Direct detection of diclofenac radical produced by ultraviolet irradiation using electron spin resonance method

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Diclofenac, a nonsteroidal anti-inflammatory drug, is commonly used as an antipyretic analgesic owing to its strong anti-inflammatory action in clinical treatment. However, diclofenac can cause injury, with gastrointestinal mucosal lesions and skin photosensitivity as the main side effects. In general, photosensitive drugs contain photosensitive chemical sites, and form free radicals under ultraviolet irradiation, leading to phototoxic reactions. Therefore, this study focuses on free radical production in photosensitive reactions of diclofenac. The free radical production mechanism of diclofenac under ultraviolet irradiation, which might result in photo-toxicity, was clarified using a direct electron spin resonance method. When diclofenac was irradiated with ultraviolet light (254 nm), diclofenac radicals were generated depending on the ultraviolet irradiation time and stably present for 30 min at room temperature. Diclofenac radicals were produced by the ultraviolet irradiation system depending on the dose of diclofenac until 2 mM. Therefore, diclofenac radicals might directly or indirectly react with various biomolecules to cause phototoxicity, other side effects, and new diclofenac pharmacology owing to its stability of diclofenac radicals.

Key Words: **ESR, diclofenac (DCF), nonsteroidal anti-inflammatory drugs (NSAIDs), UV irradiation, phototoxicity, DCF radical**

Free radicals such as superoxide ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), nitric oxide are considered to play important roles in various diseases, including acute lung injury, renal disorder with dialysis, and periodontal disease.[1–7] As controlling inflammation in these pathological states is important, nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently used as general therapeutic agents.[8,9] Although much research on NSAIDs and radicals has been conducted, the detailed reaction mechanism of NSAIDs about radical has yet to be clarified.[10–17]

Recently, NSAIDs, especially propionic acid derivatives, have shown phototoxicity.[18–20] Some drugs used to treat human diseases are known to be activated under light irradiation to cause skin rashes, including redness, swelling, and pigmentation. Phototoxic reactions are known to be among the causes of photosensitivity in these drugs, in which agents activated by photochemical reactions cause damage to biochemical components directly or through reactive oxygen species (ROS).[18,19] When a drug molecule absorbs photon energy, electrons are excited from the ground state to the excited state, depending upon the bond type and associated energy level. As the electrons enter different orbitals through photoexcitation and electron pairs are eliminated, the excited electrons have radical properties. Energy transfer from excited drug molecules to oxygen (type-II photochemical reaction) generates singlet oxygen ($^1O_2$), which might participate in the oxidation of membrane lipids and proteins, or induce DNA damage.[20,21] Furthermore, these excited drug molecules might react directly with *in vivo* molecules (such as DNA, proteins, and cell membranes) though electron or hydrogen transfer (type-I photochemical reaction).[20] Excessive ROS production in the body, can result in the oxidation of nucleic acids, proteins, sugars, and lipids to cause various biological disorders.[22–24]

Diclofenac (DCF) is an acetic acid derivative and NSAID that is often used clinically. The action of DCF is particularly strong among NSAIDs, and is characterized by the rapid suppression of pain and heat generation.[25–28] In addition to a strong action, DCF has known clinical side effects, such as causing gastrointestinal and kidney disorders, and phototoxicity.[29–35] However, the detailed mechanism of DCF phototoxicity has yet to be clarified.

Therefore, this study aimed to clarify the reaction mechanism of DCF radical ($^{\bullet}$DCF) production under ultraviolet (UV) irradiation, which can cause DCF phototoxicity, using a direct electron spin resonance (ESR) method.

Materials and Methods

Chemicals. DCF was purchased from Wako Pure Chemical Ind. (Tokyo, Japan). 1-Oxyl-2,2,6,6-tetramethyl-4-hydroxypiperidine (TEMPOL) was purchased from TOCRIS Bioscience (Ellisville, MO). Superoxide dismutase from bovine erythrocytes (SOD) was purchased from Wako Pure Chemical Ind. (Osaka, Japan). Water used in these experiments was treated to remove the trace metals by passing through Chelex 100 Resin (Bio-Rad Laboratories, Inc. Hercules, CA) after distillation.

