FIGURE 1:

F

F

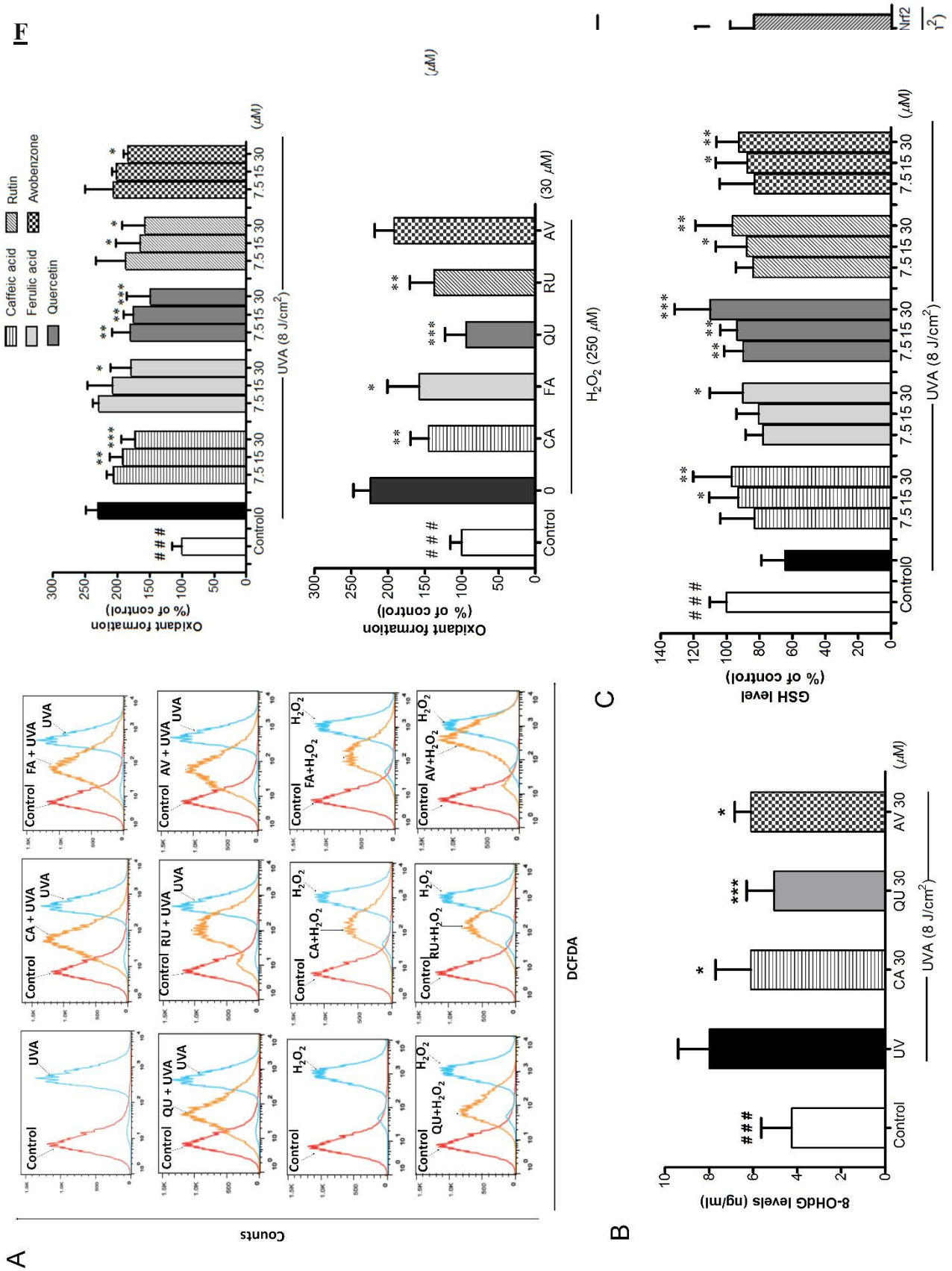
