fig. 2B, determination of malondialdehyde (MDA) content revealed that the HCD+NaCl group had the highest level of MDA (around 32 nmol), whereas the HCD alone group had a MDA content of 23 nmol. However, the ND and ND+NaCl groups showed MDA levels of 10 nmol and 18 nmol respectively, suggesting that NaCl supplementation caused elevation of oxidized species in the liver under both ND and HCD.

Discussion

High salt intake is directly related with increased incidence of hypertension and coronary artery disease. Therefore, reduced sodium intake can be beneficial, especially for people with certain medical conditions such as high blood pressure, kidney disease, and heart problems [14]. Specifically, reduction of dietary salt intake by up to 3 g per day has been shown to reduce the incidence of coronary heart disease, stroke, and myocardial infarction across populations [15].

Until now, there has been no evidence linking hypertension, atherogenesis, infertility, or male sterility with high salt uptake. In the current study, high salt treatment induced oxidation (Fig. 1) and glycation (Fig. 2) of HDL, resulting in protein aggregation and proteolytic degradation related with production of dysfunctional HDL. At the cellular level, NaCl treatment accelerated atherogenesis via LDL phagocytosis as well as impaired embryo production and survivability in zebrafish fed HCD (Fig. 3). Histological analysis revealed that infertility was due to altered spermatogenesis (Fig. 4) as well as elevated inflammation in the testis (Fig. 5). Following 21 weeks of NaCl supplementation, serum TG and GOT levels significantly increased despite body weight reduction (Table 1). Especially, serum TG levels remarkably increased in the HCD group fed NaCl to around 630 mg/dL, which was 1.7-fold higher than that of the ND group. This result suggests that the inflammatory response was aggravated by HCD and NaCl treatment, as elevation of serum TG levels has been established as an independent risk factor for inflammatory disease [37]. Further, the serum TG level is an important and independent predictor of coronary artery disease (CAD) and stroke, which might explain the link between high salt intake and incidence of coronary artery disease. Production of dysfunctional HDL following NaCl treatment also contributed

to the development of coronary artery disease. Similarly, high salt diet (final 8%, wt/wt) fed to rats for 6 weeks was shown to elevate serum glucose, TG, and cholesterol levels. High salt-fed rats also slowed elevation of oxidative stress and hypertension [38]. These reports are in good agreement with our current findings in terms of dyslipidemia. Acute elevation of serum TG levels and fatty liver changes in the high salt and HCD diet group correlated well with elevation of hepatic inflammation (Fig. 9). Further, elevated mortality of embryos and ROS production in the HCD and NaCl group was in good agreement with elevation of inflammation.

Zebrafish sperm motility was impaired by increasing the osmolarity to above 100 mM. Interestingly, high salt consumption has been shown to severely damage testicular tissue and spermatogenesis [39]. Interestingly, serum TG and glucose levels were elevated by NaCl treatment without HCD (Table 1) despite reduction of body weight. Although the mechanism of action is still unknown, increased salt uptake might impair cellular osmolarity and lipid homeostasis. Similarly, another group reported that ammonium chloride fed to rats for 3 weeks increased serum TG and decreased serum HDL-C levels with body weight reduction [40].

It has been reported that high serum cholesterol is associated with male infertility in animals and humans [16-18]. Poor semen quality as well as decreased sperm count, motility, and morphology have been observed in hypercholesterolemic rats; histopathological abnormalities in the testis are also associated with HCD [19]. However, a putative mechanism for functional change of testis by HCD consumption are still remained to be investigated.

It is well known that impairment of male sterility is related to low testosterone levels, which are caused by metabolic syndrome and insulin resistance. Increased oxidation and

glycation of lipoproteins could contribute to lipotoxicity and the inflammatory response (Fig. 1- 4), which can trigger endothelial dysfunction and arterial stiffening. Treatment of salt to HDL and LDL cause modification of function and structure as well as increased oxidation and glycation. Our research group previously reported that modification of lipoproteins increases foam cell formation in human macrophages [41, 42]. In addition, high salt diet administered to zebrafish for 12 and 24 weeks increased serum total cholesterol, TG, and glucose levels. These parameters are well known factors in vascular diseases such as atherosclerosis, hypertension, and diabetes. In the current study, high salt and/or HCD fed to zebrafish significantly reduced embryo production and testicular function.

The testis is one of the most important organs in steroidogenesis and cholesterol metabolism, which is regulated by HDL and LDL receptors [43]. LDL receptor/ApoE double KO mice, the most common animal model of atherosclerosis, display abnormal spermatogenesis and reduced testosterone levels, suggesting a direct relationship between atherosclerosis and altered spermatogenesis. Testicular interstitial tissue, which was severely damaged upon salt intake in our study (Fig. 7 and 8), is important for maintaining and supporting spermatogenesis since Leidig cells produce testosterone. Although there is no direct evidence of reduced testosterone production, spermatogenic defects and damaged interstitial tissues are most likely responsible for reduction of embryo production following NaCl treatment. Further, HCD had a synergistic effect with high salt intake. As expected, 24-weeks HCD consumption reduced embryo production (Fig. 6A), confirming previous reports of a relationship between high serum cholesterol levels and male infertility in animals and humans [16-18].

High salt diet is a severe problem as well as one of the causative factors of many

diseases, including hypertension and cardiovascular disorders. However, the correlation between fertility and high salt intake has not been well studied. Based on our observations, high salt diet increases serum cholesterol and TG levels as well as reduces testicular function. Our groups also reported that modification of HDL and LDL, especially glycation and oxidation, is associated with pro-inflammation and pro-atherogenic factors processes [22, 24, 41]. Similarly, male rats fed oxidized cholesterol diets for 14 weeks showed elevated serum LDL/oxidized LDL levels, reduced sperm counts, smaller seminal vesicles, and decreased fertility [44]. Our current result make a good agreement with a previous paper that high salt diet (8% NaCl) for 6 weeks was linked with higher oxidative stress and male sterility in rat via impairment of spermatogenesis [45].

To our knowledge, this is the first report suggesting that high salt intake is associated with infertility via degeneration of lipoprotein, testicular spermatogenesis, especially under hypercholesterolemia. High NaCl treatment *in vitro* modified lipoproteins and caused inflammation, whereas high salt consumption *in vivo* induced abnormal serum lipid profiles and spermatogenic defects in zebrafish. These results suggest that low salt uptake can ameliorate human dyslipidemia and infertility by enhancing spermatogenesis.

Acknowledgements

This work was supported by the Mid-carrier Researcher Program (2014-11049455) and the Medical Research Center Program (2015R1A5A2009124) through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning.

References

1. K. Stolarz-Skrzypek, A. Bednarski, D. Czarnecka, K. Kawecka-Jaszcz and J. A. Staessen, *Curr. Hypertens. Rep.*, 2013, 15, 122-130.

2. K. Ando and T. Fujita, *Free Radic. Biol. Med.*, 2009, 47, 213-218.

3. K. J. Aaron and P. W. Sanders, *Mayo Clin. Proc.*, 2013, 88, 987-995.

4. N. E. Miller, D. S. Thelle, O. H. Forde and O. D. Mjos, *Lancet*, 1977, 1, 965-968..

5. K. H. Cho, *BMB Rep.*, 2009, 42, 393-400.

6. P. J. Barter, S. Nicholls, K. A. Rye, G. M. Anantharamaiah, M. Navab and A. M. Fogelman, *Circ. Res.*, 2004, 95, 764-772.