Measurement of diclofenac radical ($^{\bullet}$DCF) by direct ESR method. DCF was diluted with distilled water in a quartz flat cell (160 µl) and, irradiated with UV (254 nm) using a Handy UV Lamp (SUV-6) (AS ONE, Osaka, Japan). After UV irradiation, ESR spectra were immediately recorded at room temperature in a quartz flat cell using a JEOL JES-FR 30 EX Free Radical Monitor (JEOL, Tokyo, Japan). The operating conditions of the ESR spectrometer were as follows: frequency, 9.42 GHz; field, 335.618 ± 5 mT; microwave power, 16.0 mW; modulation frequency, 100 kHz; modulation width, 0.32 mT; amplitude, 7.9 × 100; time constant, 0.3 s; and sweep time, 1 min. To identify and determine the amount of radical species $^{\bullet}$DCF, the *g* value was corrected using the internal Mn marker of the ESR instrument used in this study. The $^{\bullet}$DCF concentration was calculated from a calibration curve prepared from the integrated value of the ESR signal of TEMPOL aqueous solution, for which the spin concentration is known.

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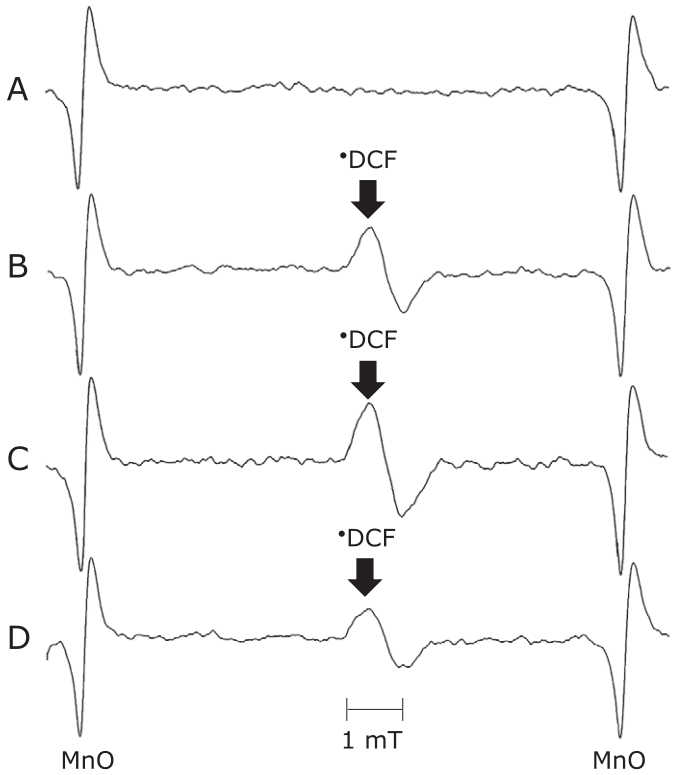

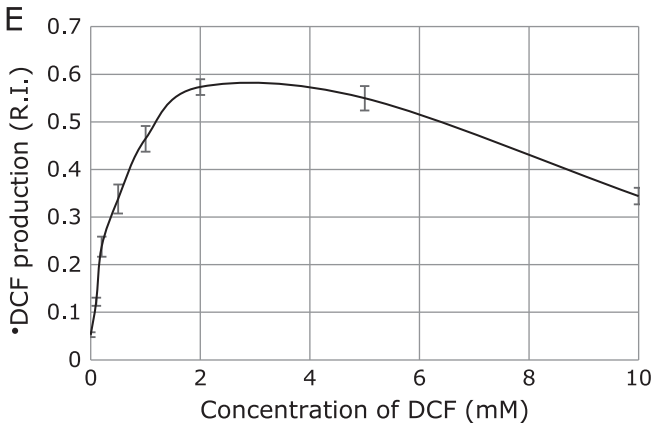

Fig. 1. ESR spectra was measured in various concentration of DCF irradiated with UV (254 nm) for 3 min. (A) Spectrum obtained in the reaction mixture of 0 mM DCF. (B) Spectrum obtained in the reaction mixture of 1 mM DCF. (C) Spectrum obtained in the reaction mixture of 2 mM DCF. (D) Spectrum obtained in the reaction mixture of 10 mM DCF. (E) •DCF production with dose-dependent manner of DCF. ESR measurement conditions were described in materials and methods. All spectra were recorded after DCF irradiation with UV (254 nm) for 3 min.

