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A COMPARATIVE STUDY OF ARTIFICIAL CEROID/ LIPOFUSCIN FROM DIFFERENT TISSUE MATERIALS OF RATS

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The artificial ceroid/lipofuscin pigments originated from different organ tissues, including liver, brain, heart, and kidney of rats, and biomaterials were studied with improved fluorometric techniques. With all tissue materials exposed under ultraviolet (UV) light, a series of similar fluorescent colors were observed under microfluorometer. Analogous fluorescence spectra were also demonstrated with a three-dimensional (3-D) front-surface fluorometric technique despite of the tissue differences. Measured with 3-D fluorometry, relatively simple lipofuscin-like fluorophores were observed from the reactions of malondialdehyde (MDA) with critical biological macromolecules, such as bovine serum albumin (BSA)

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and DNA. Our results demonstrated that the biomaterials from different tissues have a similar fate under accelerated oxidative/carbonyl stresses but may be differentiated by a fluorescence intensity ratio.

Keywords: Aging, age pigments, carbonyl stress, lipofuscin, malondialdehyde

Accumulation of age pigments and related materials have long been recognized during aging process in humans and animals both intracellularly and extracellularly. These age-related pigments, particularly lipofuscin in lysosomes, have been found to be increased with age in brain, heart, skin, as well as in other tissues that contain "long lived" proteins (Brunk & Terman, 2002; Seehafer & Pearce, 2006; Sohal, 1981; Yin, 1996).

Although lipofuscin has been studied for more than a century, a number of scientific issues still remain unsolved, such as its pathophysiological origin, definite composition, degradation modalities, extraction and estimation techniques, and the relationship of lipofuscin-like materials in different organelles (Schmucker & Sachs, 2002; Sohal, 1981; Porta, 2002; Sparrow & Boulton, 2005). Due to substantial technical difficulties, particularly the low extractability and solubility of "mature" lipofuscin-like pigments (Patro, Patro, & Mathur, 1993), systematic and comparative investigation over different tissue materials has never been achieved.

However, to overcome difficulties remained in quantification of ceroid/lipofuscin pigmentsin situ, improved fluorometric techniques have been developed in our earlier studies, in which ultraviolet (UV) irradiation was found to be a quick and practical access to produce artificial ceroid/lipofuscin pigments (Li, Liao, Wang, Sheng, & Yin, 2006; Nilsson & Yin, 1997).

In this study, the artificial ceroid/lipofuscin pigments originated from different organ tissues and biomaterials were reexamined in situ with a three-dimensional (3-D) front surface (face) spectrofluorometry, and their full fluorescence spectra were for the first time demonstrated and compared.

METHODS

Materials and Stock Solutions

Sprague-Dawley (SD) rats were provided by the Animal Department of the College of Life Sciences, Hunan Normal University.

1,1,3,3-Tetramethoxypropane (TMP), purity >98%, was obtained from Fluka Chemie (Buchs, Switzerland). Butylated hydroxytoluene

(BHT), purity >99.0%, was obtained from Sigma Chemical (St. Louis, MO, USA).

Ultrapure water (fluorescence-free) was produced through Milli-Q water purification system (Millipore, China), and its electronic resistance was always checked to be >18.2 MΩ · cm.

A fresh malondialdehyde (MDA) stock solution (10 mM) was prepared by hydrolyzing TMP, which was modified according to a method described by Kikugawa, Tsukuda, and Kurechi (1980) and as in our early studies (Li et al., 2006). Thus 0.084 ml (0.5 mmol) TMP was mixed with 1.0 ml 1.0 M HCl, and shaken at 40°C for about 2 min. After the TMP was fully hydrolyzed, the pH was adjusted to 7.4 with 6 N NaOH, and the stock solution was made up to a final of volume of 50 ml with 0.1 M sodium phosphate buffer (pH 7.4). The MDA concentration of stock solution was often checked by measuring absorbance at 267 nm using $\epsilon_{MDA} = 31500$.

Other chemicals used were all of analytic grade from BioRad (Shanghai, China).

Equipments

An Olympus BX51-TF microfluorometer (Olympus Optical, Japan) was employed for obtaining fluorescence micrographs, in which three filter combinations was installed: (1) UV excitation, excitation filter BP330–385, dichoric beamsplitter DM400, barrier filter BA420; (2) blue excitation, excitation filter BP450–480, dichoric beamsplitter DM500, barrier filter BA455; (3) green excitation, excitation filter BP510–550, dichoric beamsplitter DM570, barrier filter BA590.

