

Development of Estimation Methods of Skin Oxidation and Evaluation of Anti-Oxidative Effects of Genistein in Topical Formulations

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The objective of the present study was to establish the method of measurement of hydrogen peroxide and to estimate the anti-oxidative effect of genistein in the skin. UVB induced skin oxidation and anti-oxidative effect of genistein formulations were evaluated by determining levels of hydrogen peroxide. The mechanism involved in the determination of hydrogen peroxide is based on a color reaction between ferric ion (Fe^{3+}) and xylenol orange, often called FOX assay and subsequent monitoring of absorbance values of the reactant at 540 nm. The reaction was to some extent pH-dependent and detection sensitivity was greatest at pH 1.75. Genistein liposomal gel demonstrated better anti-oxidative effect with regard to lowering hydrogen peroxide levels elevated by UVB irradiation compared to genistein-suspended gel. A linear relationship has been observed between anti-oxidative effect of genistein and drug deposition in the skin tissue. Genistein liposomal gel resulting in the localization of the drug in the deeper skin led to improved anti-oxidative effect compared to genistein gel. The suggested method for evaluation of oxidation of the skin can be used as a tool to screen effective anti-oxidative agents and their delivery systems acting on the skin.

Key Words: Oxidation, Skin, Hydrogen peroxide, Genistein

INTRODUCTION

Isoflavonoids are widely distributed in soybean and the family of Leguminosae. The isoflavones such as genistein and daidzein are the major isoflavonoids of soybean, which occur in the form of mainly genistin and daidzin, respectively. The content and composition of isoflavonoids are varied greatly according to the types of plants. Genistein has received considerable attention because epidemiologic studies have shown that consumption of soybean-containing diets was associated with a lower incidence of certain human cancers in Asian populations [1]. *In vitro* studies further showed that such chemopreventive and antineoplastic effects were related to the anti-oxidative activity of genistein and inhibition activities on cell proliferation and angiogenesis [2-5]. Oxidative stress preventive effect of isoflavonoids is related to their phenolic structures.

It has been also demonstrated that the isoflavone significantly inhibits ultraviolet (UV) light-induced oxidative

DNA damage. Solar ultraviolet (UV) light, particularly UVB and UVA are known to produce reactive oxygen species (ROS) such as superoxide, hydroxyl radicals, hydrogen peroxide, and singlet oxygen in the cells and skin which seriously damage DNA, leading to gene mutation and abnormal cell proliferation. ROS are frequently involved in the activation of procarcinogens, for example the activation of polycyclic aromatic hydrocarbons. One of ROS, hydrogen peroxide may contribute to promote tumor progression [6].

A topical sunscreen formulation has been widely used to reduce or prevent UV-induced skin damages. Additionally, topical formulations comprising compounds with anti-oxidative activity may provide significant protection from oxidative damage to the skin. For instance, skin rejuvenation could be observed with anti-oxidative compounds *via* stimulation of synthesis of hyaluronic acid [7]. The present work was designed to examine the possibility of the prevention of UV irradiation induced oxidation of the skin by genistein formulated into topical gels. To test anti-oxidative effect of genistein in the skin we developed the method of evaluation of skin oxidation by monitoring levels of hydrogen peroxide. Also, genistein was topically administered as gel and liposomal gel formulations to study the effect of skin delivery efficiency on the anti-oxidative activity of genistein.

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ABBREVIATIONS: UV, ultraviolet; ROS, reactive oxygen species; HPLC, high performance liquid chromatography.

METHODS

Materials

Genistein (purity >94%) was provided by Rexgene Biotech Co., Ltd. (Seoul, Korea). Soya phosphatidyl choline and xylenol orange were purchased from Sigma Chemical Co. (St. Louis, MO, USA). D-Sorbitol was purchased from Duksan Pure Chemical Company (Gyeonggi-do, Korea). Ammonium ferrous sulfate was purchased from Junsei Chemical Co. (Tokyo, Japan). Pluronic F127 (poloxamer 407) was purchased from BASF Co., Ltd. (Seoul, Korea). All other chemicals and reagents were purchased from commercial sources, and were of analytical grade. Doubly distilled water was used for all experiments. Female hairless mice aged 6 weeks old were purchased from Orient Bio Inc. (Gyeonggi-do, Korea). The mice were fed the commercial diet and tap water *ad libitum*, housed under constant environment in the animal facility of Chung-Ang University.

