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Novel fluorescence-based method to characterize the antioxidative effects of food metabolites on lipid droplets in cultured hepatocytes

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Abstract

A fluorescence microscopic method for characterizing size, quantity, and oxidation of lipid droplets (LDs) in HepG2 cells was developed. LDs were induced by palmitic (PA), oleic (OA), or linoleic acids (LA), and stained with two fluorescent probes for neutral lipids and lipid peroxides. Each fatty acid increased the number of LDs and oxidized LDs (oxLDs) and the degree of LD oxidation time-dependently, as well as increased intracellular triglyceride hydroperoxides. LDs induced by LA without AAPH showed the most significant oxidation degree over PA and OA, especially in large LDs (area $\geq 3 \mu\text{m}^2$, oxLD/LD = $52.3 \pm 21.7\%$). Under this condition, two food-derived antioxidants were evaluated, and both of them significantly improved the LD characteristics. Moreover, chlorogenic acid reduced the quantity of large LDs by 74.0%–87.6% dose-dependently. The proposed method might provide a new approach to evaluate the effect of dietary antioxidants.

Keywords

Lipid droplet (LD), fluorescence microscopy, antioxidant, lipid oxidation, non-alcoholic fatty liver disease (NAFLD)

1. Introduction

Lipid droplets (LDs) are usually filled with triglyceride (TG) as their hydrophobic cores, and enclosed by a phospholipid monolayer as their hydrophilic shell, which mainly consists of phosphatidylcholine (PC).¹ It is known that LDs are induced by the accumulation of TG in the bilayer membrane of the endoplasmic reticulum.² LDs can grow by fusion, ester translocation, and neosynthesis *in situ*,³⁻⁵ resulting in a variety of size distribution. Conventionally, LDs were recognized just as the inert storage for energy. However, in recent years they have been uncovered to be a highly dynamic organelle that plays a central role in lipid and energy homeostasis.^{6,7}

In the current researches, the LD imaging by oil red O staining is generally used for lipid accumulation measurement,⁸ but the available information is rather limited. A very recent study by Zhao et al. analyzed the profiling of PCs and TGs in the LD of HepG2 cells by in-tip solvent microextraction mass spectrometry (ITSME-MS).⁹ However, since that strategy focused on single LD analysis, rather than the whole LDs in the cells, the overall characteristics of LDs in the cells was unavailable, such as their total quantity, their morphology, and their size distribution. Moreover, although the composition of lipid molecular species in LD could be known by MS, there is a lack of information on the oxidized lipids (i.e. lipid oxidation products) in these LDs so far. Therefore, a

comprehensive profiling method for all the LDs in the cells, including multiple index such as quantity, morphology, size, and oxidation degree, is to be established.

It is of great importance to focus on the oxidation in LD, because the oxidized products in LDs can reflect the oxidative stress in the whole cell. More interestingly, it is reported that an increase of reactive oxygen species (ROS) is positively correlated with an increase in the number of LDs in hepatocytes.¹⁰ These changes will lead to the dysfunction of LD homeostasis, which is considered to be a factor causing a series of metabolic syndromes, e.g. obesity and non-alcoholic fatty liver disease (NAFLD).^{11–13} ROS is also known to react with the intracellular polyunsaturated fatty acids (PUFAs) and cause lipid peroxidation in the liver of NAFLD patients.¹⁴ Moreover, microvesicular and macrovesicular steatoses are different in prognosis, suggesting a possible relationship between oxidation and size of LDs.¹⁵ Thus, to get a better understanding toward the pathophysiological conditions associated with LDs, detailed investigation on the physicochemical properties of the intact and oxidized LDs is desired.¹⁵

Researchers have been hunting for natural antioxidants for decades. The dietary-derived antioxidants are abundant in fruits, vegetables, essential oils, and so on.^{16–18} One of the most representative antioxidants in food, chlorogenic acid, is produced from crop plants, tea, and coffee beans,^{19–21} and has been revealed various beneficial effects,

including anti-diabetes, anti-obesity, anti-inflammatory, and anti-FA peroxidation.^{22,23} It is of our interest that chlorogenic acid prevents hepatic TG accumulation, and that chlorogenic acid possesses remarkable radical scavenging capacity.²⁴ However, the effect of chlorogenic acid on the physicochemical property of LDs is unavailable. The similar situation is found in another phenolic antioxidant isolated from the Pacific oyster, 3,5-dihydroxy-4-methoxybenzyl alcohol (DHMBA).²⁵ Different from chlorogenic acid, DHMBA activates nuclear factor erythroid 2-related factor 2 (Nrf2) pathway in hepatocytes, not only exerting ROS scavenging.²⁶ Activation of Nrf-2 pathway induces a battery of Nrf2-dependent genes and enzymes, such as phase II enzymes, xenobiotic transporters, and drug-metabolizing enzymes against ROS,^{24,26,27} and then attenuates hepatic steatosis, insulin resistance, obesity, and inflammation in non-alcoholic steatohepatitis (NASH)-model mice.²⁸ However, the effect of DHMBA on the physicochemical property of LDs remains to be clarified, similar to the case of chlorogenic acid.

Therefore, here we aim to establish a novel fluorescence-based method for characterizing the LDs in number, size distribution, and degree of oxidation in human hepatocytes. There, two fluorescent probes are used to analyze intact and oxidized LDs individually. TG hydroperoxides (TG-OOH) are determined in hepatocytes by LC-

MS/MS to compare with the proposed method. The above information will show the difference between the effects of chlorogenic acid and DHMBA on LDs.

2. Materials and Methods

2.1 Chemicals

SRfluor 680-phenyl, a fluorescence probe for neutral lipids, was purchased from Funakoshi Co. Ltd. (Tokyo, Japan). Liperfluo and Hoechst33342, fluorescence probes for lipid peroxides and nuclei, respectively, were purchased from Dojindo Laboratories (Kumamoto, Japan). LC-MS grade chloroform, isopropanol, methanol, and water were purchased from Wako Pure Chemical (Osaka, Japan). Ammonium formate, ammonium acetate, and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich (St. Louis, MO). The free fatty acids, namely palmitic acid (PA), oleic acid (OA), and linoleic acid (LA) were purchased from Cayman Chemical Co. (Ann Arbor, MI). 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) (oxidant) and chlorogenic acid (antioxidant) were purchased from Sigma-Aldrich. DHMBA was chemically synthesized in house as previously reported.²⁵ Other chemicals and reagents were of analytical grade and purchased from Kanto Chemical Industry (Tokyo, Japan) unless specified.