7. S. Deakin, X. Moren and R. W. James, *Arterioscler. Thromb. Vasc. Biol.*, 2007, 27, 1146-1152.

8. O. Elboudwarej, H. Hojjat, S. Safarpoor, S. Vazirian and S. Ahmadi, *Diabetes Metab. J.*, 2011, S4.

9. H. Feng and X. A. Li, *Curr. Opin. Endocrinol. Diabetes Obes.*, 2009, 16, 156–162.

10. D. Li and J. L. Mehta, *Cardiovasc. Res.*, 2005, 68, 353–354.

11. A. E. Omu, *Med. Princ. Pract.*, 2013, 22, 30-42.

12. Y. Koga, Y. Hirooka, S. Araki, M. Nozoe, T. Kishi and K. Sunagawa, *Hypertens. Res.*, 2008, 31, 2075-2083.

13. J. Y. Kim, H. H. Kim and K. H. Cho, *Cardiovasc. Toxicol.*, 2013, 13, 148-160.

14. P. Meneton, X. Jeunemaitre, H. E. de Wardener and G. A. MacGregor, *Physiol. Rev.*, 2005, 85, 679-715.

15. K. Bibbins-Domingo, G. M. Chertow, P. G. Coxson, A. Moran, J. M. Lightwood, M. J. Pletcher and L. Goldman, *N. Engl. J. Med.*, 2010, 362, 590-599.

16. R. S. Padrón, J. Más, R. Zamora, F. Riverol, M. Licea, L. Mallea and J. Rodriguez, *Int. Urol. Nephrol.*, 1989, 21, 515-519.

17. T. E. Saez Lancellotti, P. V. Boarelli, M. A. Monclus, M. E. Cabrillana, M. A. Clementi, L. S. Espínola, J. L. Cid Barría, A. E. Vincenti, A. G. Santi and M. W. Fornés, *PLoS One*, 2010, 5, e13457.

18. D. J. Palmer, T. Sullivan, M. S. Gold, S. L. Prescott, R. Heddle, R. A. Gibson and M. Makrides, *BMJ.*, 2012, 344, e184.

19. M. A. Shalaby, H. Y. el-Zorba and G. M. Kamel, *Pharmacol. Res.*, 2004, 50, 137-142.

20. M. A. Ramírez-Torres, A. Carrera and M. Zambrana, *Ginecol. Obstet. Mex.*, 2000, 68, 224-229.

21. R. J. Havel, H. A. Eder and J. H. Bragdon, *J. Clin. Invest.*, 1955, 34, 1345-1353.

22. K. H. Park, D. G. Shin, J. R. Kim and K. H. Cho, *J. Gerontol. A Biol. Sci. Med. Sci.*, 2010, 65, 600-610.

23. Jr. H. B. Brewer, R. Ronan, M. Meng and C. Bishop, *Methods Enzymol.*, 1986, 128, 223–246.

24. K. H. Park and K. H. Cho, *J. Gerontol. A Biol. Sci. Med. Sci.*, 2011, 66, 511-520.

25. H. Esterbauer, G. Striegl, H. Puhl and M. Rotheneder, *Free Radic. Res. Commun.*, 1989, 6, 67-675.

26. M. S. Blois, *Nature*, 1958, 181, 1199–2000.

27. R. P. Noble, *J. Lipid Res.*, 1968, 9, 693-700.

28. C. Nusslein-Volhard and R. Dahm, eds, Oxford University Press, 2002.

29. K. Stoletov, L. Fang, S. H. Choi, K. Hartvigsen, L. F. Hansen and C. Hall, *Circ. Res.*, 2009, 104, 952-960.

30. S. Jin and K. H. Cho, *Food Chem. Toxicol.*, 2011, 49, 1521-1529.

31. R. Kopp, I. Bauer, A. Ramalingam, M. Egg and T. Schwerte, *PLoS One*, 2014, 9, e89099.

32. R. Spence and C. Smith, *Behav. Ecol.*, 2006, 17, 779-783.

33. K. H. Park and K. H. Cho, Fish Shellfish Immunol., 2011, 31, 904–910.

34. E. Owusu-Ansah, A. Yavari, S. Mandal and U. Banerjee, *Nat. Genet.*, 2008, 40, 356-361.

35. N. A. Sabaliauskas, C. A. Foutz, J. R. Mest, L. R. Budgeon, A. T. Sidor, J. A. Gershenson, S. B. Joshi and K. C. Cheng, *Methods*, 2006, 39, 246-254.

36. N. D. Koc, N. Teksöz, M. Ural and C. Akbulut, *Elixir Aquaculture*, 2012, 46, 8117-8120.

37. P. Libby, *Circ. Res.*, 2007, 100, 299-301.

38. M. Olaleye, O. Crown, A. Akinmoladun and A. Akindahunsi, *Hum. Exp. Toxicol.*, 2013, 33, 602-608.

39. J. G. Wilson-Leedy, M. K. Kanuga and R. L. Ingermann, *Theriogenology*, 2009, 71, 1054-1062.

40. A. T. Høstmark, M. S. Lunde and A. Haug, *Lipids Health Dis.*, 2013, 12, 92.

41. K. H. Park, W. Jang, K. Y. Kim, J. R. Kim and K. H. Cho, *Biochem. Biophys. Res. Commun.*, 2010, 392, 295–300.

42. W. Jang, N. H. Jeoung and K. H. Cho, *Mol .Cells*, 2011, 31, 461-470.

43. A. C. Langheinrich, A. Paradowska, R. Kilinski, M. Kampschulte, K. Steinfeld, B. Altinkilic, K. Steger, P. Stieger, M. Bergmann and W. Weidner, *Int. J. Androl.*, 2012, 35, 562-571.

44. S. Ghanbarzadeh, A. Garjani, M. Ziaee and A. Khorrami, *Drug Res.*, 2014, 64, 510-515.

45. B. O. Iranloye, G. O. Oludare, A. O. Morakinyo, N. A. Esume and L. C. Ekeh, *J. Hum. Reprod. Sci*., 2013, 6, 267-272.

Figure legends

Fig. 1. Acceleration of LDL oxidation by NaCl and cupric ion. Electrophoretic patterns of LDL based on agarose gel electrophoresis (A) and SDS-PAGE (B). Conjugated diene detection during copper-mediated LDL oxidation. Continuous monitoring of conjugated diene production for up to 2 hours with NaCl and cupric ion (C).

Fig. 2. Oxidation of HDL by cupric ion and NaCl. Electrophoretic patterns of HDL based on agarose gel electrophoresis (A) and SDS-PAGE (B).

Fig. 3. Glycation of HDL by fructose in the presence of NaCl. Fluorospectroscopic determination of extent of glycation based on fluorescence intensity (Ex = 370 nm, Em = 440 nm). (B) Electrophoretic pattern of HDL under native conditions by agarose gel electrophoresis after 48 hours.

Fig. 4. Electrophoretic patterns of glycated HDL and LDL by SDS-PAGE (15% for HDL and 6% for LDL) with different incubation times.

Fig. 5. Cellular uptake of oxLDL in the presence of NaCl in a concentration-dependent manner. PMA-differentiated macrophages were incubated with 50 μL of oxLDL (1 mg/mL)

and NaCl in 400 μL of RPMI1640 media. Extent of cellular uptake of lipids or LDL by macrophages was compared by oil-red O staining as described in the text. Cells were photographed using a Nikon Eclipse TE2000 microscope (Tokyo, Japan) at 400 X magnification.