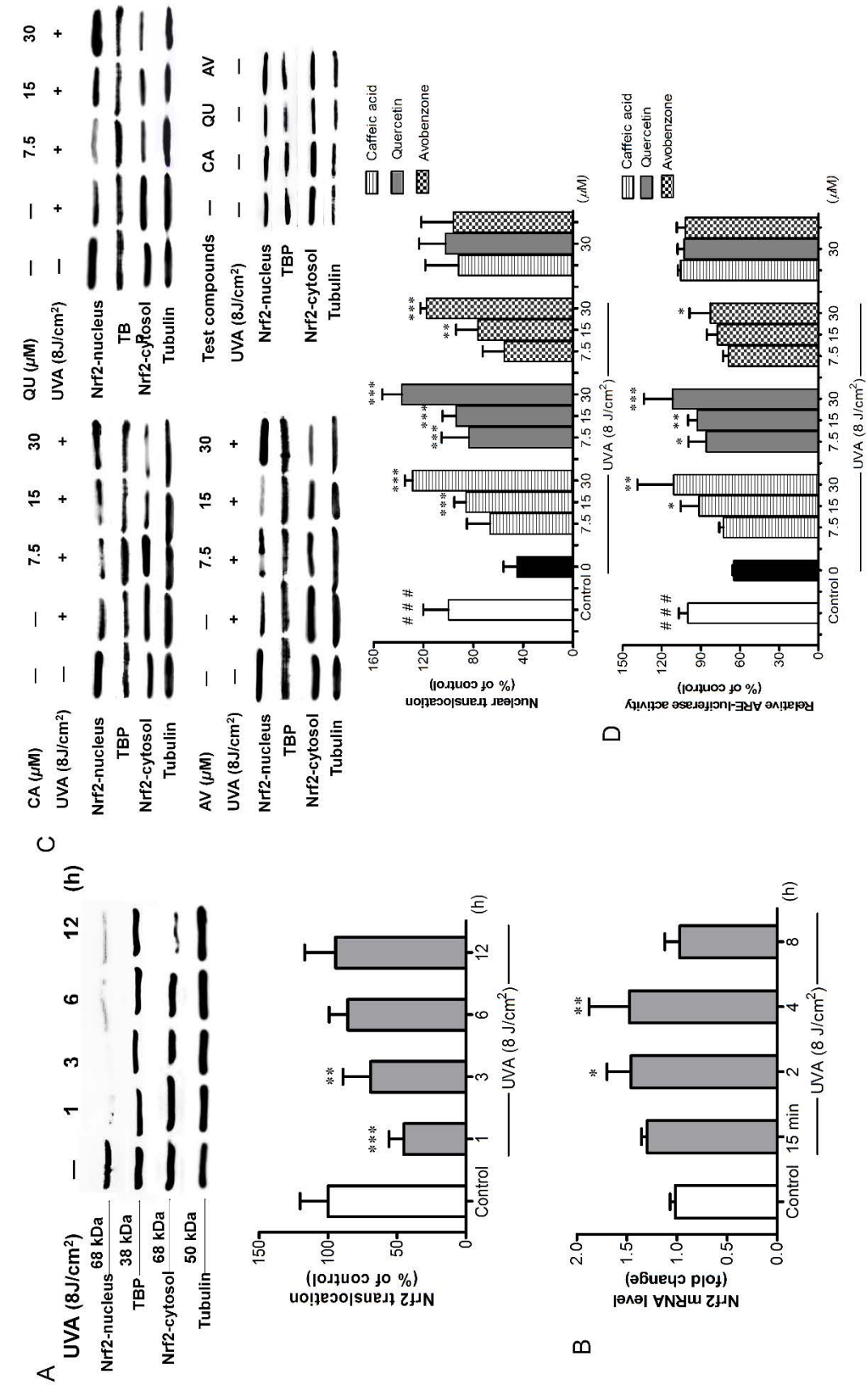