2.2 Cell culture

Human HepG2 cells were cultured in DMEM supplemented with 10% FBS, 0.225% NaHCO₃, 100 mg/mL penicillin, and 100 U/mL streptomycin at 37°C under 5% CO₂. For this experiment, 35 mm glass bottom dish (MATSUNAMI, Japan) was coated with 0.1% gelatin solution for 30 min at room temperature. The cells were cultured in 0.1% gelatin-coated glass bottom dishes with 6×10⁵ cells in 3 mL of the medium. After a 24-hour incubation, the cells were treated with 400 µM free FAs and 1 mM AAPH (pro-oxidant) or antioxidants (0–500 µM DHMBA or chlorogenic acid).

2.3 Fluorescence imaging parameters

Cells were washed in PBS, and then stained with 5 µM SRfluor 680-phenyl, 10 µM Liperfluor, and 10 µg/mL Hoechst33342 for 30 min at 37°C. After incubation, the staining buffer was replaced with serum free DMEM. Fluorescence was observed using the BZ-9000 fluorescence microscope (Keyence Co. Ltd., Osaka, Japan) equipped with the following filter sets; excitation: 360/40 nm, emission: 460/50 nm, dichroic mirror: 400 nm (blue); excitation: 470/40 nm, emission: 525/50 nm, dichroic mirror: 495 nm (green); excitation: 620/60 nm, emission: 700/75 nm, dichroic mirror: 660 nm (red). Each fluorescence were observed following fluorescence acquisition parameters: Hoechst33342 fluorescence (excitation: 350 nm, emission: 361 nm, acquisition time:

200 milliseconds, binning: 2×2 , F-stop: 1, field-of-view: $100 \mu\text{m} \times 100 \mu\text{m}$); Liperfluor
fluorescence (excitation: 487–524 nm, emission: 535–579 nm, acquisition time:
770 milliseconds, binning: 2×2 , F-stop: 1, field-of-view: $100 \mu\text{m} \times 100 \mu\text{m}$); SRfluor
fluorescence (excitation: 615–650 nm, emission: 695–770 nm, acquisition time:
300 milliseconds, binning: 2×2 , F-stop: 1, field-of-view: $100 \mu\text{m} \times 100 \mu\text{m}$).^{29–31} Three
to five visual field were randomly selected per dish, and bright field and three
fluorescence images (blue, green, and red) were obtained from each same visual field.

2.4 LC/MS analysis

Lipids were extracted from the cultured cells according to Folch et al.³² In brief, the cells
were extracted with 600 μL of ice-cold chloroform/methanol 2:1 (v/v, with 0.002% BHT
and TG 11:0/11:0/11:0 as internal standard) twice, and then dried in vacuum. The residues
were dissolved in 100 μL of methanol, and then centrifuged at 680 g under 4°C for 15 min
to remove any insoluble materials, and thereafter stored at -80°C until analysis. All
procedures were finished within 1 hour to avoid lipid degradation and auto-oxidation.

The lipid extracts were injected into a Shimadzu Prominence HPLC system (Shimadzu
Corp., Kyoto, Japan) coupled to an LTQ Orbitrap mass spectrometer (Thermo Fisher
Scientific Inc., Waltham, MA) under ESI-positive mode. The detail parameters are shown
in supplementary material. The extracted ion chromatograms (EICs) were drawn within

the mass tolerance of 5.0 ppm, and the LC/MS identification of lipid molecules were on the basis of their HRMS data compared with our in-house library,^{33,34} as well as their retention behavior on the reversed-phase LC column. Peak extraction, EIC peak area integration, and semi-quantitation from the raw data were utilized by Xcalibur 2.2 (Thermo-Fisher Scientific Inc.).

2.5 Statistical analysis

In all experiments, cells were cultured 3 dishes for each group. All the data were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test and expressed as means $\pm$ SD. *P* values less than 0.05 were considered to be statistically significant.

3 Results and Discussion

3.1 The workflow of intact and oxidized LD imaging analyses

The scheme for image analysis is shown in **Fig. 1**. Acquired images were analyzed by ImageJ 1.50i software.^{35,36} To calibrate the length in ImageJ, pixel length of scale bar in the same magnification image was measured by using "Measure" command. SRfluor and Liperfluor fluorescence, and bright field images were obtained (**Figs. 1-A1, B1, and C1**), and then binarized based on the threshold set with reference to "RenyiEntropy" operation (**Figs. 1-A2, B2, and C2**). To exclude non-specific fluorescence and also to obtain the

positive image of oxidized LDs (oxLDs), an intersectional image was obtained from binarized images of bright field, SRfluor, and Liperfluor in the same visual field by using “Add” operation in Image Calculator of ImageJ (**Figs. 1-A2, B2, C2, and D**). To obtain an image of non-oxidized LDs (non-oxLDs), **Fig. 1-C2** was inverted by using “Invert” command (**Fig. 1-C3**), and an intersectional image was obtained from the images shown in **Figs. 1-A2, B2, and C3** by using “Add” operation (**Fig. 1-E**).

To obtain the number of oxLDs and non-oxLDs, the images (**Figs. 1-D and E**) were analyzed using “Analyze Particles” command. The images obtained with Hoechst33342 of the same visual field were also binarized and analyzed using “Analyze Particles”. The number of nuclei was used for normalization of oxLDs and non-oxLDs as the number of cells. According to Wang et al.² and Cohen et al.³⁷, LDs smaller than 3 μm^2 and those not less than 3 μm^2 were defined as small and large LDs, respectively. The number of LD was defined as the sum of the numbers of non-oxLDs and oxLDs.

3.2 Evaluation of oxidation in the FA-induced LDs

3.2.1 Effects of FAs in the presence of AAPH

LDs were induced in the HepG2 cells by PA, OA, or LA in the presence of AAPH (positive control). The fluorescence images for the PA-loaded cells are shown in **Fig. 2-A**. The number of LDs and oxLDs significantly increased at 48 h ($P < 0.01$) (**Figs. 2-B1**

and **B2**). The degree of LD oxidation as estimated by the oxLD/LD ratio was significantly increased at 8 h in small LDs ($16.2 \pm 4.5\%$) and at 24 h in large LDs ($74.2 \pm 7.6\%$) (**Fig. 2-B3**). In the OA-loaded cells (**Fig. 3-A**), a significant increase was observed in the numbers of small LDs and small oxLDs at both 24 h ($P < 0.001$) and 48 h ($P < 0.01$),^{7,29} and in the numbers of large LDs and large oxLDs at 48 h ($P < 0.05$) (**Figs. 3-B1 and B2**). The degree of LD oxidation in small and large LDs significantly increased at both 24 h ($62.8 \pm 4.4\%$ and $94.7 \pm 2.7\%$) and 48 h ($46.7 \pm 9.4\%$ and $89.1 \pm 11.1\%$) ($P < 0.001$) (**Fig. 3-B3**). In the LA-loaded cells, a significant increase was observed in the numbers of small LDs at 24 h ($P < 0.05$) and small oxLDs at 48 h ($P < 0.001$), and in the numbers of large LDs at 24 h ($P < 0.05$) and large oxLDs at 48 h ($P < 0.001$) (**Figs. 4-B1 and B2**). The degree of LD oxidation significantly increased in small LDs at 48 h ($50.0 \pm 15.9\%$) ($P < 0.001$) and in large LDs at both 24 h ($41.5 \pm 15.9\%$) ($P < 0.01$) and 48 h ($86.9 \pm 6.0\%$) ($P < 0.001$) (**Fig. 4-B3**).