Fig. 6. Embryo production and survival. Embryo production number per mating from 13-21 weeks (A). Embryonic survivability after fertilization for 96 hours in system water (B). Detection of reactive oxidized species (ROS) in embryos based on DHE staining (C). Quantification of ROS from fluorescence intensity (D). *, $p < 0.05$; $p < 0.01$

Fig. 7. Testicular histology. A. H&E staining of frozen testis tissues from zebrafish fed a normal diet (ND), 10% NaCl diet (NaCl), high cholesterol diet (HCD), and high salt plus high cholesterol (NaCl plus HCD) for 21 weeks. Arrow indicates empty interstitial space in seminiferous tubules. B. Spermatogonia (SG), spermatocytes (SC), spermatids (ST), and spermatozoa (SZ) in normal testis tissue. C. Threshold modified images of panel A for measurement of empty intra/inter spaces in seminiferous tubules. Black area indicates empty space. C. Graph shows the average value of B. * $p < 0.05$ from ANOVA.

Fig 8. Testicular histology and DHE staining. Top panel shows H&E staining of paraffin-embedded testis tissues from each treatment group as indicated (A). Bottom panel shows representative pictures of DHE and DAPI staining for detection of ROS and DNA in the nucleus, respectively. Quantification of fluorescence area by computer assisted morphometry

(B).

Fig. 9. Histological analysis of hepatic tissue.

A. Representative photos for infiltrated inflammatory cells were visualized by Hematoxylin and Eosin (H&E) staining. Representative photos for comparison of fatty liver changes in zebrafish depending on consumed NaCl between normal diet (ND) and high cholesterol diet (HCD), as visualized by Oil-red O staining (scale bar 100 μm). ROS production was compared by DHE staining. Infiltration of inflammatory cells was visualized by DAPI staining.

B. Quantification of Oil-red O-stained area and fluorescence area by computer-assisted morphometry.

Table 1. Parameters of zebrafish after 21 weeks of NaCl treatment with normal diet (ND) and high cholesterol diet (HCD).

	ND [1] Control (n=50)	ND 10% NaCl (n=50)	HCD [2] Control (n=50)	HCD 10% NaCl (n=50)
Body weight (mg)	512 ± 39[a, 3]	459 ± 34[b]	671 ± 40 [c]	567 ± 31 [d, **]
Weight (mg)/height (mm)	13 ± 1[a]	12 ± 1 [a]	17 ± 2 [b]	14 ± 1 [ab]
Total cholesterol (mg/dL)	160 ± 2 [a]	240 ± 21 [b, **]	623 ± 7 [c]	740 ± 7 [d, ***]
Triacylglyceride (mg/dL)	347 ± 2 [a]	368 ± 21 [a]	397 ± 2 [a]	630 ± 7 [b, ***]
Glucose (mg/dL)	88 ± 1 [a]	258 ± 13 [b, ***]	206 ± 10 [c]	291 ± 10 [d, **]
GOT (Karmen/mL)	164 ± 8 [a]	281 ± 7 [b]	351 ±17 [c]	496 ± 20 [d, **]
GPT (Karmen/mL)	26 ± 2 [a]	32 ± 2 [b]	35 ± 6 [b]	43 ± 2 [c, *]

[1] ND, normal diet, Tetrabit®: Tetrabit (47.5% crude protein, 6.5% crude fat, 2.0% crude fiber, 10.5% crude ash, containing vitamin A [29,770 IU/kg], vitamin D3 [1860 IU/kg], vitamin E [200 mg/kg], and vitamin C [137 mg/kg]).

[2] HCD, high cholesterol diet, Tetrabit + 4% cholesterol

[3] The mean values not sharing a common letter in the same row are significantly different between groups (p<0.05).

GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase

*, p <0.05 versus control; **, p <0.01 versus control; ***, p <0.005 versus control.

Fig. 1.

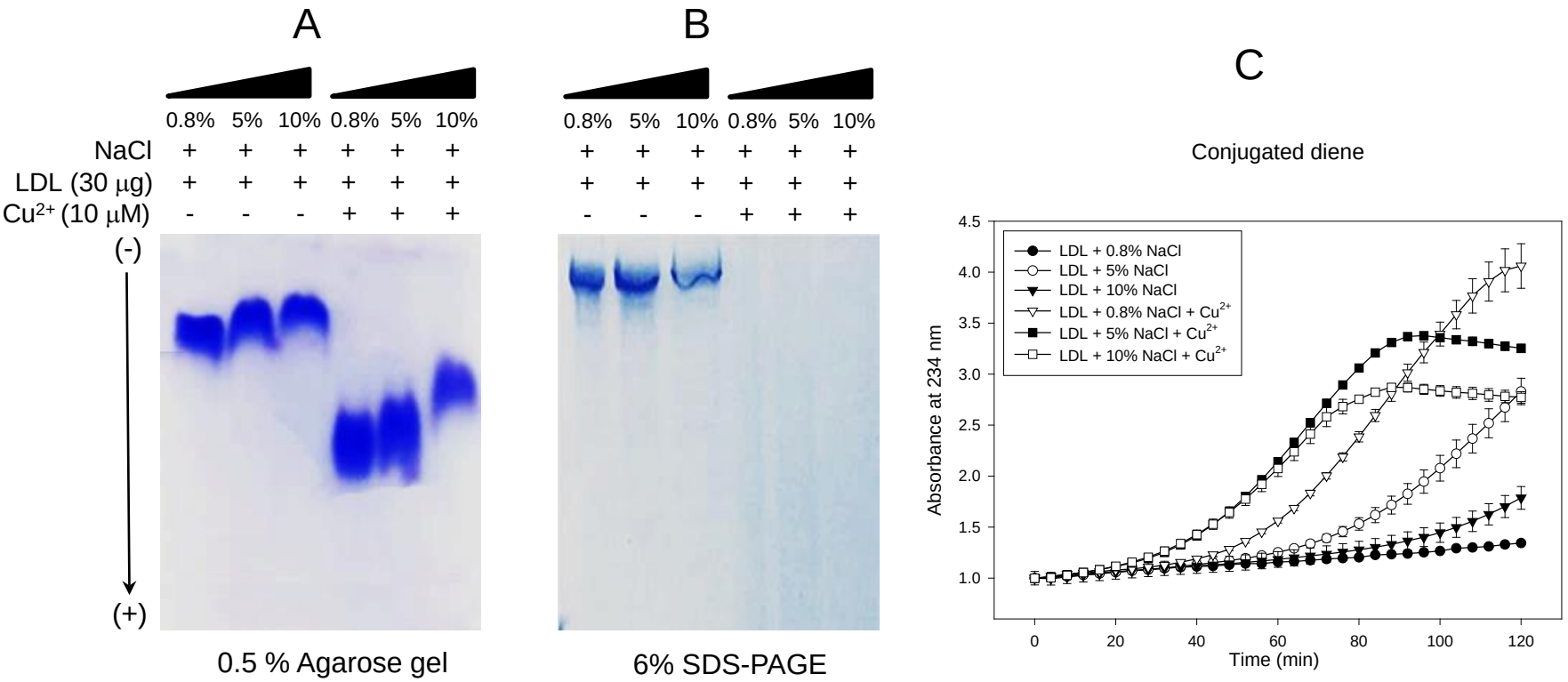


Fig. 2.