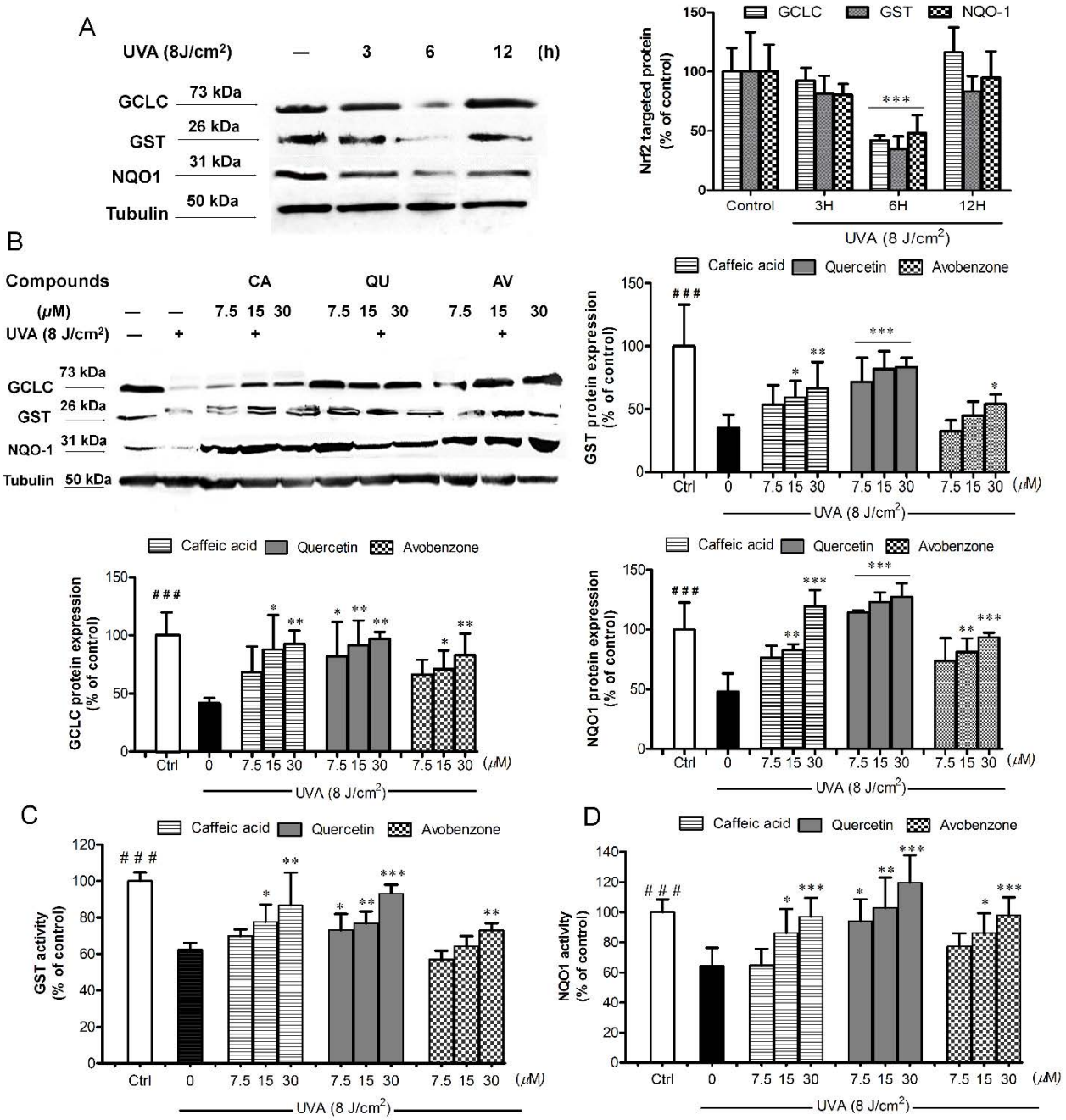