PE LS-50B fluorescence spectrophotometer (Perkin-Elmer, Norwalk, USA) installed with a standard photomultiplier (R-628) was applied for the measurement of age-related fluorophores.

Mortar, homogenizer, and quartz cuvettes were all soaked in 50% HNO_3 overnight to avoid possible fluorescent contaminants.

Preparation and Assay of Ceroidllipofuscin-Like Age Pigment

According to our early study (Nilsson & Yin, 1997) to prepare typical artificial ceroid/lipofuscin, male SD rats (about 200 g) were starved for 24 h before they were sacrificed for experiments. After bleeding, different tissues of rat including liver, brain, heart, and kidney were harvested, cut into small pieces, and washed with normal saline (with 0.01% BHT) for 3 times on ice bath. The different tissue materials were homogenized in phosphate-buffered saline (PBS) (0.1 M,

pH 7.4) and centrifuged at $1000 \times g$ for 5 min to clean out deposited tissue debris. The suspensions was then transferred into Petri dishes (without lids) and exposed under UV light in a laminar air flow bench for up to 24 h to allow rapid peroxidation to take place. The resulted artificial ceroid/lipofuscin-like materials (dried) were collected and were either dispersed on quartz slides for microfluorometric examination or inserted in a quartz holder of the front-surface accessory for spectrofluorometric assay (for technique details, see Li et al., 2006).

Reactions of MDA with BSA and DNA

Bovine serum albumin (BSA) (0.1%) were incubated with MDA (20 μM) in PBS (0.1 M, pH 7.4) at 37°C for 24 h.

DNA (0.1%) was incubated with MDA (10 mM) in PBS (0.1 M, pH 7.4) at 37°C for 48 h.

Reaction products were measured by the PE LS-50B fluorescence spectrophotometer with both excitation and emission slits at 10 nm.

RESULTS

Using Olympus BX51-TF microfluorometer, the microfluorometric examination of artificial ceroid/lipofuscin-like age pigments is presented in Figure 1. The black and white microphotographs of the artificial ceroid/lipofuscin were shown as control graphs (Figure 1, column I). Strong blue, yellow, and red fluorescence were observed corresponding with exciter combinations with band-pass (BP) filter of 330 to 385, 450 to 480, and 510 to 550 nm, respectively (Figure 1, columns II to IV). UV exposure of different tissue materials for about 1 day induced ceroid/lipofuscin-like materials in all and showed strong age pigment-related fluorescence (Figure 1, columns II to IV), whereas a relative weaker red fluorescence were seen with the third exciter combination (Figure 1, column IV).

Applying front-surface accessory on PE LS-50B for spectrofluorometric assay, the fluorescence data of the artificial ceroid/lipofuscin pigments before and after UV irradiation were demonstrated in the 3-D fluorescence contour maps (Figures 2 to 5). The autofluorescence spectra of the samples before UV irradiation are shown in Figures 2*A* to 5*A* as controls. In each figure, noting a very strong protein-related peak at 280/350 nm, its fluorescence intensity was over the top limit (>1000). In the control figures, however, there were also some age pigment-related fluorescence maxima at about 350/455

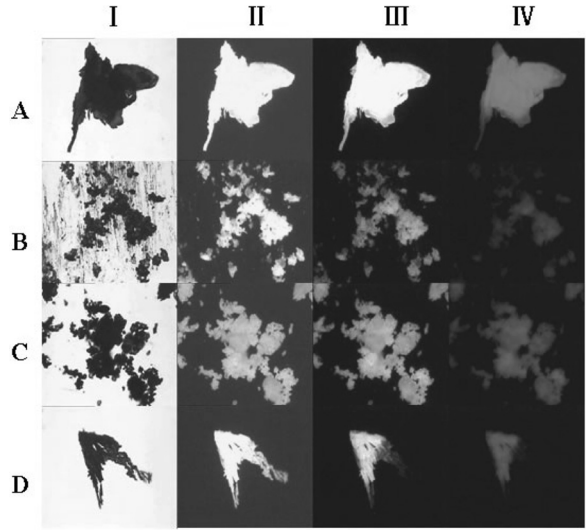

Figure 1. Microfluorometric examination of artificial ceroid/lipofuscin with different exciter combinations. The rows A, B, C, and D denote UV-exposed materials from liver, brain, heart, and kidney, respectively. The columns indicate exciter combinations: column I, control (under white light); column II, UV exciter (BP 330 to 385 nm); column III, blue exciter (BP 450 to 480 nm), and column IV, yellow exciter (BP 510 to 550 nm).