The LC/MS characteristics of the TG-OOH species are listed in **Table S1 of Supplementary Materials**. The cellular levels of these TG-OOH species are listed in **Table S2**. Changes of total TG-OOH in the cells treated with FAs in the presence of AAPH are compared (**Figure 5**). In the PA- and OA-loaded cells, total TG-OOH significantly increased at 24 h (10.3 ± 0.7 fold and 23.94 ± 10.4 fold vs. 0 h) (**Figs. 5-A**

and **B**), and then remarkably decreased at 48 h. On the other hand, in the LA-loaded cells, TG-OOH significantly and strikingly increased at 48 h (215.3 ± 27.3 fold vs. 0 h) (**Fig. 5-C**).

Liperfluo is reported to react specifically with lipid peroxides and to be useful in detection of intracellular lipid peroxides.^{30,31} TG is a less polar lipid, and therefore, is located in the core of LDs in cell. Further, in our proposed method, the signal of Liperfluo came from lipid hydroperoxide in LDs, since non-specific signals had been excluded in the process of intersection (**Fig. 1-D**). Moreover, the increase of TG-OOH also supported this assumption: the significant increase of TG-OOH in the OA- and LA-loaded cells (**Fig. 5**) was associated with the significant increase of small oxLDs (**Figs. 3-B2 and 4-B2**).

3.2.2 Effects of FAs in the absence of AAPH

In the LA-loaded cells (**Fig. S1-C**), a significant increase was observed in the numbers of small LDs at 4 h - 48 h ($P < 0.01$) and of small oxLDs at 8 h - 48 h ($P < 0.01$), and in the numbers of large LDs and large oxLDs at 8 h - 48 h ($P < 0.05$) (**Figs. 6-C1 and C2**). The degree of LD oxidation in small and large LDs significantly increased at 4 h - 48 h ($25.6 \pm 6.6\%$ - $38.0 \pm 3.4\%$ and $52.3 \pm 21.7\%$ - $72.2 \pm 12.4\%$) ($P < 0.01$) (**Fig. 6-C3**). Although the similar trend was observed in the PA- and OA-loaded cells (**Figs. 6-A and B**), the process proceeded more slowly than that in the LA-induced cells (**Fig. 6-C**).

It is of interest that the LA-induced LDs were oxidized the most slowly among the three FAs in the presence of AAPH, but the most quickly in the absence of AAPH (Figs. 2, 3, 4, and 6). To explain this discrepancy, we speculate that the LA incorporated in the LA-induced LDs as TG served as a reservoir of oxidative stress caused by AAPH. On the other hand, in the absence of AAPH, the higher desaturation in LA than that in PA and OA might have resulted in the increased susceptibility of the LA-induced LDs to oxidation (Figs. 6-A3, B3, and C3). On the basis of these findings, LD oxidation seems to depend on both the fatty acyl composition of TG and the strength of oxidative stress.³⁸

According to the present study, LDs can be oxidized in the cells, and therefore, can initiate and promote radical chain reactions. Moreover, LDs can provide fuels to continue the reactions, which results in intense, prolonged, and expanding oxidative reactions in the cells. Hence, it is highly possible that the LD-involved radical chain reaction exhausts the cellular antioxidant system, causing irreversible damages to the cell. A previous study reported that NAFLD model rats experienced complications with chronic depletion of hepatic glutathione (GSH), leading to a decrease of ROS scavenging activity.³⁹

3.3 Evaluation of the effects of food-derived antioxidants on LDs by the proposed method

Because of the high susceptibility of the LA-induced LDs to oxidation (Fig. 6-C), we

investigated the effects of antioxidants on the LA-induced LDs in the absence of AAPH. DHMBA represents indirect antioxidants, and chlorogenic acid represents direct antioxidants.²⁷ Their structures are shown in **Fig. 7**. DHMBA decreased both the number of oxLDs and the degree of oxidation (**Fig. 8-A**). On the other hand, chlorogenic acid decreased large LDs and large oxLDs (**Figs. 8-B1 and B2**). However, it did not decrease the degree of LD oxidation (**Fig. 8-B3**). The discrepancy in the effect on LDs between the two compounds might be explained by their different antioxidative mechanisms.

DHMBA has been reported to protect hepatocytes *in vitro* from oxidative stress,⁴⁰ and improve pathological and metabolic changes in the liver of NASH model mice.²⁸ The increased expression of quinone reductase and glutathione reductase, induced *via* activation of Nrf2 pathway by sulforaphane, are reported to maintain for more than 120 hours.⁴¹ Thus, in our present study, DHMBA reduced the degree of oxidation in LDs possibly through the activation of Nrf2-pathway (**Figs. 8-A2 and A3**). Chlorogenic acid, on the other hand, serves as a direct antioxidant exerting radical scavenging activity.^{24,26} Although the radical scavenging activity of chlorogenic acid is 3.1-fold stronger than that of DHMBA,²⁶ the radical scavenging activity of direct antioxidants finish quickly due to the oxidation of themselves.⁴² In addition, chlorogenic acid suppresses fatty acid synthesis and enhance β -oxidation.^{40,43} Thus, in this study, chlorogenic acid decreased the number

of large LDs and oxLDs possibly through TG hydrolysis rather than antioxidative effects (Figs. 8-B1 and B2). The proposed method could be a useful tool for future research on the interaction between antioxidants and LDs.