Statistical analysis. The statistical significance of the difference was determined by an unpaired Student's *t* test. Data are expressed as means ± SE. Differences between groups were considered statistically significant at the level of $p<0.05$.

Result

ESR spectra and production mechanisms of •DCF. A prominent ESR spectrum of •DCF was observed after UV irradiation (254 nm) for 3 min in the absence of H_2O_2 (Fig. 1A–D). This represents the first report of •DCF detection using a direct ESR method at room temperature. •DCF increased until 2 mM DCF in the reactive mixture and then gradually decreased to 10 mM DCF in the reactive mixture (Fig. 1E). When DCF (0.1 mM) was irradiated with UV (254 nm), the intensity of •DCF radical generation increased with increasing UV irradiation time (Fig. 2). Increased the amount of •DCF production depending on UV irradiation time could be also observed with other concentrations (0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10 mM) of DCF (data not shown). To evaluate the •DCF production mechanism, we attempted to reduce the amount of dissolved oxygen in the •DCF production system by bubbling with N_2. With reduced dissolved oxygen in the DCF solution (10 mM), the ESR peak of •DCF significantly decreased in intensity, but did not disappear (Fig. 3). These results indicated that •DCF was generated even when the oxygen concentration was low.

Time course of •DCF. To study the lifetime of •DCF, the amount of •DCF was measured after UV irradiation (254 nm) for 3 min. The •DCF peak height obtained by the direct ESR method gradually decreased, but did not disappear, for 30 min (Fig. 4). •DCF was found to be a longer life radical in this instance than $O_2^{\bullet-}$ and •OH.

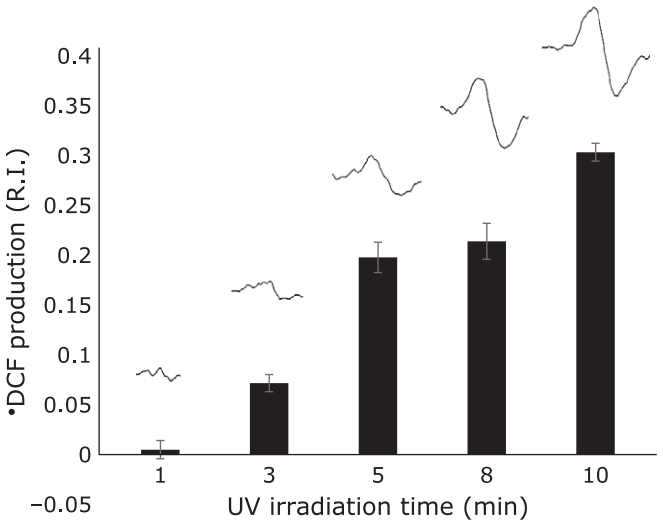

Fig. 2. •DCF production with time dependent manner of UV irradiation. ESR signal intensity of •DCF were obtained in the reaction system of DCF (0.1 mM), irradiated with UV (254 nm). ESR measurement condition were as described in materials and methods. Amounts of •DCF are expressed as a relative intensity (R.I.) by normalization of the •DCF signal height to the standard signal intensity of manganese oxide (MnO) and are the means ± SD of three independent experiments ($n = 3$). The waveform and R.I. of the ESR spectra from the •DCF spin adducts at each UV irradiation time was shown on the figure. Intensity: mean ± SD.

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Reactivity of •DCF with $O_2^{\bullet-}$. To study the effect of $O_2^{\bullet-}$ on •DCF production, the amount of •DCF was measured after UV irradiation (254 nm) for 5 min. The relative intensity of •DCF measured using the direct ESR method increased with the addition of superoxide dismutase (SOD; 100 U/ml) (Fig. 5). This result confirmed that •DCF was more likely to be generated without $O_2^{\bullet-}$.