and 280/455 nm, although they were quite inconspicuous. Similar fluorescence characteristics from ceroid/lipofuscin materials of different tissues were observed (Figures 2*B* to 5*B*). The apparent fluorescence maxima (peaks) of excitation and emission (Ex/Em) from different samples were listed as follows: liver, 280/450 and 390/455 nm (Figure 2*B*); brain, 280/450 and 395/455 nm (Figure 3*B*); heart, 280/455, 390/455, and 390/590 nm (Figure 4*B*); and kidney, 280/450, 390/460, and 390/590 nm (Figure 5*B*). Although the liver and brain samples showed no apparent fluorescence peak at the 390/590 nm, they all implied a tendency to form peaks in this area.

The reaction adducts of MDA + BSA and MDA + DNA showed simple fluorescence peaks when measured with 3-D spectrofluorometry (not using front-surface accessory for these aqueous samples) (Figures 6 and 7). The main peaks of the BSA + MDA product were at 280/460, 395/460 nm (Figure 6), and a single peak of the DNA + MDA product was at 390/460 nm (Figure 7).

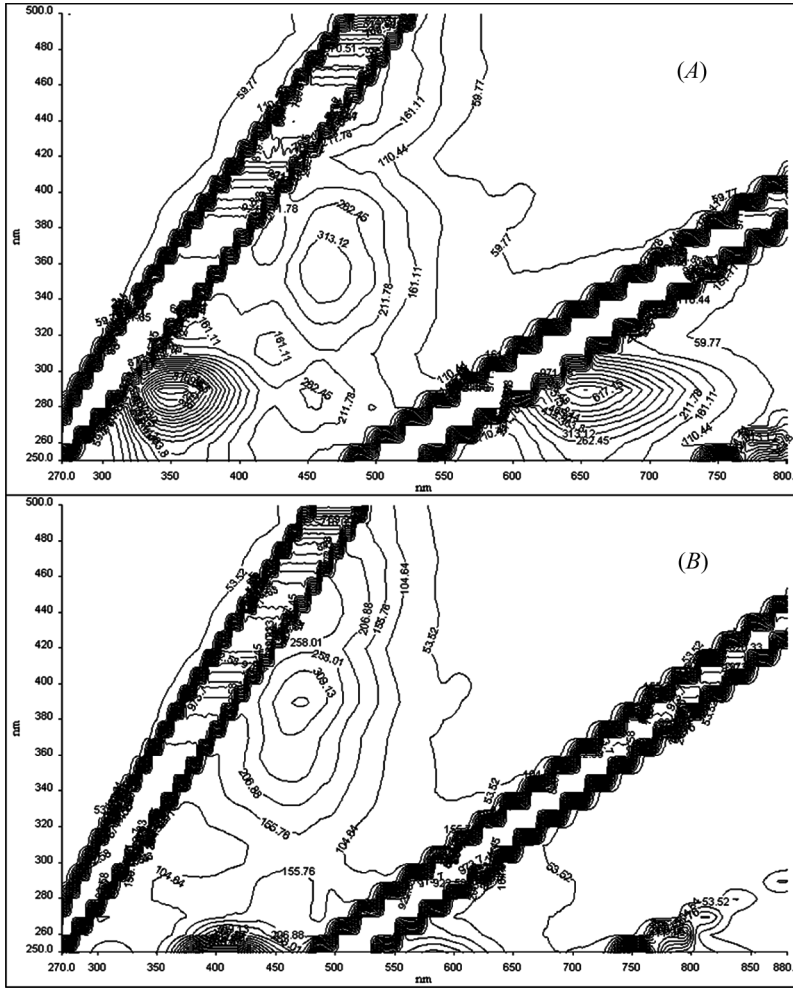

Figure 2. The front surface spectrofluorometric technique detected 3-D fluorescent contour map of the liver materials before and after UV irradiation. (*A*) Before UV irradiation; (*B*) UV-irradiated for 24 h.