It should be noted that the present study has mainly technical aspect, however LD metabolism is concerned with ROS, ER stress, mitochondrial function, and lipogenic and lipolytic enzymes,^{44,45} which might be also involved in LD oxidation. It is expected to get a better understanding of LD metabolism that clarification of the detailed interaction among the oxidation of LDs, ROS, ER stress, and mitochondrial function. For another limitation, the result with primary cells might be different from those with the cultured tumor cells,⁴⁶ like HepG2 cell used in the present study. Thus, the present method might not perfectly represent the physiological conditions in liver. Although the difference of the result between primary cells and HepG2 cells should be verified in the future, HepG2 cells are more suitable for stable screening of antioxidants than primary cells, because HepG2 is readily available and easy to grow. Thus, our proposed method using HepG2 cells could promise the utility as a globally usable method.

In conclusion, the proposed imaging method can provide detailed physicochemical information of LDs in hepatocytes. This method might be useful to explore antioxidant foods and drugs for prevention and alleviation of health disorders involving LD

268 accumulation and lipid oxidation.

269

270 **Abbreviations Used**

271 AAPH, 2,2'-azobis (2-amidinopropane) dihydrochloride; BHT, butylated
272 hydroxytoluene; LA, linoleic acid; LC, liquid chromatography; LD, lipid droplet; MS,
273 mass spectrometry; NAFLD, non-alcoholic fatty liver disease; OA, oleic acid; oxLD,
274 oxidized lipid droplet; PA, palmitic acid; PC, phosphatidylcholine; ROS, reactive oxygen
275 species, TG, triglyceride; TG-OOH, triglyceride hydroperoxide.

276

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282

283 **Supporting Information**

284 LC/MS data of the identified triacylglycerol hydroperoxides and their relative
285 intensities are listed in the Supplementary Materials.

Conflict of interest

The authors declare no competing financial interests.

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Figure captions

Figure 1. Scheme of the protocol for image analysis. Three types of images (A1, B1, and C1) were binarized based on the threshold set with reference to “RenyiEntropy” algorithm. In binarized images (A2, B2, and C2), white area means positive area of each fluorescent. By using Image Calculator of ImageJ to obtain intersectional image, a common white area for LD (A2), SRfluor (B2) and Liperfluo (C2) was obtained. An intersectional image was shown as the oxidized LDs (oxLDs) in image (D). C3 was inverted from C2 and shown as Liperfluo negative area. By using Image Calculator of ImageJ to obtain intersectional image, a common white area for LD (A2), SRfluor (B2) and Liperfluo negative (C3) was obtained. An intersectional image was shown as the non-oxidized LDs (non-oxLDs) in image (E). D and E were analyzed for the number of white area by ImageJ and shown as the number of oxLD and non-oxLD. Total LDs means the total of oxLDs and non-oxLDs, the total of the number of oxLDs and non-oxLDs were shown as the number of LDs.

Figure 2. Lipid accumulation and oxidation in HepG2 cells treated with PA. Cells were seeded in glass bottom dishes and treated with 400 μ M PA and 1 mM AAPH. After

0–48 h of incubation, the cells were stained with SRfluor® 680 Phenyl (red; neutral lipids), Liperfluo (green; lipid peroxides), and Hoechst33342 (blue; nuclei). A: Fluorescence images were acquired using a fluorescence microscope. The scale bar shown in each image is 10 μm . B: quantification of the number of LDs and oxLDs, and the degree of LD oxidation as calculated by the ratio oxLD/LD. ImageJ software was used for quantification of fluorescence images. The results are presented as the number of LD (B1), the number of oxLD (B2), and degree of oxidation (B3) for small ($< 3 \mu\text{m}^2$) and large ($\geq 3 \mu\text{m}^2$) LDs. Columns and bars represent the mean $\pm$ SD ($n = 3$). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Figure 3. Lipid accumulation and oxidation in HepG2 cells treated with OA. LD stains and data processing were done as described in the explanation of **Fig. 2**. Columns and bars represent the mean $\pm$ SD ($n = 3$). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Figure 4. Lipid accumulation and oxidation in HepG2 cells treated with LA. LD stains and data processing were done as described in the explanation of **Fig. 2**. Columns and bars represent the mean $\pm$ SD ($n = 3$). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Figure 5. Change fold of intracellular TG-OOH in the FA-loaded cells in the presence of AAPH. Cells were loaded 400 μ M PA (A), OA (B), and LA (C) with 1 mM AAPH. After 0–48 h of incubation, the whole cell lipid was extracted. TG-OOH was detected by LC/MS which condition was described in material and method section. Columns and bars represent the mean $\pm$ SD (n = 3). **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Figure 6. Quantification of the number of LDs and oxLDs, and the degree of LD oxidation as calculated by the ratio oxLD/LD. Cells were treated with 400 μ M PA (A1-3), OA (B1-3), and LA (C1-3) in the absence of AAPH. After fluorescence staining, the cells were observed using a fluorescence microscope. ImageJ software was used for quantification of fluorescence images. The results are presented as the number of LD (A1, B1, and C1), the number of oxLD (A2, B2, and C2), and degree of oxidation (A3, B3, and C3) for small (< 3 μ m²) and large ($\geq$ 3 μ m²) LDs. Columns and bars represent the mean $\pm$ SD (n = 3). **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Figure 7. Lipid accumulation and oxidation in HepG2 cells treated with LA and antioxidants. Cells were seeded in glass bottom dishes and treated with 400 μ M LA and 0, 125, 250 and 500 μ M of the following antioxidants: DHMBA (A) and chlorogenic acid

484 (B). After 8 h of incubation, the cells were stained with SRfluor® 680 Phenyl (red),
485 Liperfluo (green), and Hoechst33342 (blue). Fluorescence images were acquired using a
486 fluorescence microscope. The scale bar shown in each image is 10 μm .

487

488 **Figure 8.** Effects of antioxidants on lipid accumulation and oxidation of LA-induced
489 LDs in the absence of AAPH. Cells were treated with 400 μM LA and 0, 125, 250 and
490 500 μM of the following antioxidants: DHMBA (A) and chlorogenic acid (B). After
491 fluorescence staining, the cells were observed using a fluorescence microscope. To
492 quantify the number of LDs and oxLDs, fluorescence images were analyzed with ImageJ
493 software. The results are presented as the number of LD (A1 and B1), the number of
494 oxLD (A2 and B2), and degree of oxidation (A3 and B3) for small ($< 3 \mu\text{m}^2$) and large
495 ($\geq 3 \mu\text{m}^2$) LDs. Columns and bars represent the mean $\pm$ SD (n = 3). * $P < 0.05$, ** $P < 0.01$.