A
Counts
DCFDA
Control
UVA
CA + UVA
FA + UVA
QU + UVA
RU + UVA
AV + UVA
H2O2
CA+H2O2
FA+H2O2
QU+H2O2
RU+H2O2
AV+H2O2
Caffeic acid
Ferulic acid
Quercetin
Rutin
Avobenzone
Oxidant formation (% of control)
UVA (8 J/cm2)
H2O2 (250 μM)
B
8-OHdG levels (ng/ml)
GSH level (% of control)
C
Control
UV
CA 30
QU 30
AV 30
Control0
Nrf2

FIGURE 4:


A
UVA (8J/cm²)
Nrf2-nucleus
68 kDa
TBP
38 kDa
Nrf2-cytosol
68 kDa
Tubulin
50 kDa
— 1 3 6 12 (h)
B
Nrf2 mRNA level (fold change)
Control 15 min 2 4 8 (h)
UVA (8 J/cm²)
Nrf2 translocation (% of control)
Control 1 3 6 12 (h)
UVA (8 J/cm²)
C
CA (μM) — — 7.5 15 30
UVA (8J/cm²) — + + + +
Nrf2-nucleus
TBP
Nrf2-cytosol
Tubulin
AV (μM) — — 7.5 15 30
UVA (8J/cm²) — + + + +
Nrf2-nucleus
TBP
Nrf2-cytosol
Tubulin
QU (μM) — — 7.5 15 30
UVA (8J/cm²) — + + + +
Nrf2-nucleus
TB
Nrf2-cytosol
Tubulin
Test compounds — CA QU AV
UVA (8J/cm²) — — — —
Nrf2-nucleus
TBP
Nrf2-cytosol
Tubulin
Caffeic acid
Quercetin
Avobenzone
Nuclear translocation (% of control)
Control 0 7.5 15 30 7.5 15 30 7.5 15 30 (μM)
UVA (8 J/cm²)
D
Caffeic acid
Quercetin
Avobenzone
Relative ARE-luciferase activity (% of control)
Control 0 7.5 15 30 7.5 15 30 7.5 15 30 (μM)
UVA (8 J/cm²)


A
UVA (8J/cm²)
—
3
6
12
(h)
GCLC
73 kDa
GST
26 kDa
NQO1
31 kDa
Tubulin
50 kDa
GCLC
GST
NQO-1
Nrf2 targeted protein (% of control)
Control
3H
6H
12H
UVA (8 J/cm²)
B
Compounds
CA
QU
AV
(μM)
UVA (8 J/cm²)
GCLC
GST
NQO-1
Tubulin
Caffeic acid
Quercetin
Avobenzone
GST protein expression (% of control)
GCLC protein expression (% of control)
NQO1 protein expression (% of control)
Ctrl
0
7.5 15 30
(μM)
C
D
GST activity (% of control)
NQO1 activity (% of control)

HIGHLIGHTS

- Depletion of Nrf2 could stimulate melanogenesis under UVA-mediated oxidative stress.
- A single dose of UVA irradiation resulted in time-dependent alterations of Nrf2 nuclear accumulation and its target antioxidant proteins including GCLC, GST and NQO1 B16F10 cells.
- Antioxidant and UVA blocking compounds could effectively provide an early protection against UVA-induced melanogenesis in correlation with their antioxidant potentials through indirect regulatory effect on Nrf2-ARE pathway.
- To avoid excessive activation of Nrf2, which could harm the cells, indirect modulation of Nrf2-ARE pathway to promote redox balance by photoprotective compounds with antioxidant or sunscreen properties may provide a pharmacological insight for protection against photooxidative damage and hyperpigmentation.