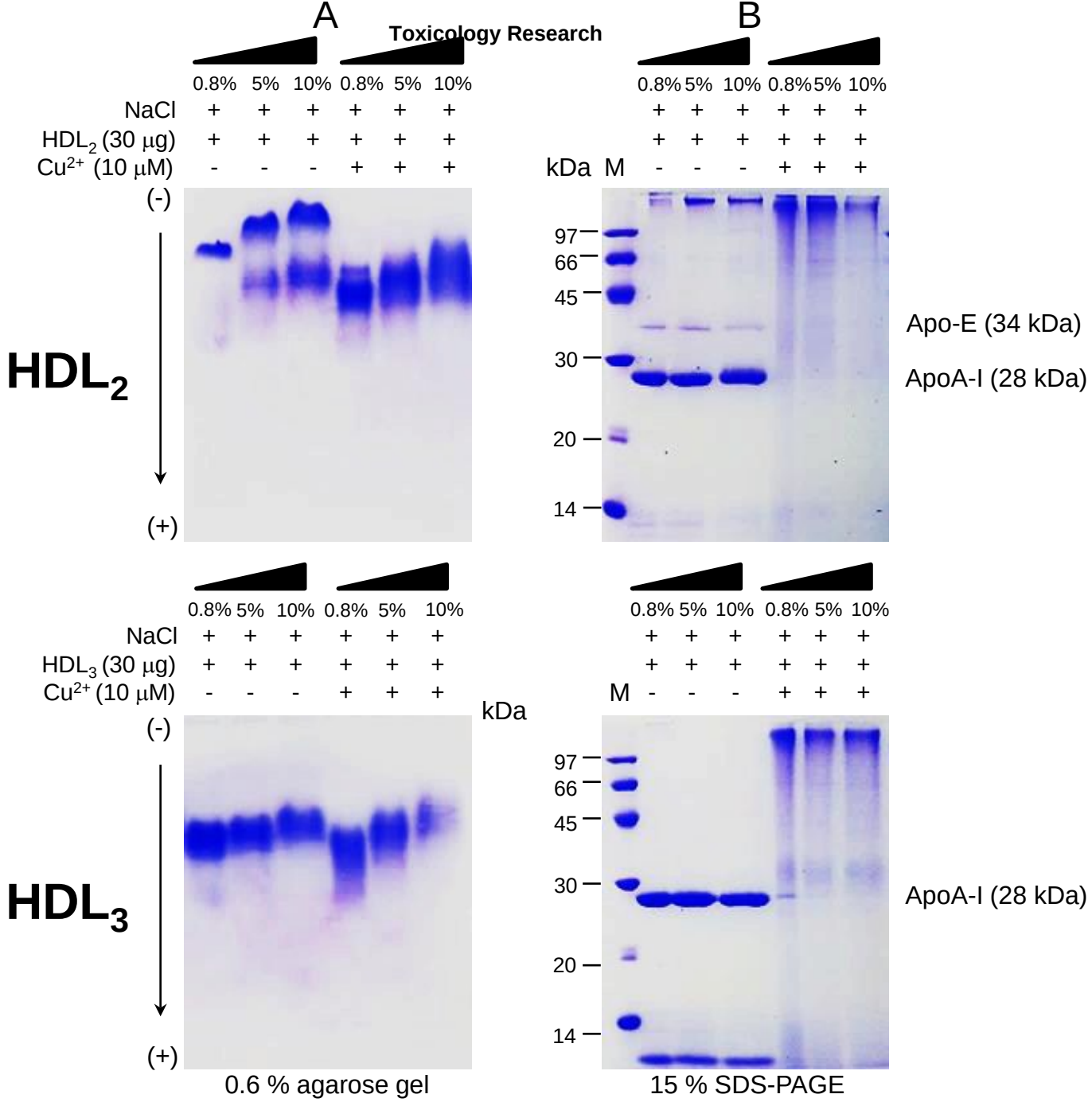


Fig. 3

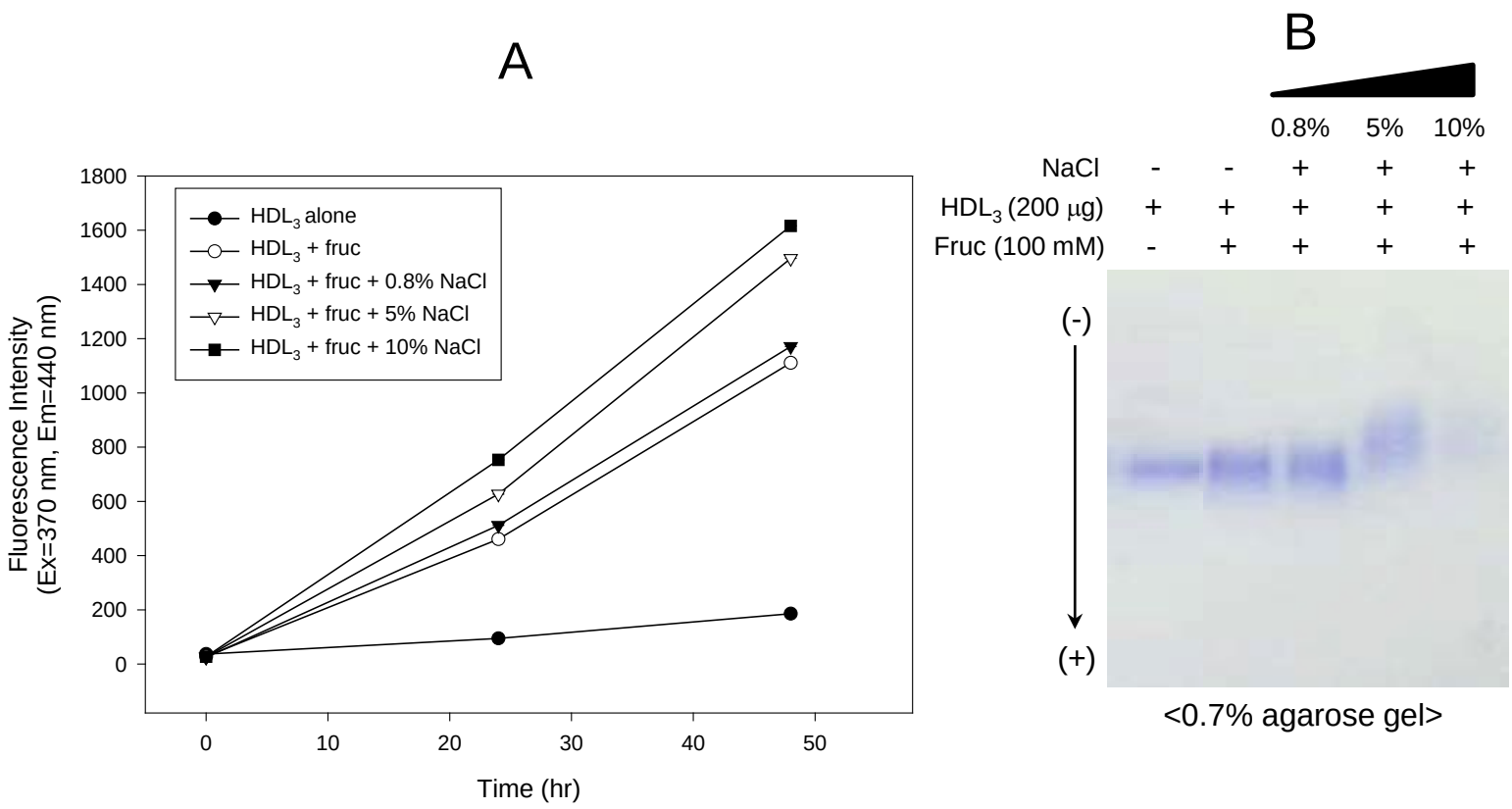


Fig. 4.