Characteristics of •DCF. The spin adducts of radical species •DCF were identified, and their amounts determined. A sharp single P_1 signal was obtained at a g value of 2.0038 when DCF (1 mM) was irradiated with UV light (254 nm) for 10 min (Fig. 6). These results showed that •DCF detected under UV irradiation was an oxygen radical. The amount of radicals at this time was determined to be 5.39×10^7 spins. The •DCF concentration in this system was calculated, to be 1 μM comparison with the spins of TEMPOL radicals. The •DCF concentration was significantly higher, indicating that, •DCF might cause tissue injury directly.

Discussion

In this study, we obtained direct evidence that DCF forms extremely stable •DCF under UV irradiation. This represents the first evidence of stable •DCF detection in a UV irradiation system using direct ESR method.

Kawaguchi *et al.*[36] previously estimated that the photolysate of DCF was cyclized and radicalized at the center of the molecule by LC-NMR analysis, which was in agreement with our results. LC-NMR analysis provides detailed information on substance structure, but information on unpaired electrons is difficult to

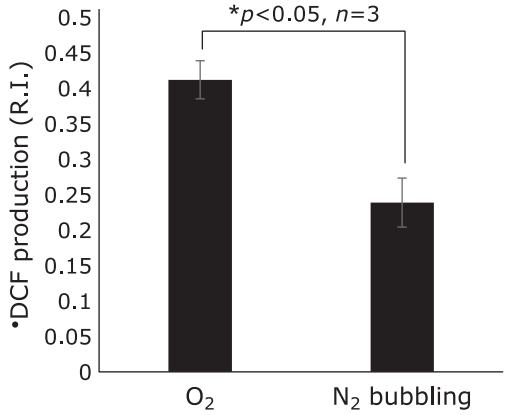

Fig. 3. Effect of dissolved O_2 on •DCF formation. For the treatment of DCF (1 mM), dissolved oxygen was reduced with N_2 bubbling. Each of those substituted with N_2 and those not substituted with N_2 low concentration of O_2 was irradiated with UV (254 nm) for 3 min, and then measured by ESR. ESR measurement conditions were as described in materials and methods. Intensity: mean ± SD. *p<0.05.

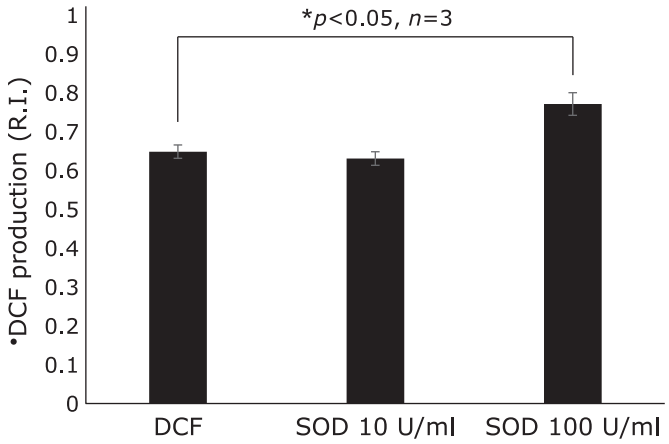

Fig. 5. Effects of superoxide dismutase (SOD) on •DCF formation. After adjustment of DCF (final conc. 1 mM), SOD (final conc. 10 U/ml, 100 U/ml) was added removing the $O_2^{\bullet-}$. Each sample with SOD was irradiated with UV (254 nm) for 5 min, and then measured by ESR. ESR measurement conditions were as described in materials and methods. Intensity: mean ± SD. *p<0.05.