Preparation of liposomal gel formulation of genistein

Liposomes were composed of soya phosphatidyl choline and prepared by conventional rotary evaporation and extrusion method. Genistein and lipid were dissolved in the mixture of methanol and chloroform (1 : 1). Solvents were later removed by rotary vacuum evaporation (Rotary Evaporator, Ambala, India). Lipid films were then hydrated with distilled water by gently vortex mixing. Then, the hydrated lipid film was sonicated for 50 sec and sonication was repeated 5 times. Finally, manual extrusion was carried out 9 times through a 200 nm polycarbonate membrane (Millipore, USA). Genistein concentration in the liposomal solution was 1.6 ± 0.2 mg/ml when 30 mM of lipid and 15 w/v% of genistein were used. Separately, a poloxamer 407 gel was prepared by simply solubilizing poloxamer in water (40%) at 4°C. Then, genistein liposomal gel was produced by mixing the poloxamer gel and genistein loaded liposomes (1 : 1).

Preparation of genistein suspended gel

Genistein was suspended in water (i.e., genistein suspension) and this was used in the preparation of the poloxamer gel at 4°C.

In vivo UVB induced skin oxidation and anti-oxidative effect of genistein formulations

Hairless mice were randomly grouped with four mice in each group. Genistein formulated into gel and liposomal gel was applied on the dorsal skin and the amount of the drug applied was 2.7 mg as genistein. Then, 1 hour after the drug treatment, UVB rays were irradiated to the mice for 250 seconds. The distance between the UV lamp (306 nm, 20 W) and the dorsal skin of mice was approximately 40 cm. Mice were exposed to three times every other day UVB of 15 kJ/m^2 [8]. Mice were sacrificed 1 hour after the last UV exposure and full thickness dorsal skins were excised (2 cm in diameter) and analyzed for skin oxidation and anti-oxidative effect of genistein formulations.

Determination of levels of hydrogen peroxide in hairless mice skin

Analysis method to quantitate levels of hydrogen peroxide in the skin was developed as an index of skin oxidation after slight modification of previously reported procedures elsewhere [8-15]. The excised skin was mixed with salt solution (pH 7.4) containing 137 mM NaCl, 5 mM KCl, 8.5 mM Na_2HPO_4 , 0.8 mM MgCl_2 , 5 mM glucose, and 5 mM NaN_3 . The skin mixture was minced with a homogenizer (10,000 rpm, 40 sec, 5 times). The sample was incubated at 37°C for 2 hours. After incubation, tissue debris was removed by centrifugation at 3,000 rpm for 3 min. The supernatant was placed in glass tube and mixed with 6 ml of acetonitrile to deproteinize the sample, followed by centrifugation again at 3,000 rpm for 10 min. After this, a 100 μl of the supernatant was taken and mixed with 900 μl of the reaction solution containing 250 μM ammonium ferrous sulfate, 100 μM xylenol orange and 100 μM sorbitol in 25 mM H_2SO_4 . Then, it was kept at room temperature for 30 min. The absorbance of each sample at 540 nm was measured by a spectrophotometer and concentrations of hydrogen peroxide were calculated using the standard curve and the concentration was expressed as μM of hydrogen peroxide per cm^2 of skin. The pH value of the reaction solution was 1.75 and this value was selected following the evaluation of the effect of pH of the reaction solution on the absorbance value at 540 nm.

Skin deposition of genistein

Genistein formulations were applied on hairless mouse dorsal skin and the animals were left for 3 hours to allow the drug to be absorbed transdermally. The hairless mice were sacrificed and the dorsal skins were excised into a round shape (2 cm in diameter). The dorsal skin surface was washed five times with phosphate buffered saline to remove attached drug on the surface. And then, dorsal skin surface was cut into small pieces. The tissue was homogenized with 5 ml of methanol solution for 5 min. After extraction of genistein with methanol for 12 hours, followed by centrifugation for 10 min at 3,000 rpm, the amount of genistein was determined by HPLC [16].

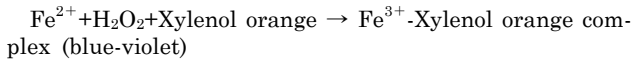
Statistical analysis

All reported data are mean \pm SD. Statistical significance was checked by Student's t-test and $p < 0.05$ was considered to be significantly different unless otherwise indicated.

RESULTS AND DISCUSSION

Development of assay method of hydrogen peroxide in the skin tissue

Prior to study on anti-oxidative effect of genistein formulated into poloxamer gel and poloxamer liposomal gel, the analytical method which quantitates the amount of hydrogen peroxide, an indicator of skin oxidation, in the hairless mouse skin was needed. The mechanism involved in the determination of hydrogen peroxide is based on a color reaction between ferric ion (Fe^{3+}) and xylenol orange, often called FOX assay as summarized in below [8-15].