DISCUSSION

The artificial ceroid/lipofusin-like pigments produced from different tissues of rats showed similar fluorescent properties after irradiation (Ex/Em, 390/455, 280/455, 390/590 nm). It appeared that, although the materials were collected from different tissues, from different

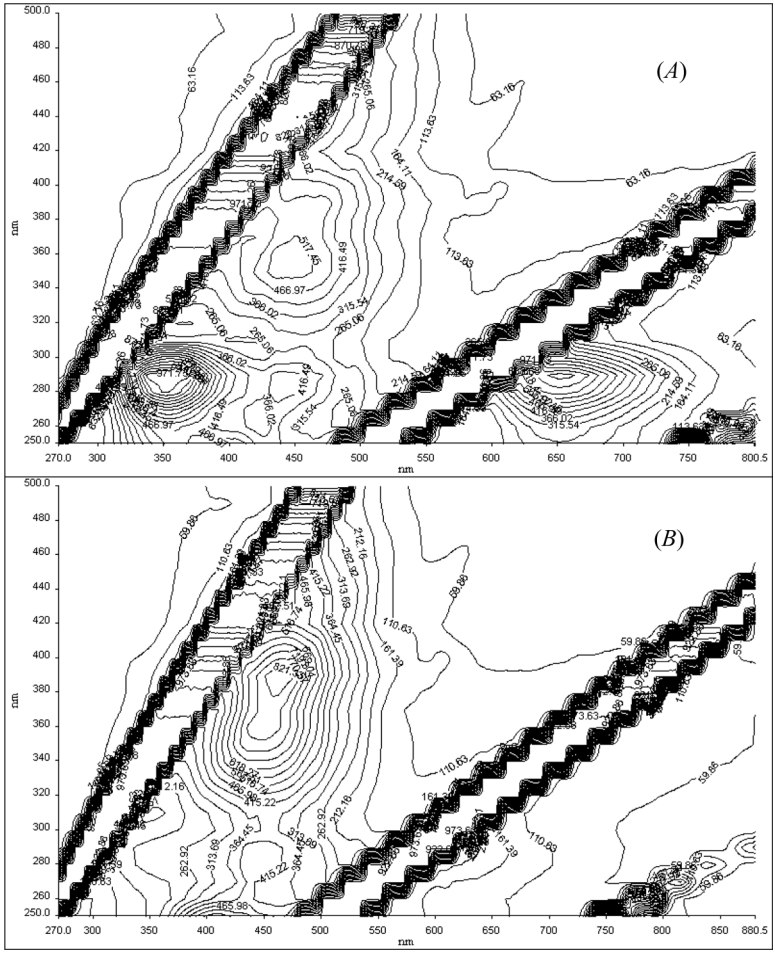

Figure 3. The front surface spectrofluorometric technique detected 3-D fluorescent contour map of the brain materials before and after UV irradiation. (*A*) Before UV irradiation; (*B*) UV-irradiated for 24 h.

biological components, and may pass through different biochemical processes, the final composition and the biochemical structure of the end products seemed to have some common fluorometric haracteristics.

Up to date, the mechanisms of age pigment formation were reported mainly based on conjugation/cross-linking-related biochemical aggregations (Björkerud, 1964; Brunk & Terman, 2002;