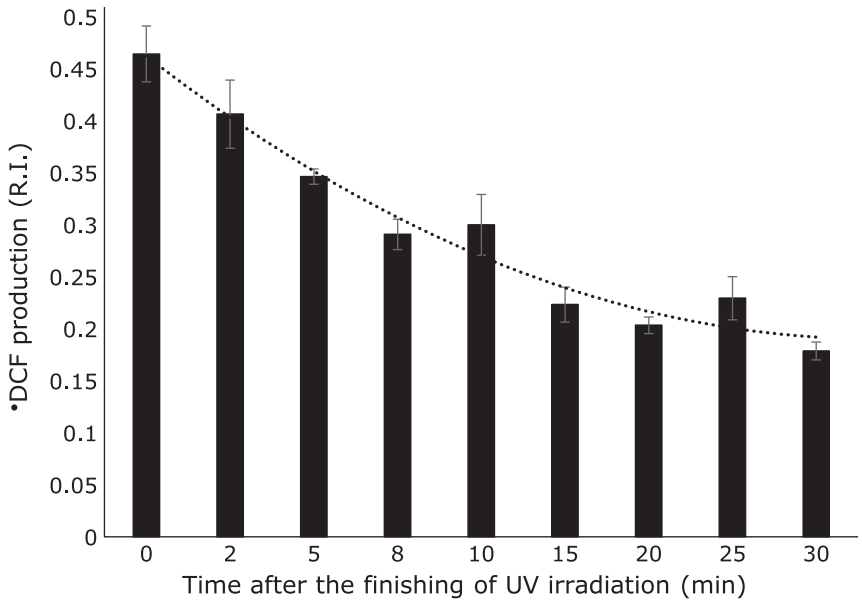

Fig. 4. Time course of •DCF spin adducts generation from the finishing of the UV irradiation. The ESR spectrum of •DCF spin adducts were obtained from the finishing of the irradiating DCF (1 mM) with UV (254 nm) for 3 min (n = 3). ESR measurement conditions were as described in materials and methods. Intensity: mean ± SD.

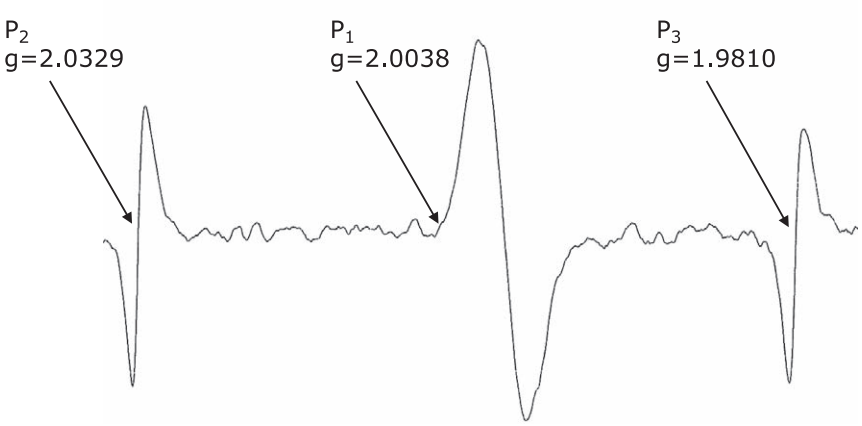

Fig. 6. ESR spectrum and *g* value of ˙DCF spin adducts. The ESR spectrum and *g* value from ˙DCF spin adducts were obtained in the system of DCF (1 mM), irradiated with UV (254 nm) for 10 min. P_1 is a single signal derived from ˙DCF. P_2 is the 3rd signal and P_3 is the 4th signal of internal Mn marker of ESR. ESR measurement conditions were as described in Materials and Methods.

obtain using this method. Therefore, in this study, we elucidated the mechanism of ˙DCF formation using a direct ESR method.

Our results, showed that ˙DCF generation was dependent on the DCF concentration until 2 mM, but on the UV irradiation time, and that the radical species might be an alkoxyl radical or a nitrogen radical based on the *g* value (2.0038). In order to identify radical species, it need to be further examination of *g* value using more useful internal marker as well as of the hyperfine structure of the ESR spectrum with smaller modulation width.

Assuming that ˙DCF is an alkokyl radical, we showed that electron transfer from ˙DCF to O_2 hardly occurred because the amount of DCF production decreased with N_2 bubbling [see equation (1)].

$$\text{˙DCF} + O_2 \rightleftarrows \text{DCF} + O_2^{\bullet-} \quad (1)$$

Furthermore, we showed that electron transfer from ˙DCF to $O_2^{\bullet-}$ occurred because the amount of ˙DCF produced increased with the addition of SOD [see equation (2)]. Besides, when ascorbic acid (ASA) as a scavenger of ˙OH and $O_2^{\bullet-}$ was added to the complete reaction mixture, ASA strongly suppressed ˙DCF production. Therefore, it might be suggested that ˙OH play important role in the production of ˙DCF (data not shown).