Hydrogen peroxide which is index of skin oxidation was extracted from the skin. Ferrous ion (Fe^{2+}) is oxidized to form ferric ion (Fe^{3+}) by hydrogen peroxide. This ferric ion (Fe^{3+}) is then reacted with xylenol orange and thereby blue-violet color is produced in a concentration of hydrogen peroxide dependent manner. The produced color is monitored at 540 nm.

It is our observation that the reported method did not provide sensitivity enough to measure changes in the levels of hydrogen peroxide before and after UV exposure as well as changes made by genistein formulations. Moreover, the reported method uses trichloroacetic acid as a deproteinizing solvent but it interfered with the sample at suggested absorbance, 540 nm. As can be seen in Fig. 1, various concentrations of trichloroacetic acid showed absorbance values at 540 nm, probably disturbing the measurement of hydrogen peroxide. However, acetonitrile did not exhibit any considerable absorbance values at 50 and 100% concentrations. Acetonitrile (100%) was however selected as a suitable solvent for the precipitation of protein components in the supernatant of skin tissue homogenates to increase precipitation efficiency and to maintain the hydrogen peroxide concentration as high as possible.

To further improve the sensitivity of spectroscopic measurement of hydrogen peroxide we examined the effect of pH of the reaction solution at different pH values such as pH 1.5, 1.75 and 2.5. Fig. 2 shows the relationship between concentration of hydrogen peroxide and absorbance values measured at 540 nm. The absorbance values were greatest

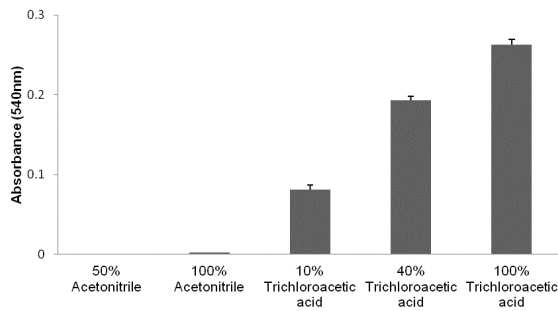


Fig. 1. Influence of deproteinization solvents in absorbance of reaction solution at 540 nm. Mean±SD (n=3).

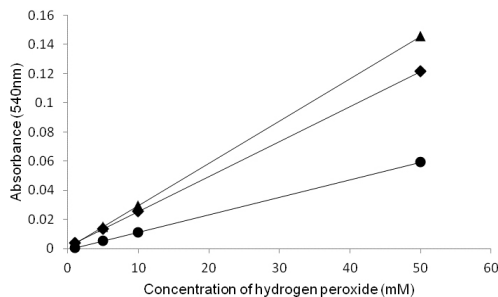


Fig. 2. Calibration curve of hydrogen peroxide constructed in different pH conditions. ●, pH 1.5; ◆, pH 2.5; ▲, pH 1.75.

at the same hydrogen peroxide concentrations when the pH of the reaction solution was adjusted to be at pH 1.75.

Evaluation of anti-oxidative property of genistein

Exposure of the skin to UV generates reactive oxygen species (ROS) such as hydrogen peroxide [17] and causes adverse effects by oxidative stress such as DNA damage [18] and suppression of the immune system of the skin [19]. In Fig. 3, concentration of hydrogen peroxide increased after UVB irradiation. This means UVB ray induced the oxidative stress in the skin. When genistein was topically administered to the skin, it led to a decrease in levels of hydrogen peroxide in the skin. This inhibitory activity of genistein may be attributed by the possible mechanism proposed by Wei et al. [8]. Topically administered genistein could possibly be transported across the skin and depending on the formulation employed the skin transport of genistein could be changed. The transdermally absorbed genistein was distributed mainly in the epidermal and dermal tissues and thereby, the drug intervened directly on the oxidation process of the skin caused by UVB irradiation. In another study, topical administration of 10 micromoles of genistein to mouse dorsal skin substantially inhibited UVB-induced hydrogen peroxide production by 40~50% [11]. The formulation used in this study also influenced the anti-oxidative efficacy of genistein. As illustrated in Fig. 3 the inhibition effect of genistein in liposomal gel formulation was greater than that obtained with genistein suspended gel. The main reason for this may be the difference in the permeation efficiency between the formulations tested. However there was no statistical difference in decreasing levels of hydrogen peroxide between two formulations.

Skin deposition of genistein

Basically, gel formulations were used in the in vivo anti-oxidative studies since this type of dosage form can easily be applied on the skin with limited dosing areas. Fig. 4 demonstrates the effect of gel formulations on the skin deposition of genistein. More drug was retained in the skin when the drug was administered in the liposomal gel. Liposomes have shown numerous to increase drug permeation across the skin tissue and thus are well known permeation enhancers [20,21].