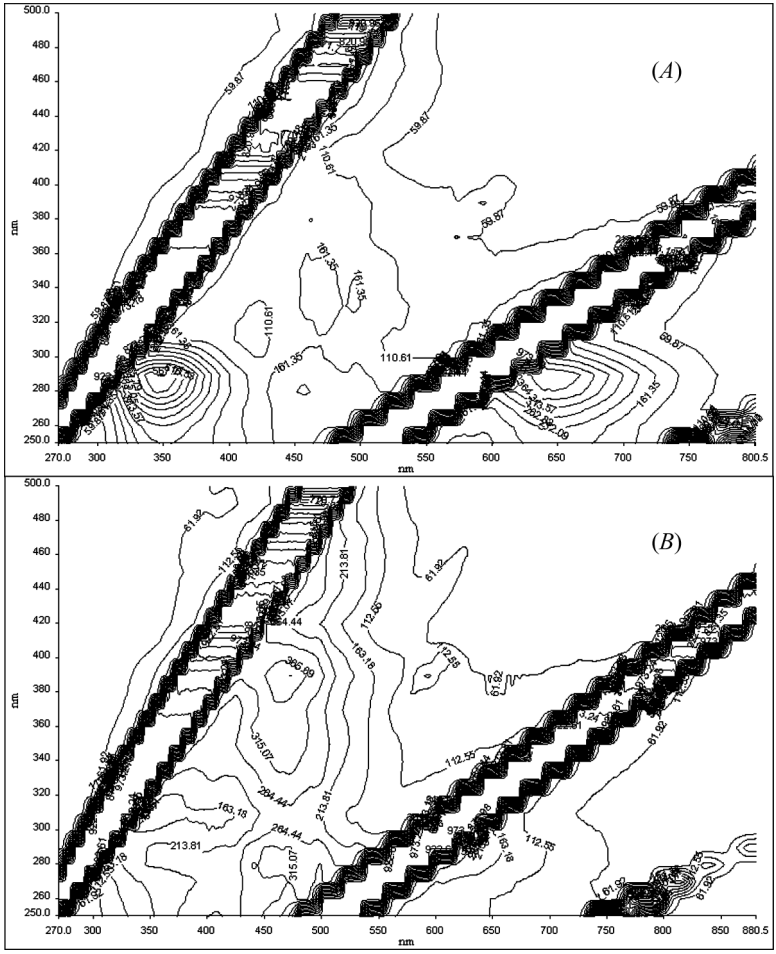

Figure 4. The front surface spectrofluorometric technique detected 3-D fluorescent contour map of the heart materials before and after UV irradiation. (*A*) Before UV irradiation; (B) UV-irradiated for 24 h.

Siakotos & Koppang, 1973; Sohal, 1981; Yin, 1996). A few biochemical side reactions, such as oxidation, glycation, and carbonylation, has been found as the critical biochemical processes that may result in the formation of the ceroid/lipofuscin-related fluorophores (Sohal, 1981; Terman & Brunk, 2004; Yin, 1992; Yin, 1996). Despite the basically common biochemical processes, the pathophysiological defending, metabolizing, and repairing systems in different organ

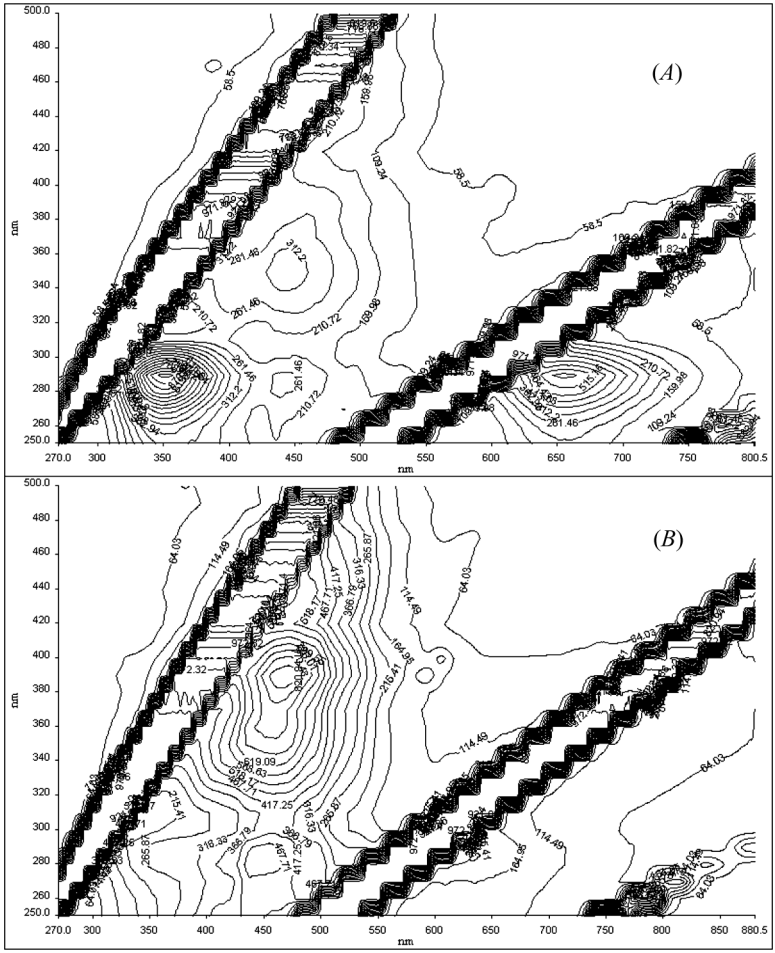

Figure 5. The front surface spectrofluorometric technique detected 3-D fluorescent contour map of the kidney materials before and after UV irradiation. (*A*) Before UV irradiation; (B) UV-irradiated for 24 h.