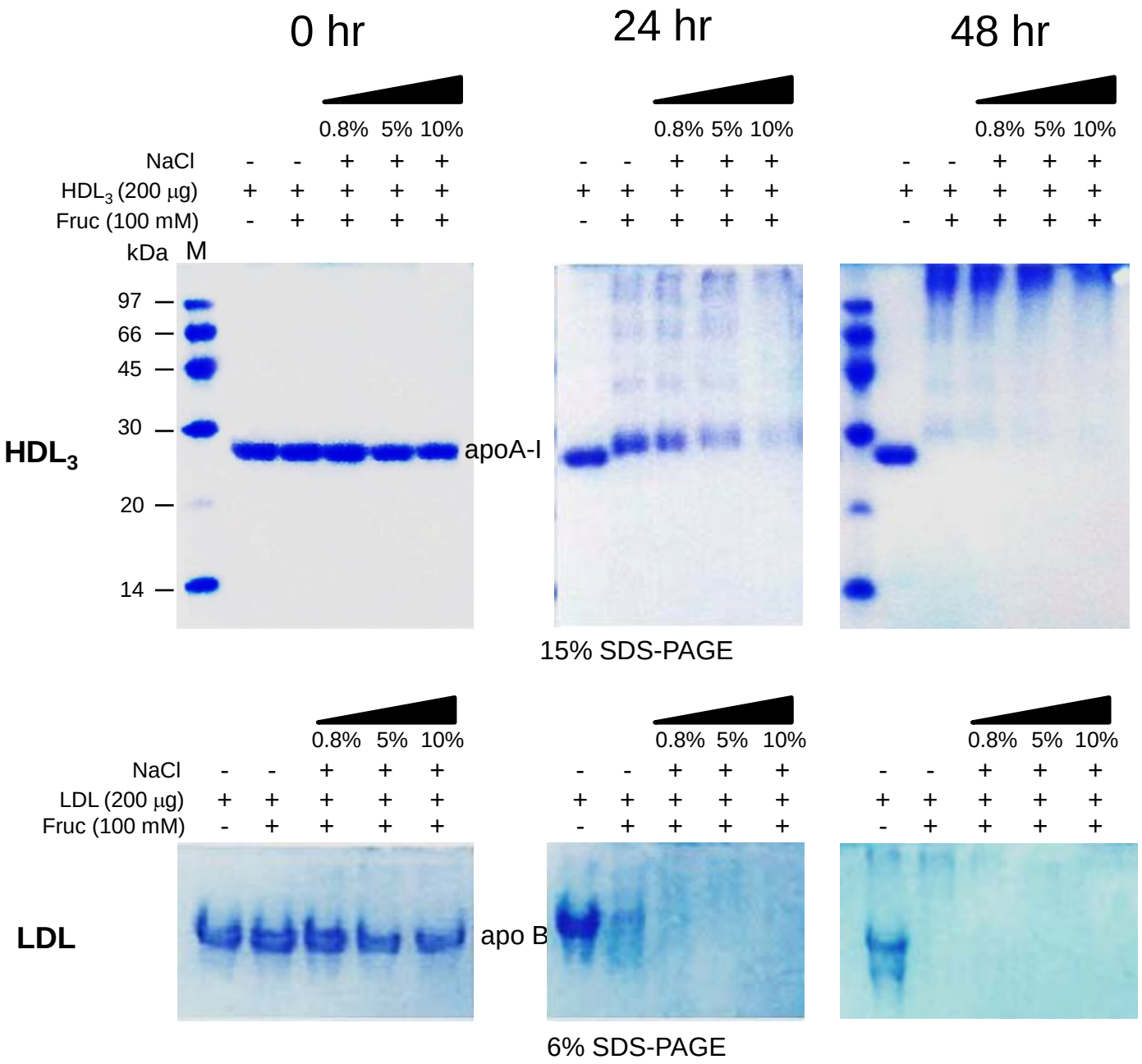
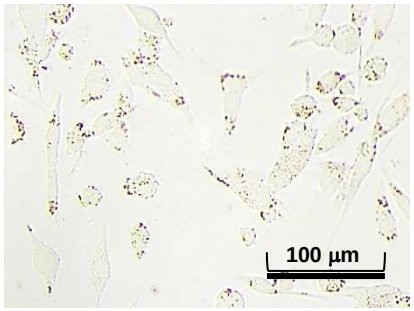


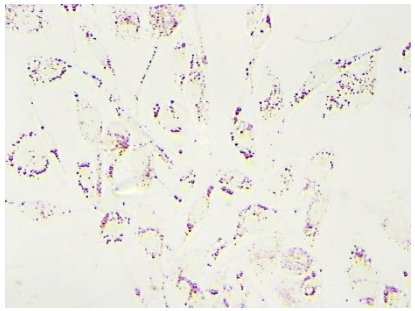
Fig. 5.