Figure graphics

Figure 1

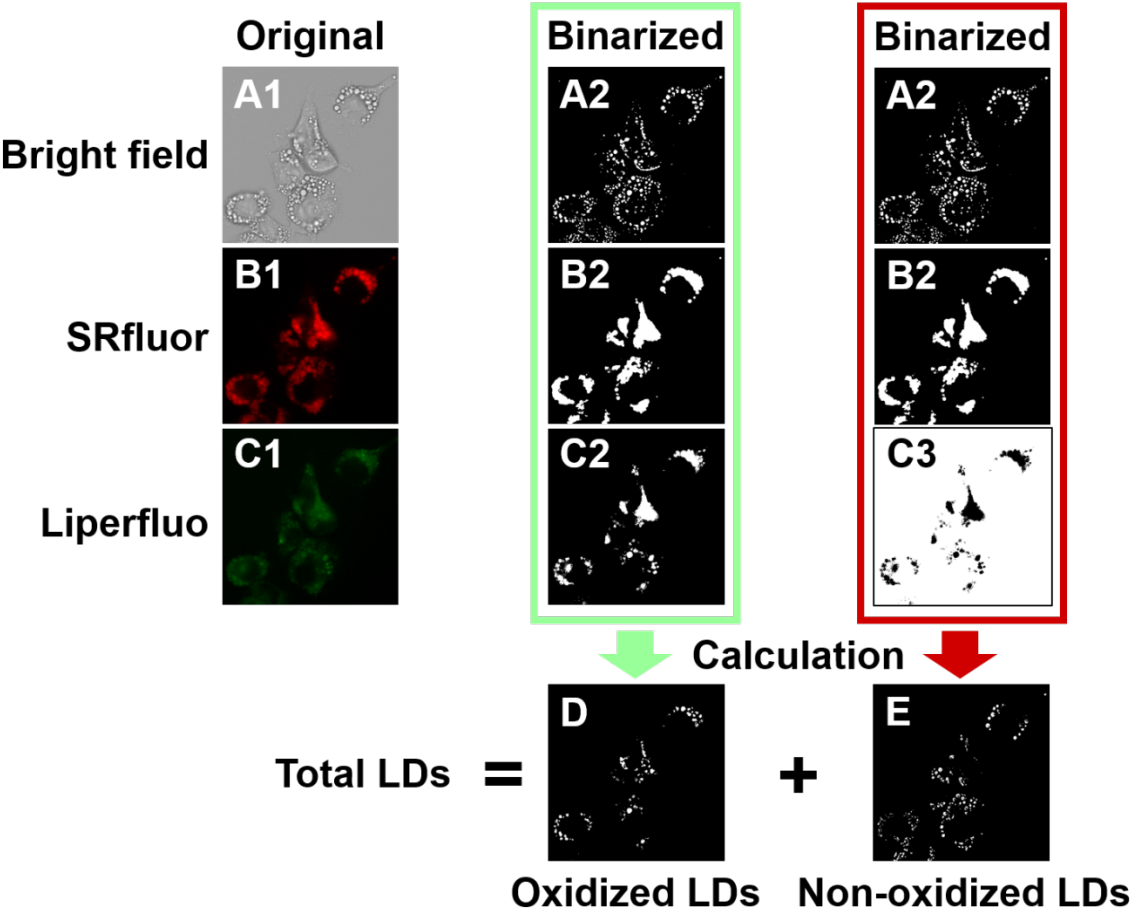


Figure 2

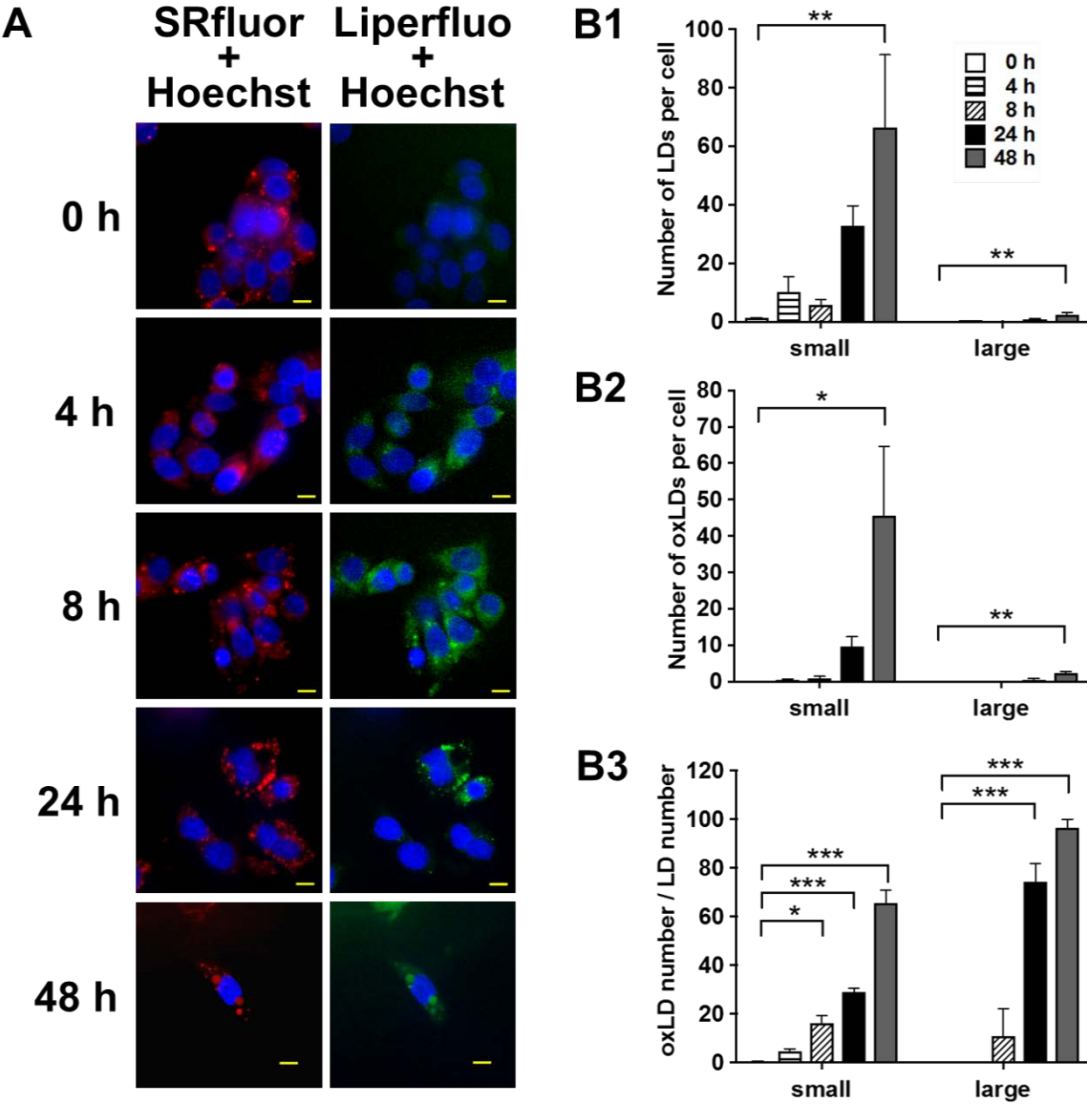


Figure 3