$$\text{˙DCF} + O_2^{\bullet-} + 2H^+ \rightarrow \text{DCF} + H_2O_2 \quad (2)$$

Accordingly, ˙DCF can be assumed to exist for a unusually long time compared with other radicals. Free radicals with unpaired electrons are known to be very unstable, for example, $O_2^{\bullet-}$ and ˙OH have half-lives of 10^{-6} and 10^{-9} s, respectively. In this study, the stability of ˙DCF did not change, even at low O_2 concentrations, suggesting that ˙DCF would be present not only in blood vessels with relatively high O_2 levels, but also in tissues with low O_2 levels. Therefore, it is possible that ˙DCF acts as a toxic radical, both in the periphery and in the blood vessels. In contrast, ˙DCF, reducing radicals, scavenge oxidative radicals and act as proactive.

In any other possible knowledge, DCF is a secondary amine, which generally act as an electron donor. It is possible that the excited state of DCF may reduce molecular oxygen to produce the DCF radical cation and $O_2^{\bullet-}$. The DCF radical cation may undergo deprotonation to produce ˙DCF, which is a nitrogen radical. In such a case, molecular oxygen act as an oxidant to generate ˙DCF. Nitrogen bubbling may decrease the oxidant, i.e., molecular oxygen, leading to the decrease of ˙DCF production. SOD converts $O_2^{\bullet-}$ to molecular oxygen and water. The oxidant (molecular oxygen) to produce ˙DCF may be recycled in the presence of SOD, leading to the increase of ˙DCF production. In future examination, the hyperfine structure of the ESR spectrum of ˙DCF will provide detailed information about the electronic structure as well as production mechanism of ˙DCF.

˙DCF generation *in vivo* is still unknown, which indicate the need for further studies. We propose that ˙DCF is generated *in vivo* because in a previous study by Miura[37] and Muraoka[38] showed that NSAIDs react with peroxidase to possibly generate NSAID radicals (˙NSAID). Previously, for both the intended and side effects of NSAID inhibition of cyclooxygenase (COX), the main mechanism has been considered to involve changes in the balance of eicosanoids.[39–41] However, this report suggests that radical formation, not only by DCF, but also by other NSAIDs is needed for physiological effects other than COX inhibition.

In conclusion, ˙DCF might generate via one-electron reduction of DCF *in vivo* or in human. Furthermore, the generated ˙DCF might affect the various diseases where DCF is frequently used for treatment,[1–7] such as inflammation, cancer, or orthopedic disorders. Therefore, it was suggested that our detected ˙DCF might have important roles in various diseases.

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Conflict of Interest

No potential conflicts of interest were disclosed.

References

1 Sato K, Kadiiska MB, Ghio AJ, *et al*. *In vivo* lipid-derived free radical formation by NADPH oxidase in acute lung injury induced by lipopolysaccharide: a model for ARDS. *FASEB J* 2002; **16**: 1713–1720.

2 Sato K, Corbett J, Mason RP, Kadiiska MB. *In vivo* evidence of free radical generation in the mouse lung after exposure to *Pseudomonas aeruginosa* bacterium: an ESR spin-trapping investigation. *Free Radic Res* 2012; **46**: 645–655.