Figure 1

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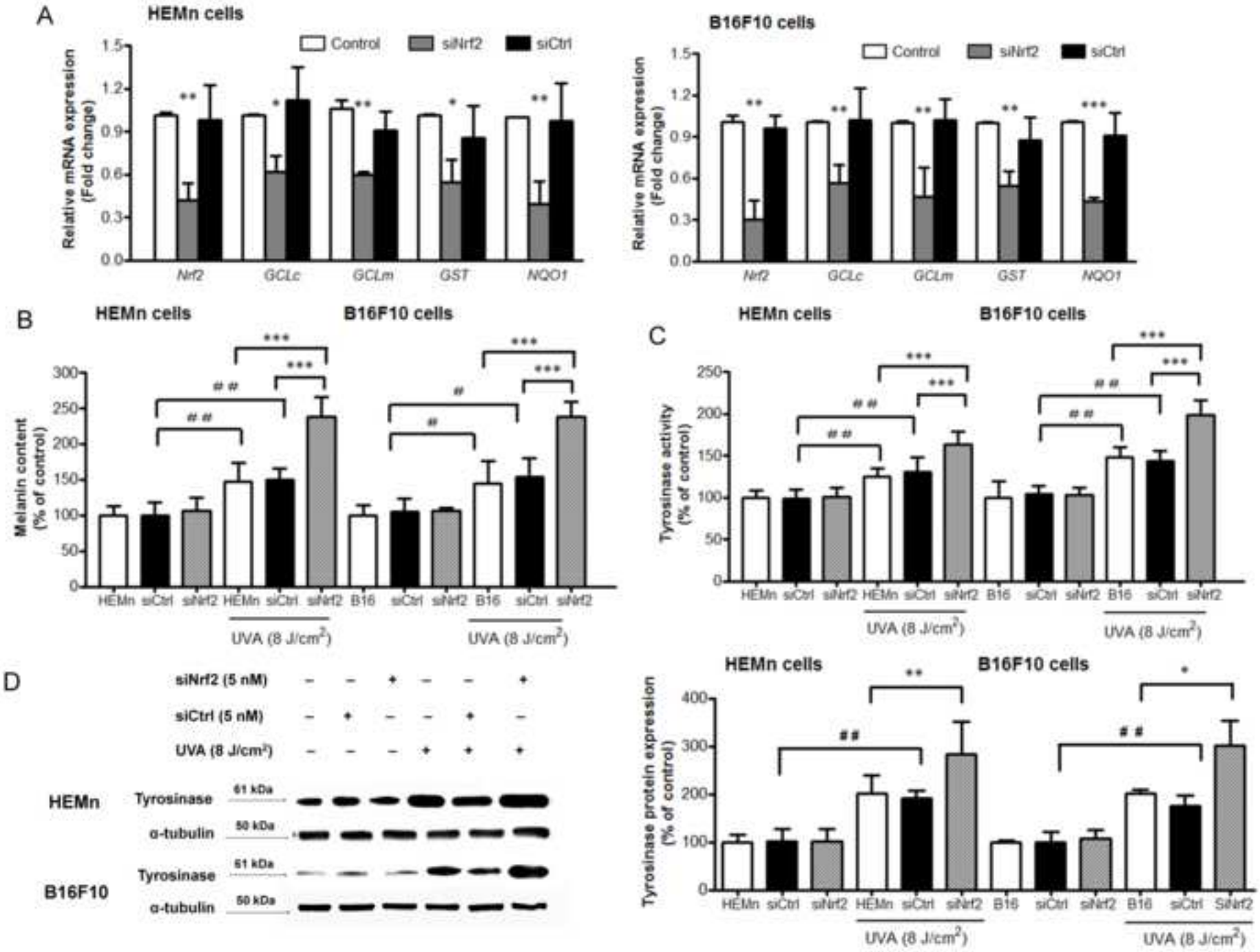