0.6% NaCl



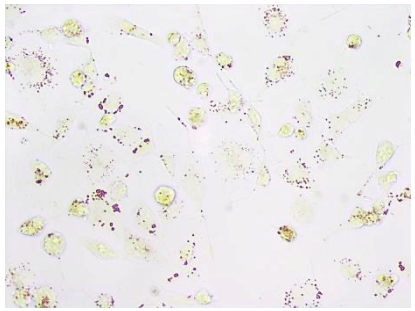
2.3×10^5

0.8% NaCl



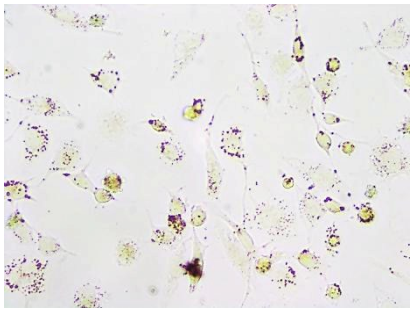
2.3×10^5

5% NaCl



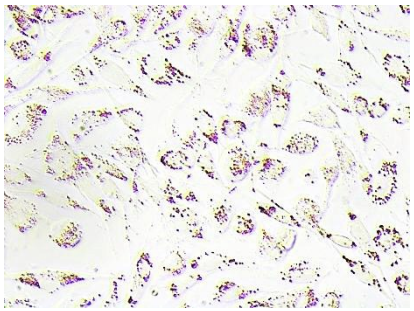
1.7×10^5

10% NaCl



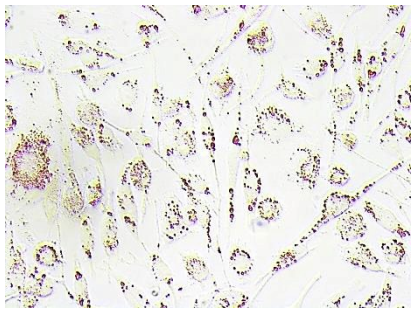
1.3×10^5

oxLDL + 0.6% NaCl



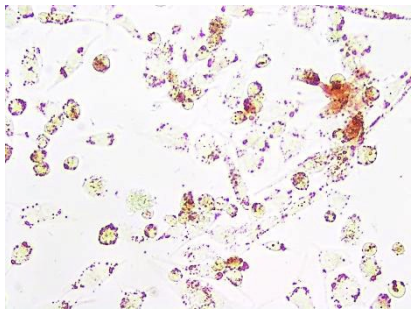
2.4×10^5

oxLDL + 0.8% NaCl