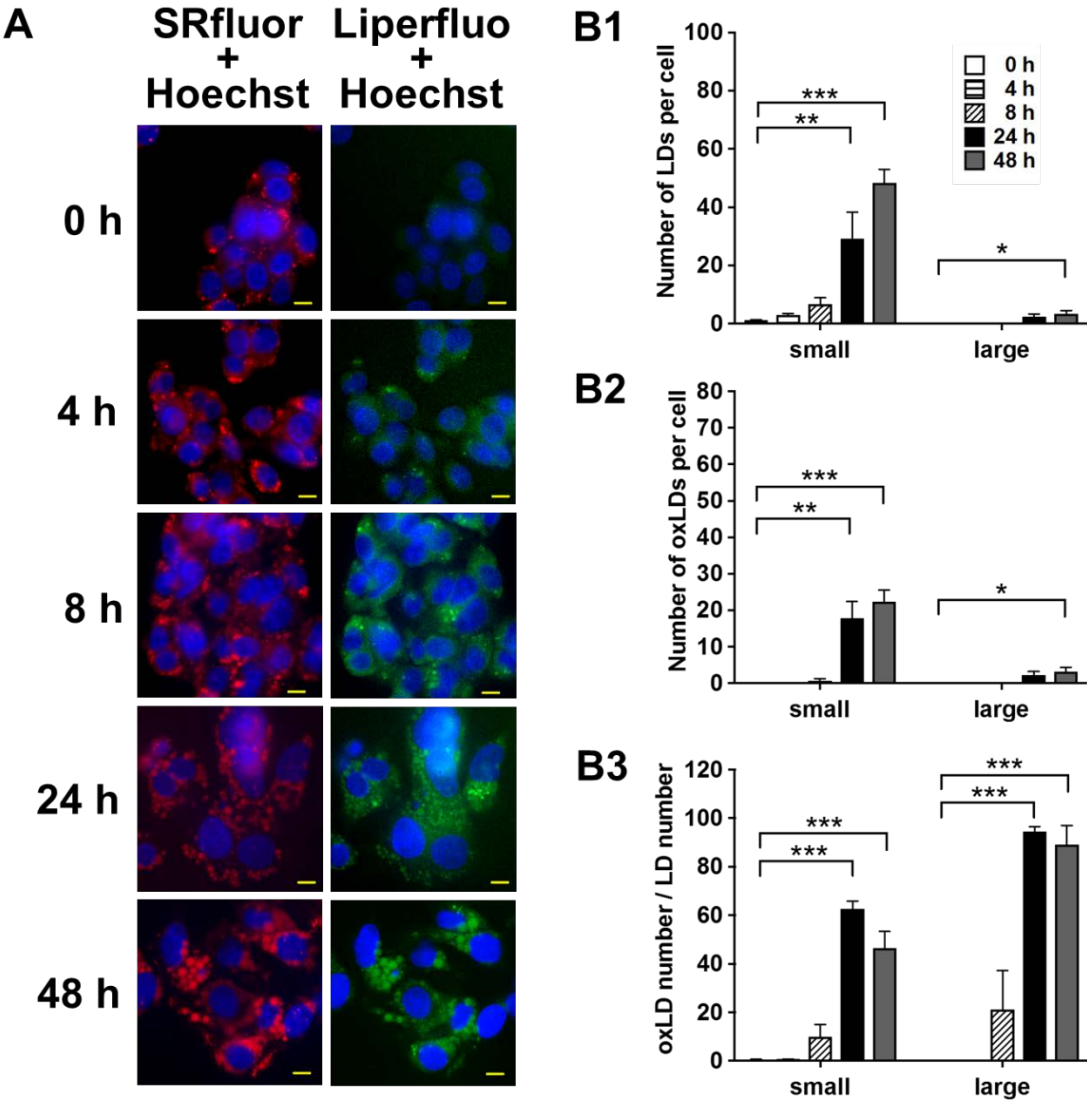


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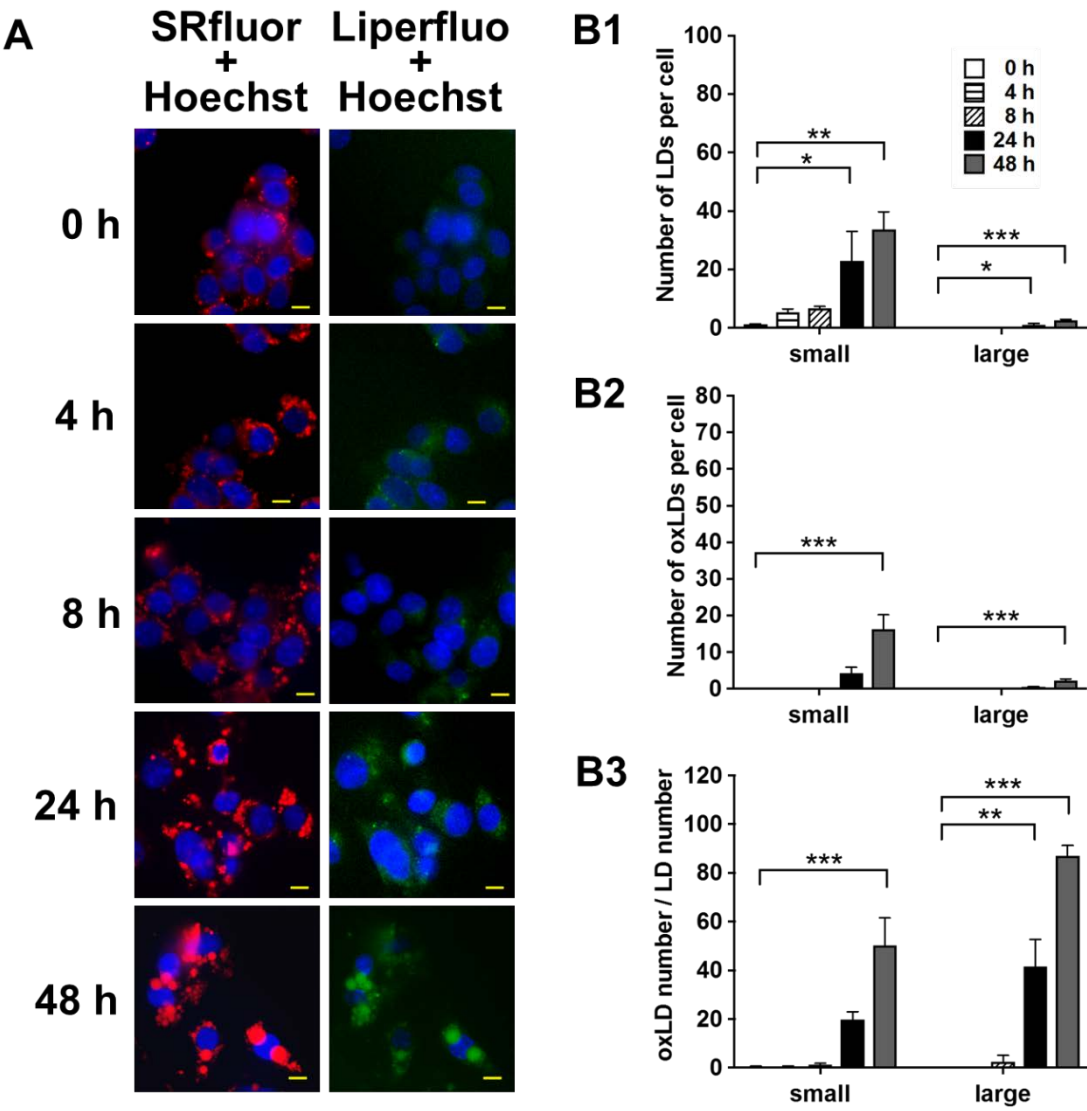