Figure 2

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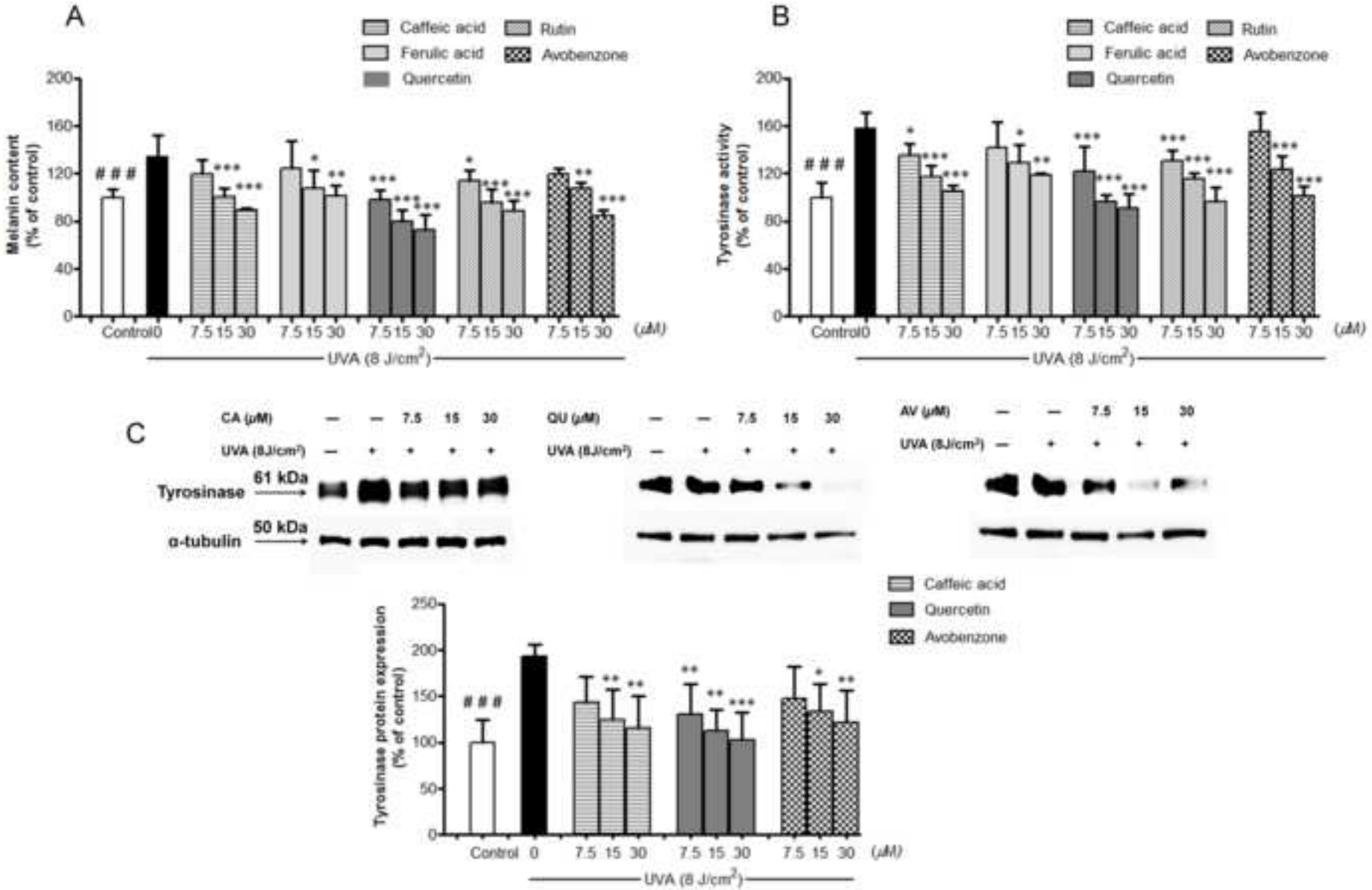