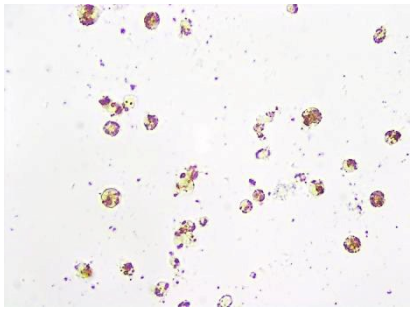
2.3×10^5

oxLDL + 5% NaCl

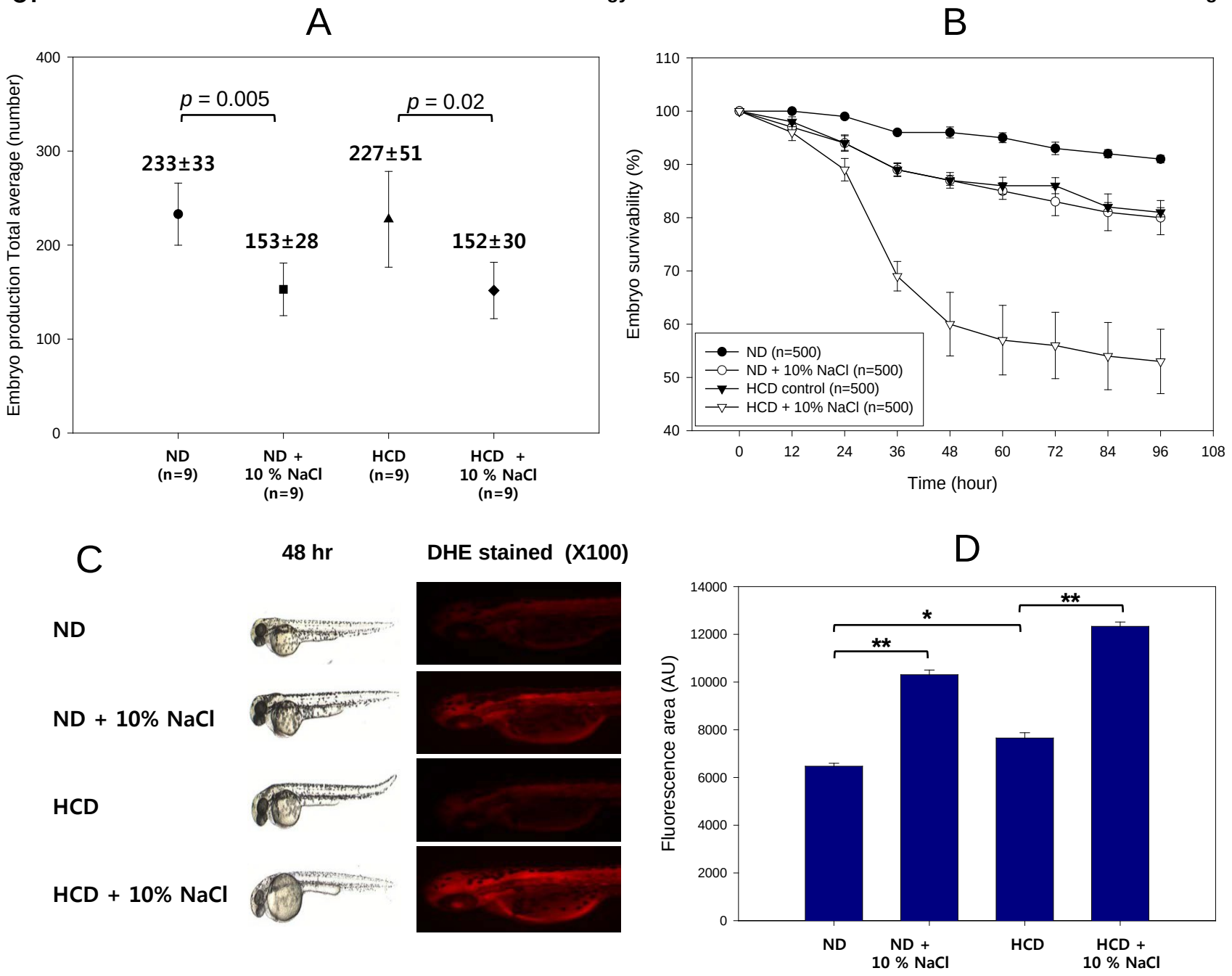


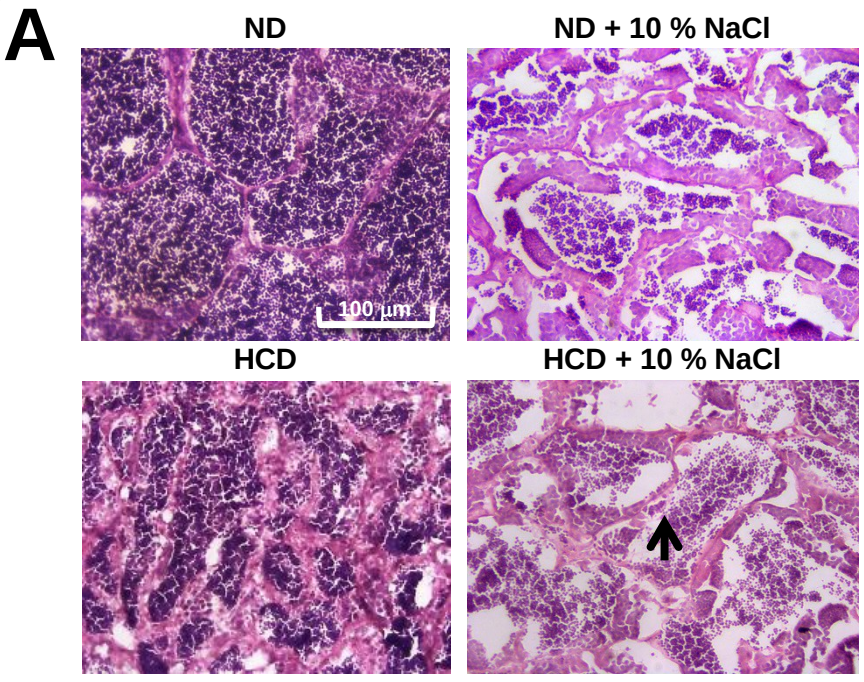
1.6×10^5

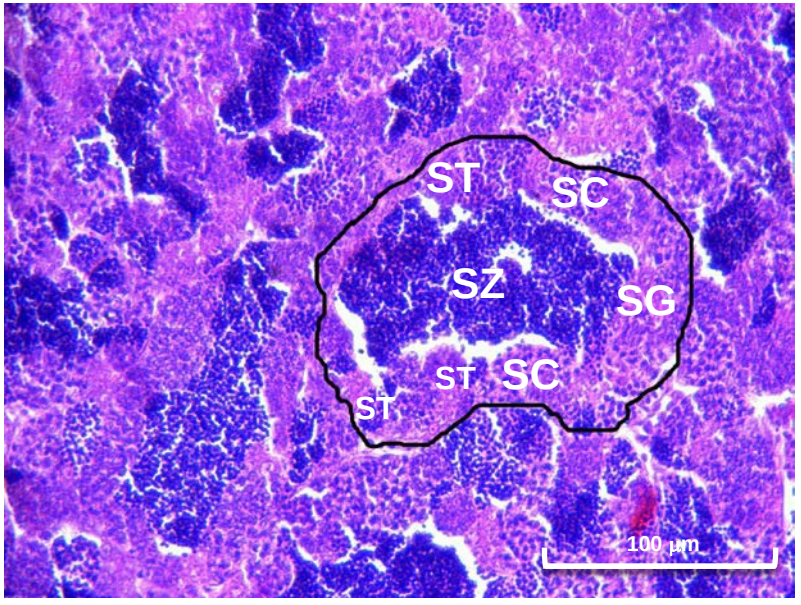
oxLDL + 10% NaCl

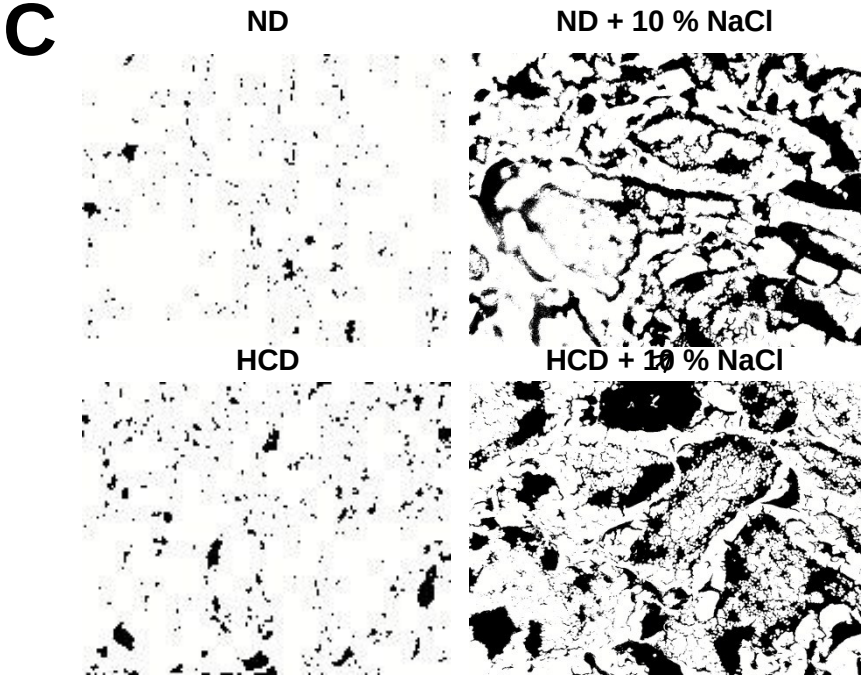


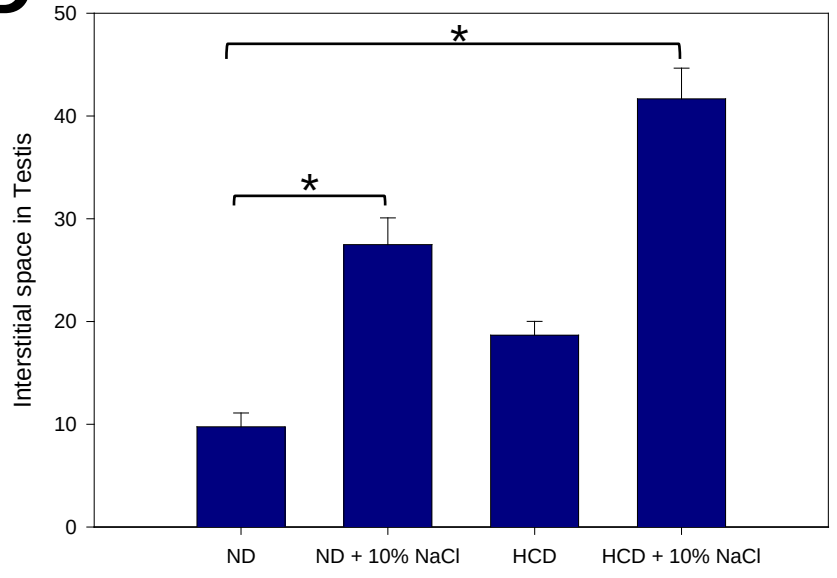
2.1×10^4











Testis (Paraffin section)