Figure 5

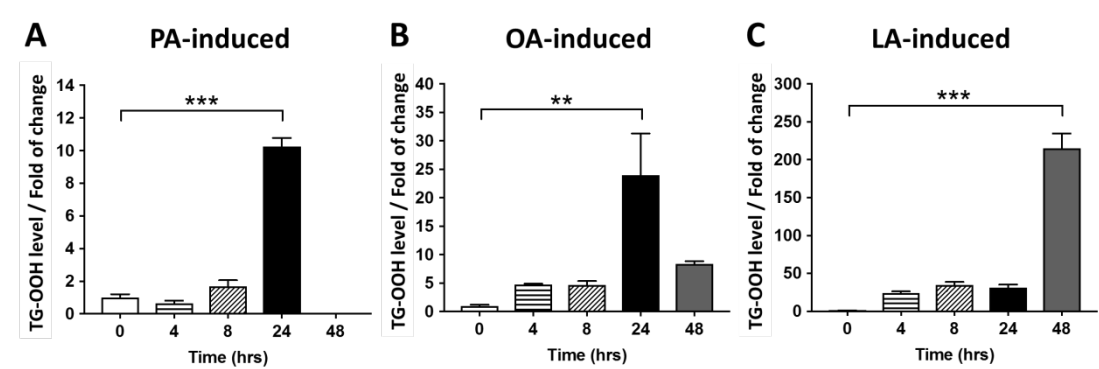


Figure 6

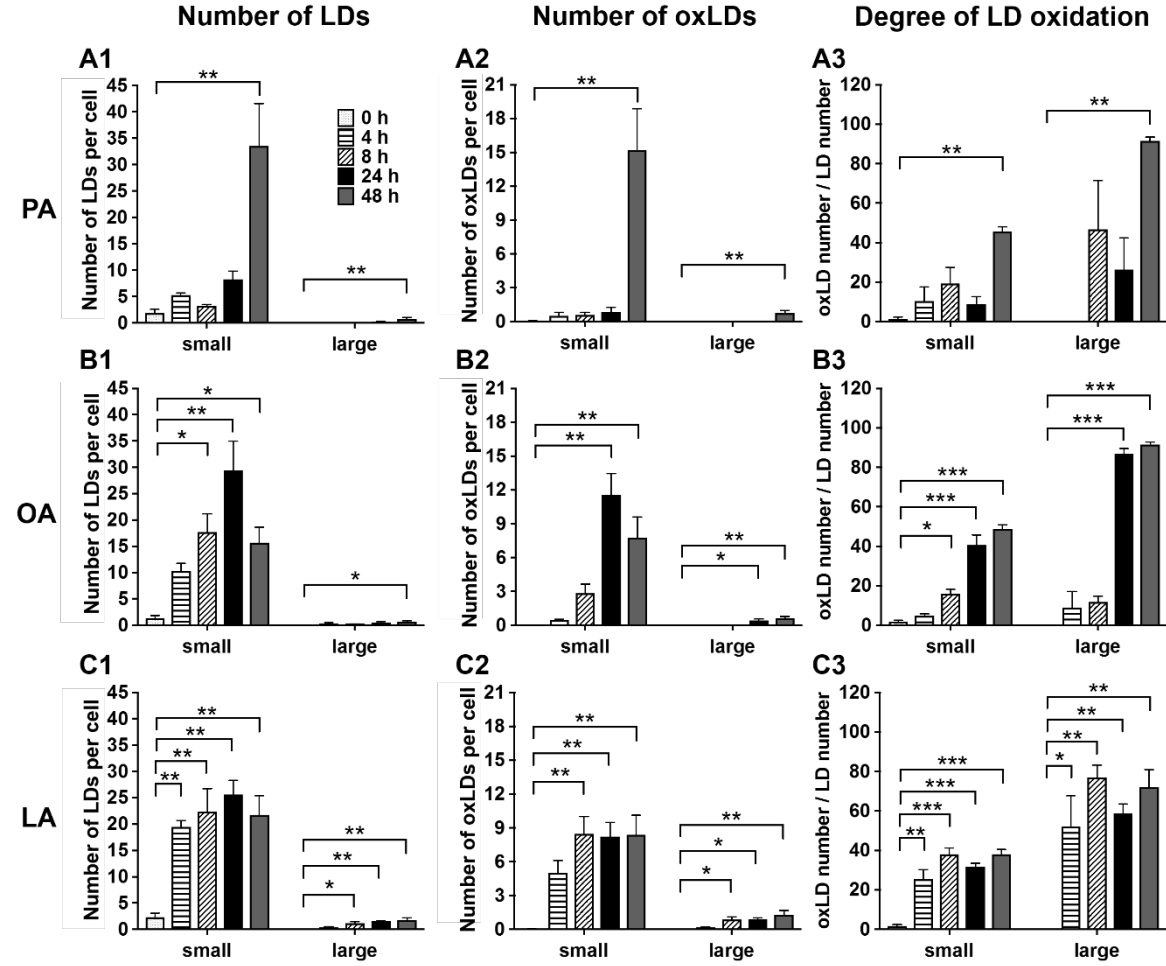


Figure 7