Figure 3

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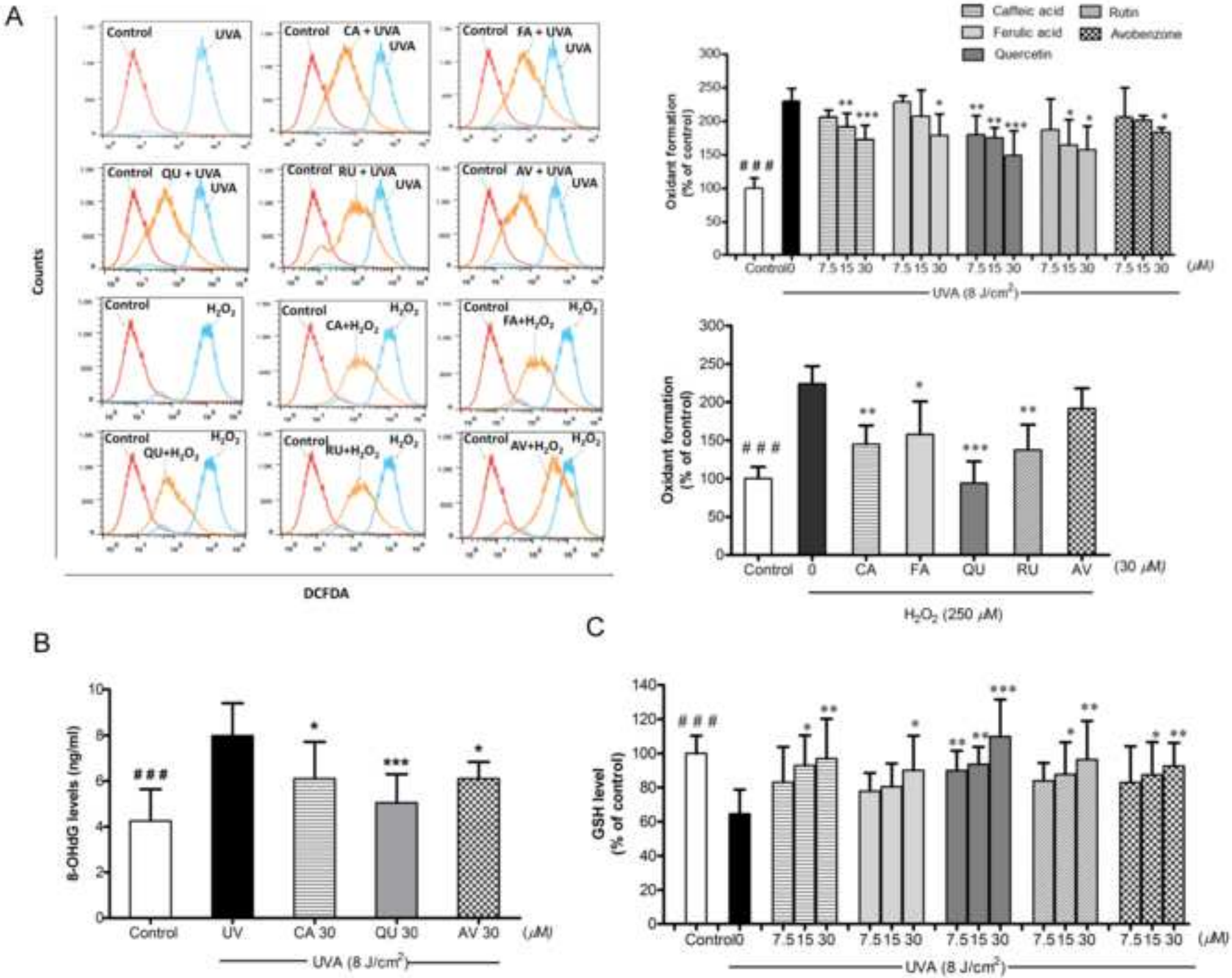