tissues are largely different in vivo, thus resulting in tissue-specific characteristics of age pigments. Due to different defending and recovering systems in different organ tissues, the formation of ceroid/lipofuscin-related materials and cross-linkages may build up versatile age pigment-related complex, which may appear differently in different tissues, such as the formation of lens cataract, various amyloids, atherosclerotic plaques, and organ fibrosis in various chronic diseases

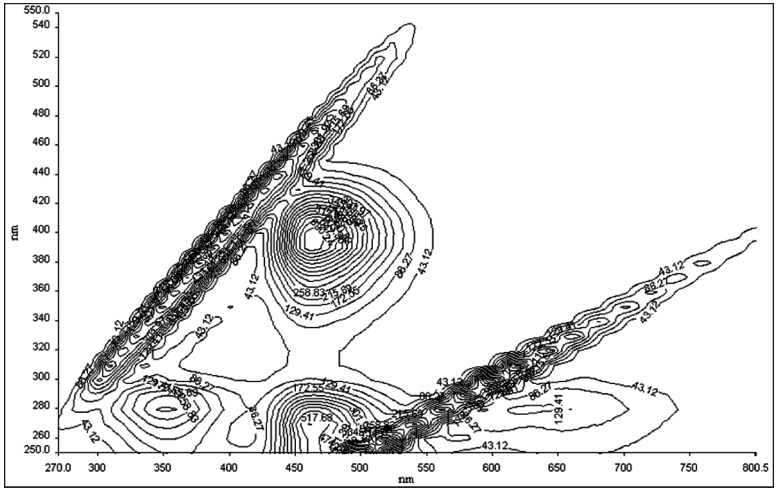

Figure 6. The 3-D fluorescent contour map of the MDA + BSA reaction product. BSA (0.1%) was incubated with MDA (20 μM) in PBS (0.10 M, pH 7.4) at 37°C for 24 h.

(Rozanowska et al., 2004; Seehafer & Pearce, 2006; Yin, 1996; Yin & Chen, 2005).

In our irradiation system, however, when the oxidative/carbonyl stress was intensified in a non-defending model, the artificial ceroid/lipofuscin pigments were found to be formed analogously among different tissues, and were found similar in fluorescent color. The strong color of fluorescence was observed from yellow-green to orange-red under microfluorometer, largely due to a high concentration-dependent shift of autofluorescence as we described before (Yin, 1994). Following such irradiation-induced stresses, our investigation demonstrated for the first time the general tendency of the fate of biological macromolecules under neither biotic nor enzymatic protection conditions. With an advantage of our front-surface 3-D measurement showing epifluorescence (escaping condensation-induced quenching effect), reliable full fluorescence spectra of artificial ceroid/lipofuscin from different organ tissues were obtained. These spectra matched very well with advanced lipoxidation end-products (ALEs) and advanced glycation end-products (AGEs), as reported in the literature (Yin, 1996; Seehafer & Pearce, 2006).

Our other data of 3-D contour maps (Figures 6 and 7, not using front-surface accessory) showed that the fluorescence peaks of the artificial ceroid/lipofuscin can also be found in the products from