3 Ueda A, Nagai K, Hirayama A, Saito C, Yamagata K. Peritoneal dialysis

doi: 10.3164/jcbn.19-91

preserves residual renal function and reduces oxidative stress during the initial period of dialysis therapy. *Adv Perit Dial* 2017; **33**: 18–21.
4 Hirayama A, Nagase S, Gotoh M, *et al*. Reduced serum hydroxyl radical scavenging activity in erythropoietin therapy resistant renal anemia. *Free Radic Res* 2002; **36**: 1155–1161.
5 Komatsu T, Lee M-C. Oxidative stress and periodontal disease in Down syndrome. In: Ekuni D, Battino M, Tomofuji T, Putnins EE, eds. *Studies on Periodontal Disease Oxidative Stress in Applied Basic Research and Clinical Practice*, New York: Human Press Springer, 2014; 211–214.
6 Komatsu T, Lee MC, Miyagi A, *et al*. Reactive oxygen species generation in gingival fibroblasts of Down syndrome patients detected by electron spin resonance spectroscopy. *Redox Rep* 2006; **11**: 71–77.
7 Yoshida A, Yoshino F, Makita T, *et al*. Reactive oxygen species production in mitochondria of human gingival fibroblast induced by blue light irradiation. *J Photochem Photobiol B* 2013; **129**: 1–5.
8 Tanaka K, Ishihara T, Sugizaki T, *et al*. Mepenzolate bromide displays beneficial effects in a mouse model of chronic obstructive pulmonary disease. *Nature Commun* 2013; **4**: 2686–2698.
9 Hirayama A, Okamoto T, Kimura S, *et al*. Kangen-karyu raises surface body temperature through oxidative stress modification. *J Clin Biochem Nutr* 2016; **58**: 167–173.
10 Ghiselli A, Laurenti O, De Mattia G, Maiani G, Ferro-Luzzi A. Salicylate hydroxylation as an early marker of *in vivo* oxidative stress in diabetic patients. *Free Radic Biol Med* 1992; **13**: 621–626.
11 Zentella de Piña M, Saldaña-Balmori Y, Hernández-Tobías A, Piña E. Nonsteroidal antiinflammatory drugs lower ethanol-mediated liver increase in lipids and thiobarbituric acid reactive substances. *Alcohol Clin Exp Res* 1993; **17**: 1228–1232.
12 Takayama F, Egashira T, Yamanaka Y. Effect of diclofenac, a non-steroidal anti-inflammatory drug, on lipid peroxidation caused by ischemia-reperfusion in rat liver. *Jpn J Pharmacol* 1994; **64**: 71–78.
13 Gupta SK, Joshi S. Role of naproxen as anti-oxidant in selenite cataract. *Ophthalmic Res* 1994; **26**: 226–231.
14 Bilodeau JF, Wang M, Chung FL, Castonguay A. Effects of nonsteroidal antiinflammatory drugs on oxidative pathways in A/J mice. *Free Radic Biol Med* 1995; **18**: 47–54.
15 Muraoka S, Miura T. Inactivation of creatine kinase during the interaction of mefenamic acid with horseradish peroxidase and hydrogen peroxide: participation by the mefenamic acid radical. *Life Sci* 2003; **72**: 1897–1907.
16 Saito R, Tamura M, Matsui H, *et al*. Qing Dai attenuates nonsteroidal anti-inflammatory drug-induced mitochondrial reactive oxygen species in gastrointestinal epithelial cells. *J Clin Biochem Nutr* 2015; **56**: 8–14.
17 Ito H, Matsui H, Hirayama A, Indo HP, Majima HJ, Hyodo I. Reactive oxygen species induced by non-steroidal anti-inflammatory drugs enhance the effects of photodynamic therapy in gastric cancer cells. *J Clin Biochem Nutr* 2016; **58**: 180–185.
18 Ferguson J, Addo HA, McGill PE, Woodcock KR, Johnson BE, Frain-Bell W. A study of benoxaprofen-induced photosensitivity. *Br J Dermatol* 1982; **107**: 429–441.
19 Epstein JH, Wintroub BU. Photosensitivity due to drugs. *Drugs* 1985; **30**: 42–57.
20 Pathak MA. Molecular aspects of drug photosensitivity with special emphasis on psoralen photosensitization reaction. *J Natl Cancer Inst* 1982; **69**: 163–170.
21 Foote CS, Shook FC, Abakerli RB. Characterization of singlet oxygen. *Methods Enzymol* 1984; **105**: 36–47.
22 Sodum RS, Fiala ES. Analysis of peroxynitrite reactions with guanine, xanthine, and adenine nucleosides by high-pressure liquid chromatography with electrochemical detection: C8-nitration and -oxidation. *Chem Res Toxicol* 2001; **14**: 438–450.