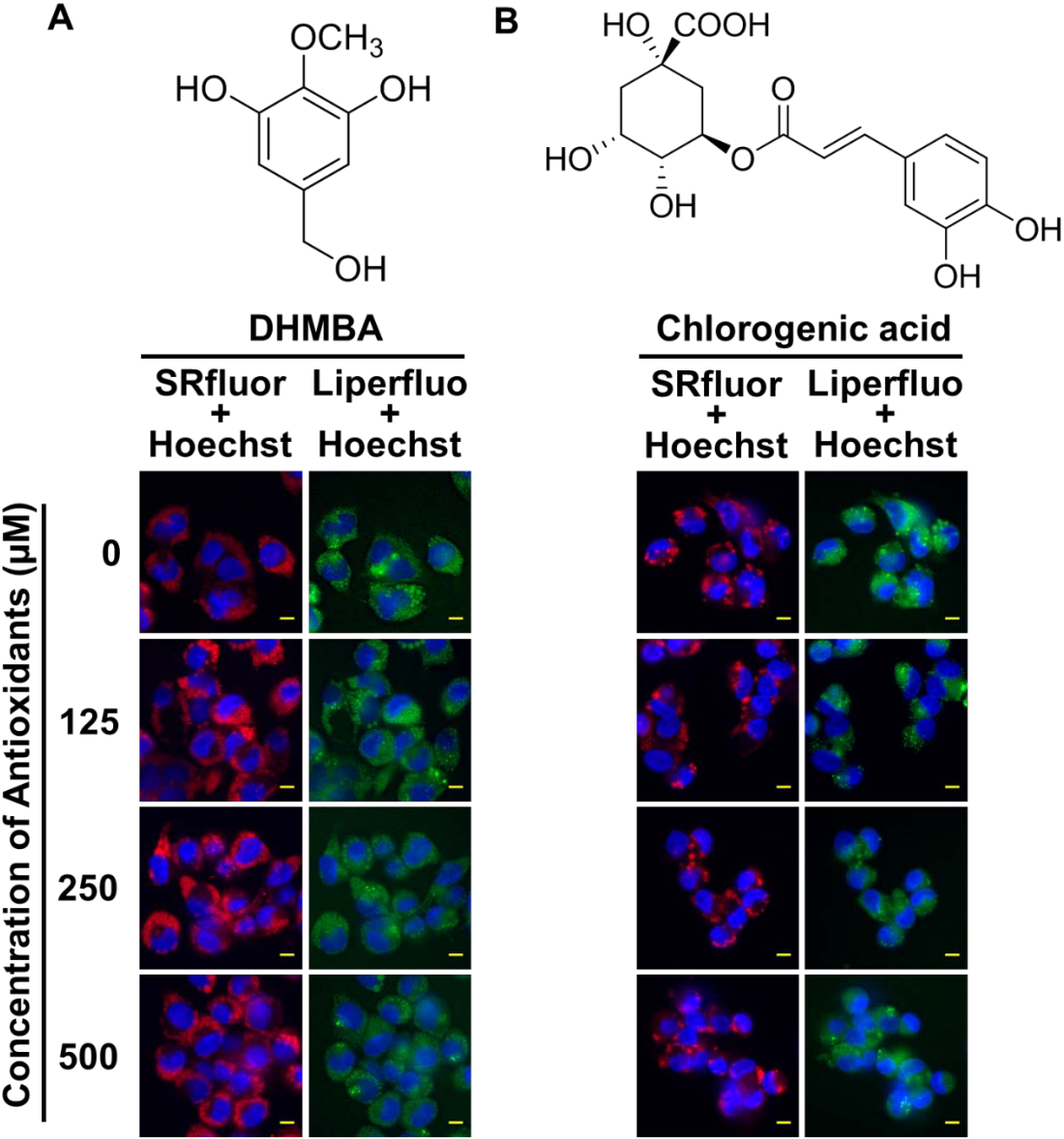
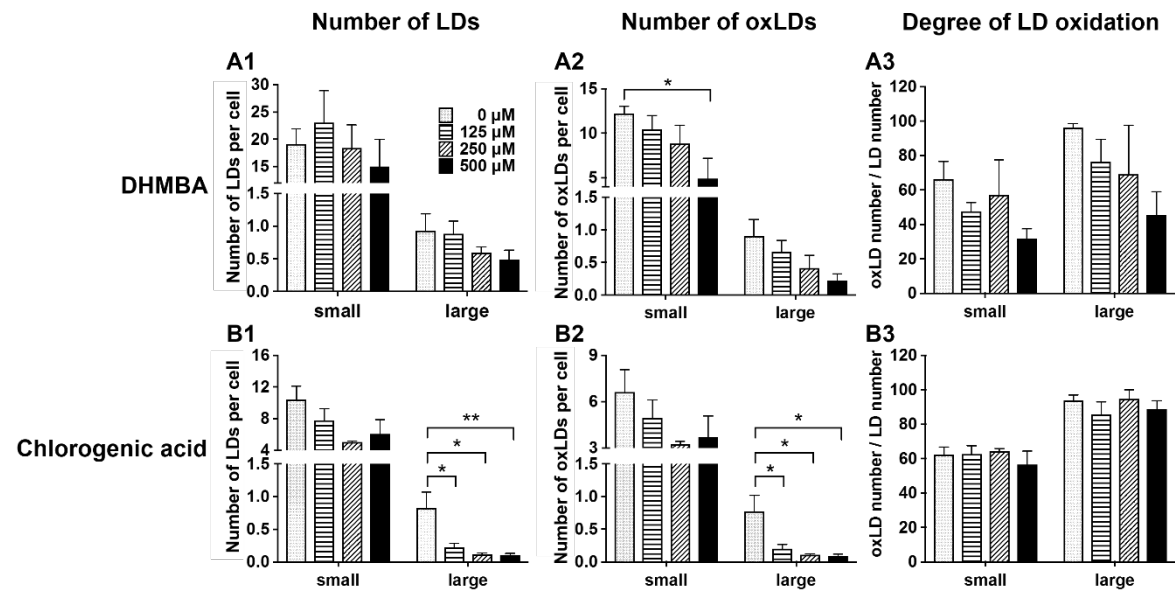


Figure 8



Abstract Graphics