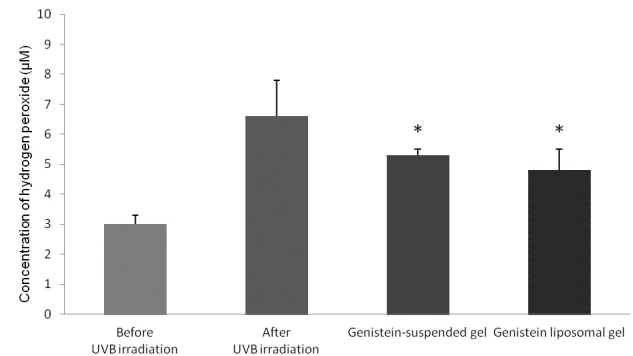


Fig. 3. Levels of hydrogen peroxide in the hairless mouse skin. Genistein from genistein-suspended gel and liposomal gel significantly reduced the increased concentration of hydrogen peroxide caused by UVB irradiation (p<0.05). Mean±SD (n=3).

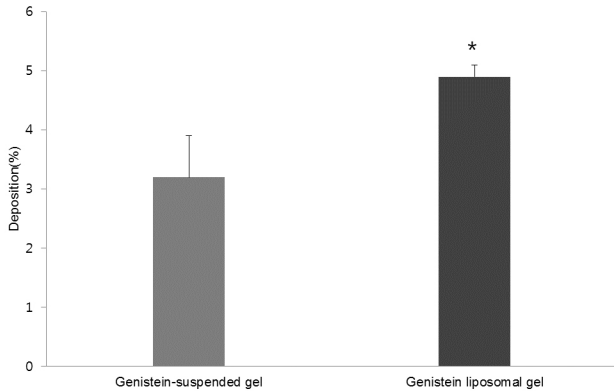


Fig. 4. Percentages of genistein retained in the hairless mouse dorsal skin. Statistically more genistein was retained in the skin from liposomal gel, compared to genistein-suspended gel ($p < 0.05$). Mean \pm SD (n=3).

Correlation between skin deposition and anti-oxidative activity of genistein

Almost linear relationship between skin deposition and anti-oxidative activity of genistein from gel formulations was observed (Fig. 5). Activity values were obtained from differences between levels of hydrogen peroxide measured after UVB irradiation without drug treatment and levels of hydrogen peroxide measured after drug treatment three times. This result indicates that to obtain better anti-oxidative effect of genistein in the prevention of skin oxidation caused by UV irradiation the drug should be delivered to the deeper area of the skin. Since the drug was not solubilized in the gel formulation, drug permeation across the skin was considerably limited. However, genistein in liposomal gel was partly solubilized in the hydrophobic domain of the liposomal carriers. Thus, drug diffusion across the skin was easier from the liposomal gel than that from genistein suspended gel. It is, therefore, considered that drug carriers need to be carefully selected to fully utilize the anti-oxidative effect of genistein.

From the results obtained it was evident that intrinsic anti-oxidative activity of genistein was observed in the skin by delivering transdermally the drug. Increased levels of hydrogen peroxide by UVB irradiation to the skin was reduced by genistein applied to the skin in the form of drug-suspended gel and liposomal gel. The anti-oxidative effect was changed according to the delivery carrier. Liposomal gel was found to be efficient carrier for genistein which led to greater anti-oxidative effect in the prevention of skin oxidation caused by UVB irradiation. There was a linear relationship between drug deposition and anti-oxidative effect of genistein confirming that drug delivery efficiency is the major determinant to exhibit anti-oxidative effect of the drug in the skin.

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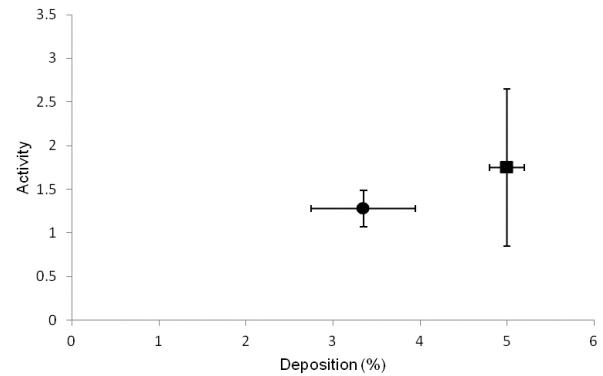


Fig. 5. Correlation between skin deposition and anti-oxidative activity of genistein from formulations. Activity values are differences between levels of hydrogen peroxide measured after UVB irradiation without drug treatment and levels of hydrogen peroxide measured after drug treatment three times. ●, Genistein-suspended gel; ■, Genistein liposomal gel. Mean \pm SD (n=3).

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