23 Stoyanovsky DA, Tyurina YY, Tyurin VA, *et al*. Thioredoxin and lipoic acid catalyze the denitrosation of low molecular weight and protein *S*-nitrosothiols. *J Am Chem Soc* 2005; **127**: 15815–15823.
24 Totter JR. Spontaneous cancer and its possible relationship to oxygen metabolism. *Proc Natl Acad Sci U S A* 1980; **77**: 1763–1767.
25 Cryer B, Feldman M. Cyclooxygenase-1 and cyclooxygenase-2 selectivity of widely used nonsteroidal anti-inflammatory drugs. *Am J Med* 1998; **104**: 413–421.
26 Menassé R, Hedwall PR, Kraetz J, *et al*. Pharmacological properties of diclofenac sodium and its metabolites. *Scand J Rheumatol Suppl* 1978; **22**: 5–16.
27 Tsurumi K, Hiramatsu Y, Yamaguchi A, Hayashi M, Shibuya T. Anti-inflammatory action of N-(2,6-dichlorophenyl)-o-aminophenylacetic acid, its sodium salt, N-(2,6-dichlorophenyl)-anthranilic acid and its sodium salt. 2. On subacute inflammation. *Nihon Yakurigaku Zasshi* 1973; **69**: 319–334.
28 Stacher G, Steinringer H, Schneider S, Mittelbach G, Winklehner S, Gaupmann G. Experimental pain induced by electrical and thermal stimulation of the skin in healthy man: sensitivity to 75 and 150 mg diclofenac sodium in comparison with 60 mg codeine and placebo. *Br J Clin Pharmacol* 1986; **21**: 35–43.
29 Somerville K, Faulkner G, Langman M. Non-steroidal anti-inflammatory drugs and bleeding peptic ulcer. *Lancet* 1986; **1**: 462–464.
30 Langman MJS. Peptic ulcer complications and the use of non-aspirin non-steroidal anti-inflammatory drugs. *Adverse Drug Reaction Bulletin* 1986; **120**: 448–451.
31 Savage RL, Moller PW, Ballantyne CL, Wells JE. Variation in the risk of peptic ulcer complications with nonsteroidal antiinflammatory drug therapy. *Arthritis Rheum* 1993; **36**: 84–90.
32 Ungprasert P, Cheungpasitporn W, Crowson CS, Matteson EL. Individual non-steroidal anti-inflammatory drugs and risk of acute kidney injury: a systematic review and meta-analysis of observational studies. *Eur J Intern Med* 2015; **26**: 285–291.
33 Ng LE, Vincent AS, Halliwell B, Wong KP. Action of diclofenac on kidney mitochondria and cells. *Biochem Biophys Res Commun* 2006; **348**: 494–500.
34 Becker L, Eberlein-König B, Przybilla B. Phototoxicity of non-steroidal anti-inflammatory drugs: *in vitro* studies with visible light. *Acta Derm Venereol* 1996; **76**: 337–340.
35 Encinas S, Bosca F, Miranda MA. Phototoxicity associated with diclofenac: a photophysical, photochemical, and photobiological study on the drug and its photoproducts. *Chem Res Toxicol* 1998; **11**: 946–952.
36 Kawaguchi K, Nakano T, Kimura K. How do you use liquid chromatography-nuclear magnetic resonance (LC-NMR) effectively for structural determinations of pharmaceutical impurities and metabolites? *Chromatography* 2011; **32**: 171–179.
37 Miura T. Direction of strategic use: a new classification of non-steroidal anti-inflammatory drugs based on reactivity with peroxidase. *Yakugaku Zasshi* 2013; **133**: 681–689.
38 Muraoka S, Miura T. Metabolism of non-steroidal anti-inflammatory drugs by peroxide: implication for gastrointestinal mucosal lesions. *Yakugaku Zasshi* 2007; **127**: 749–756.
39 Bunting S, Moncada S, Vane JR. The prostacyclin--thromboxane A2 balance: pathophysiological and therapeutic implications. *Br Med Bull* 1983; **39**: 271–276.
40 Sanak M, Simon HU, Szczeklik A. Leukotriene C4 synthase promoter polymorphism and risk of aspirin-induced asthma. *Lancet* 1997; **350**: 1599–1600.
41 Higashi N, Taniguchi M, Mita H, Higashi A, Akiyama K. Aspirin-induced urticaria and angioedema, but not bronchoconstriction, associated with cysteinyl leukotriene overproduction in 2 patients with asthma. *J Allergy Clin Immunol* 2002; **110**: 666–667.