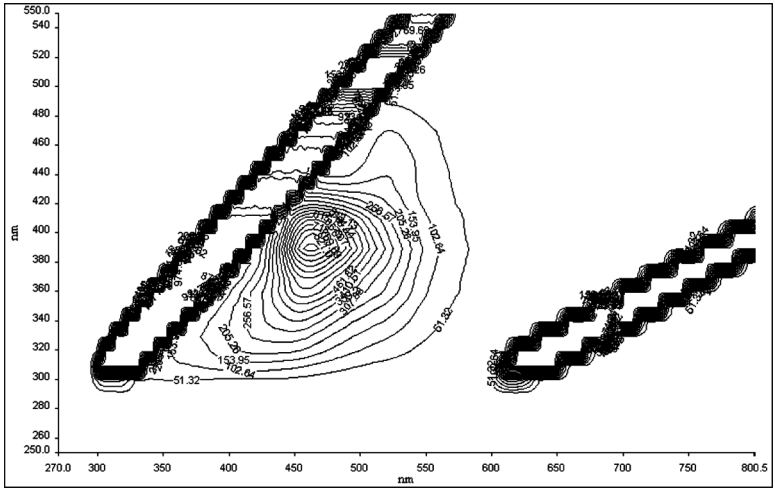

Figure 7. The 3-D fluorescent contour map of the MDA + DNA reaction product. DNA (0.1%) was incubated with MDA (10 mM) in PBS (0.10 M, pH7.4) at 37°C for 48 h.

MDA + BSA and MDA + DNA reactions. As an important unsaturated dialdehyde from lipid peroxidation, MDA reacted easily with different biological molecules and formed complex with a typical age pigment-like 3-D fluorescence contour map using the developed estimation technique (Li et al., 2006). Our data again confirmed that the carbonyl stress played a critical role in the formation of ceroid/lipofusin-like pigments. However, in the formation course of the artificial age pigments, BSA reacted more readily than DNA with MDA (shorter time, and with a lower concentration of MDA) to form age pigment-like fluorescent substances (Figures 6 and 7).

The formation of lipofuscin can be quantified with the developed fluorometric technique, particularly during the "growing period" of lipofuscin. However, a relatively similar spectral pattern of fluorescence was observed by "mature" artificial lipofuscin. In order to have a better quantitative differentiation of different tissue materials, an intensity ratio of ceroid/lipofuscin fluorescence versus protein fluorescence was presented (Figure 8), showing clear differences of different tissue materials. Whereas ceroid/lipofuscin of brain and liver showed higher values of fluorescence ratio, the fluorescence ratio from heart materials are the lowest, probably due to the specificity of the heart muscle materials.

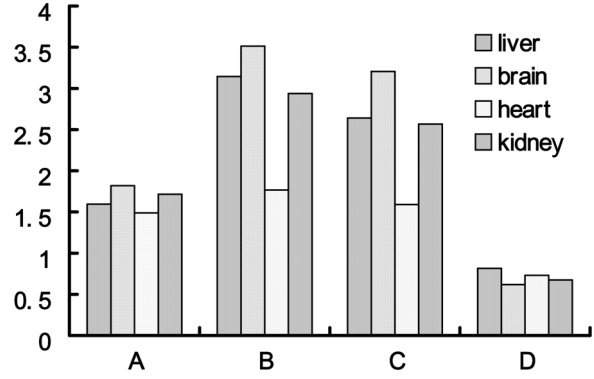

Figure 8. The quantitative comparison of ceroid/lipofuscin from different tissues of rats after 24 h UV irradiation. Data were calculated from the values of Figures 2*B*, 3*B*, 4*B*, and 5*B* and expressed as the fluorescent intensity ratio of individual ceroid/lipofuscin versus protein fluorescence (Ex280/Em350 nm). A: 280/450 versus 280/350 nm; B: 390/460 versus 280/350 nm; C: 360/462 versus 280/350 nm; D: 390/590 versus 280/350 nm.

Besides MDA, other unsaturated carbonyls, such as acrolein and 4-hydroxynonenal (4-HNE), may also react with biomaterials contributing to the fluorescent formation of ceroid/lipofuscin. The reaction between 4-HNE and proteins have been described before in other studies (Itakura et al., 2000). The major lipofuscin-like fluorophore derived from HNE and lysine, were reported as hydroxyiminodihydropyrrole (HIDP) with fluorescence characteristics similar to those of the oxidized low-density lipoprotein (LDL) (360/430 nm). Although a systematic investigation using different carbonyls in related field has been carried out recently in our laboratory, those data are to be presented in separate papers in the future so as to provide a clearer focus for this article.

In summary, with the front surface 3-D fluorometric technique, the full color and spectra of ceroid/lipofuscin-like pigments from different organ tissues and biomaterials were investigated. The general tendency of aging-related pigment formation in biological system and the common fluorescence characteristics following accelerated oxidative/carbonyl stresses were demonstrated.

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