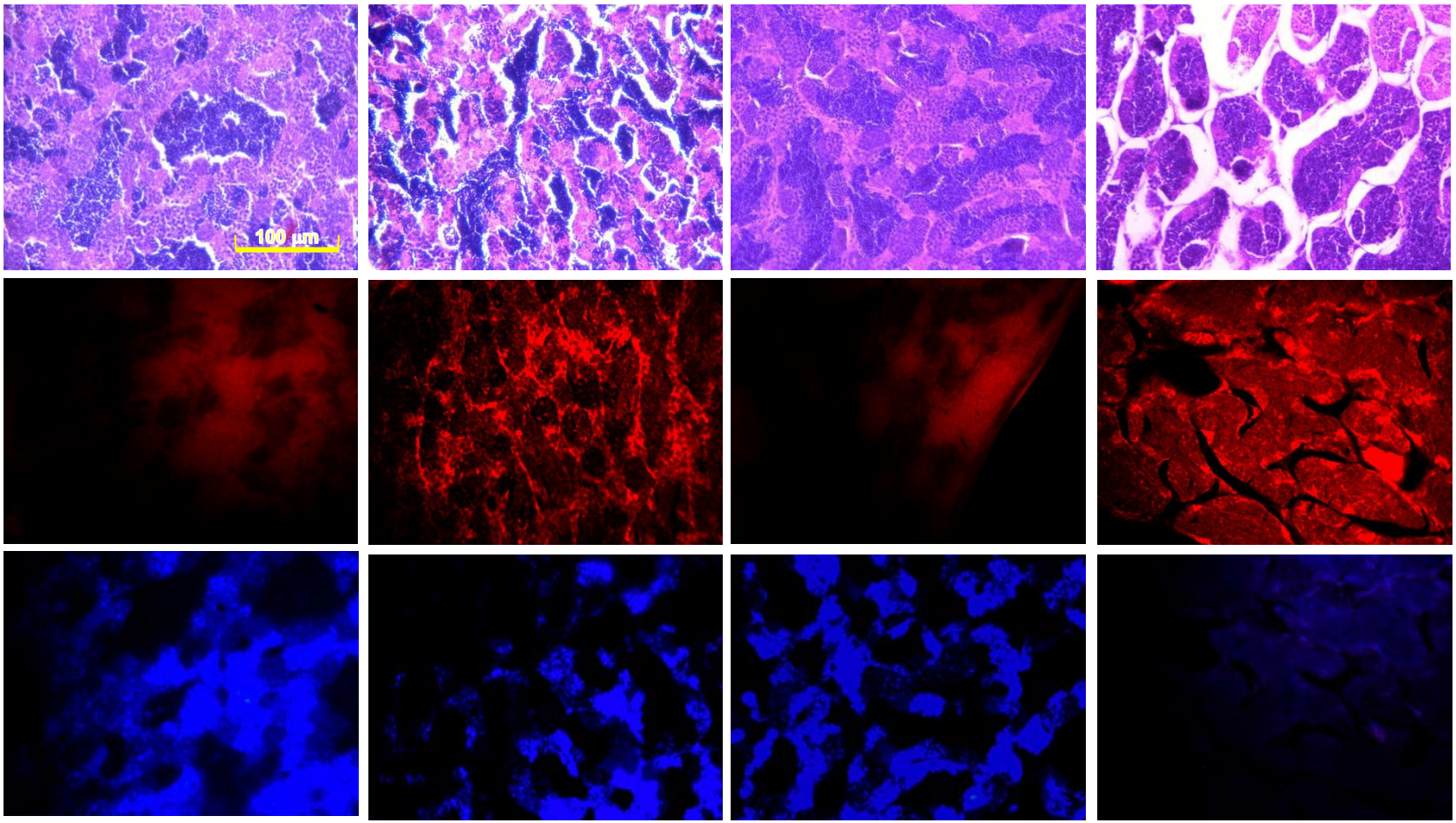
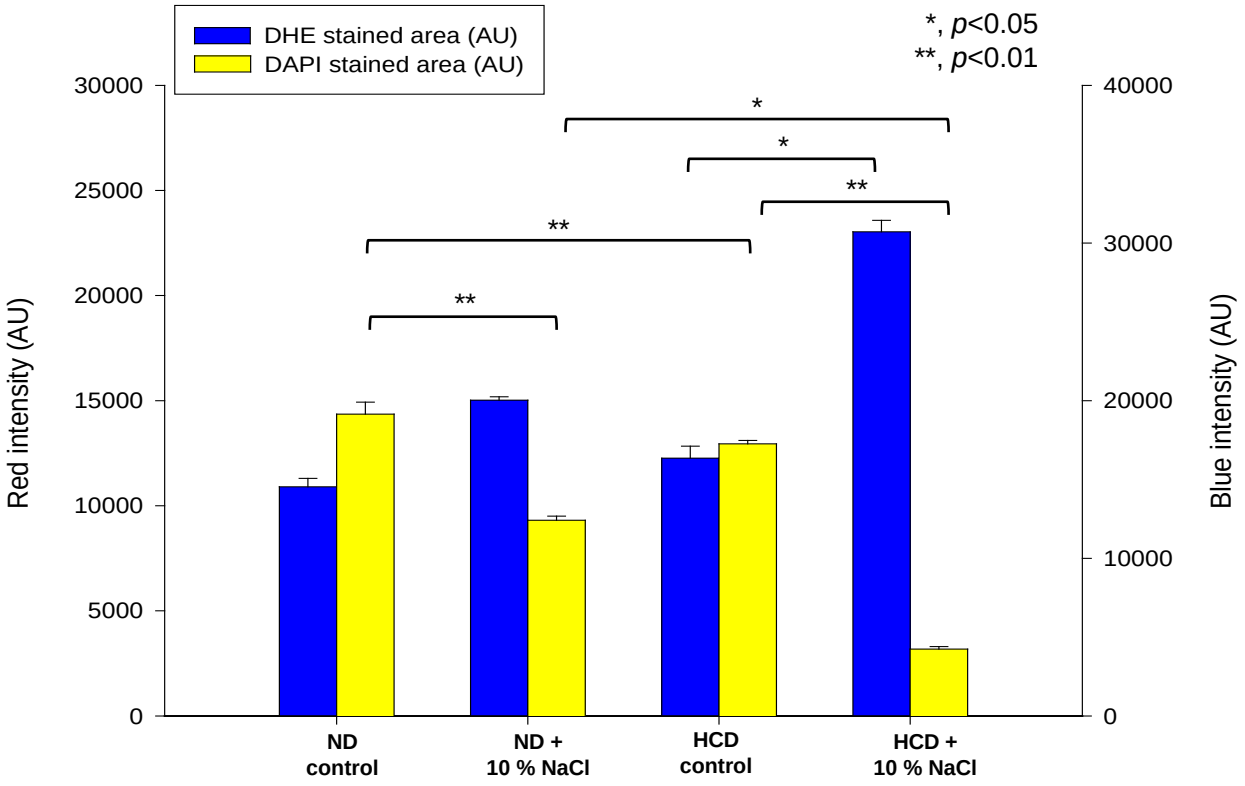


Fig. 8B



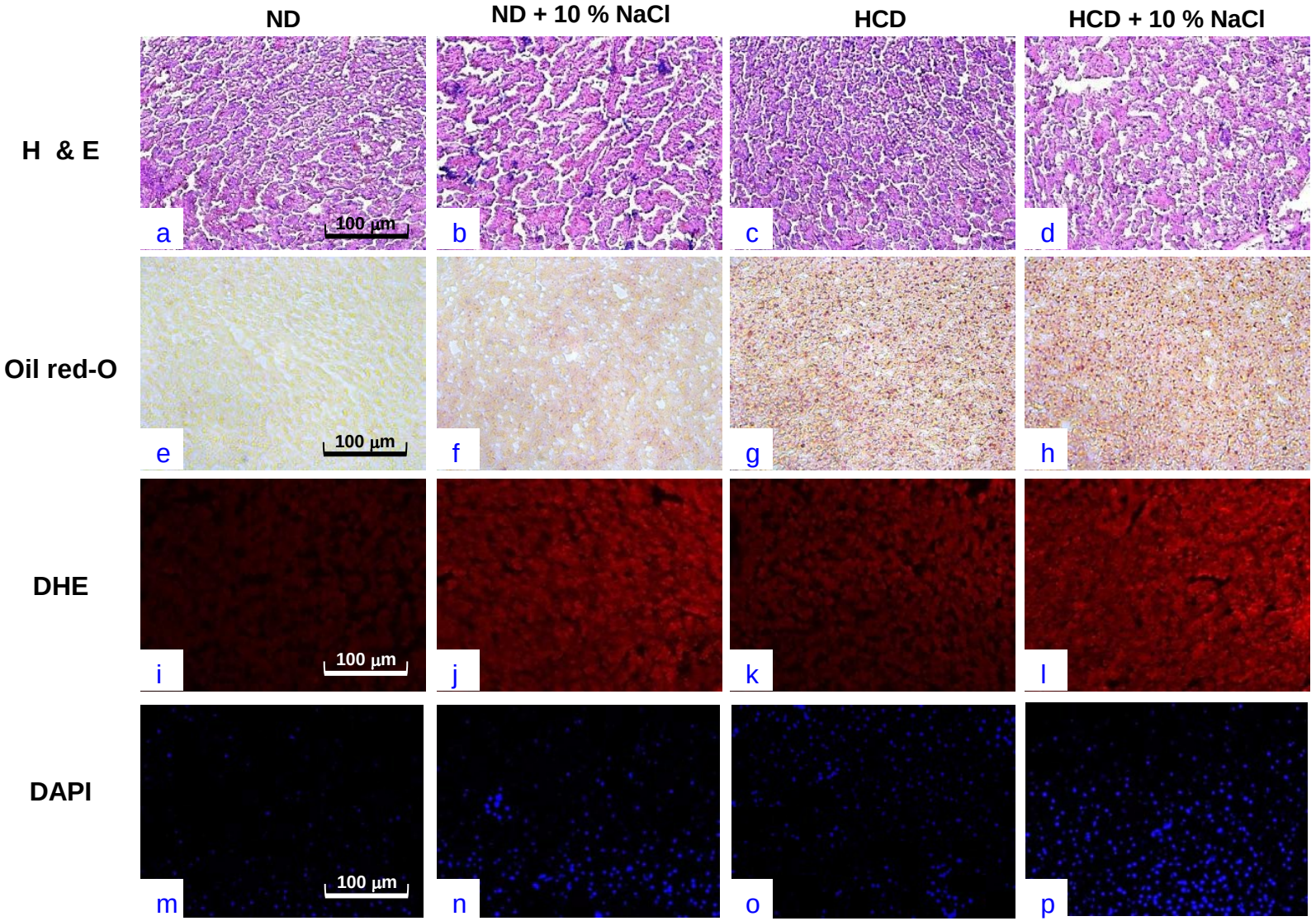


Fig. 9B