Figure 4

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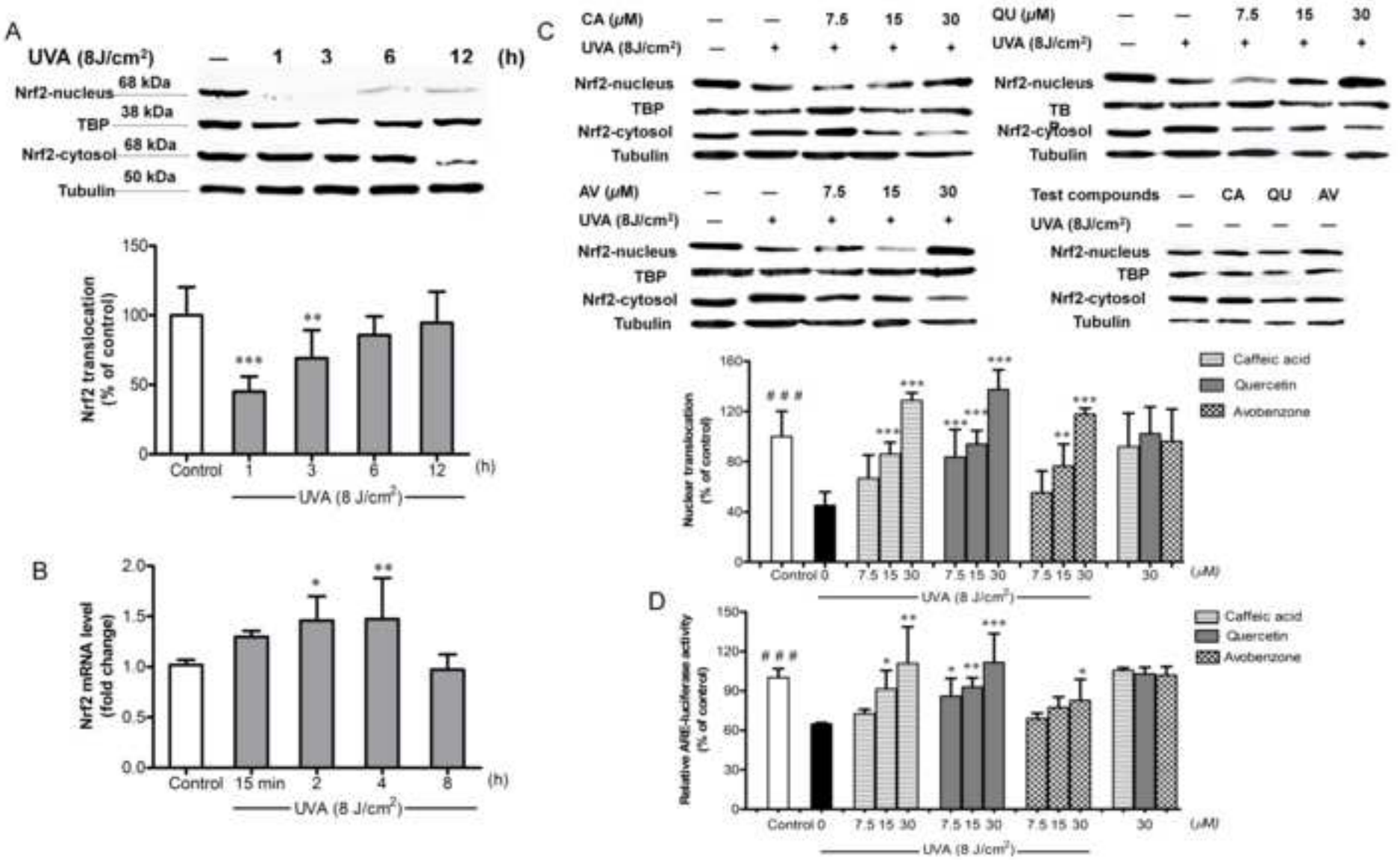

Figure 5

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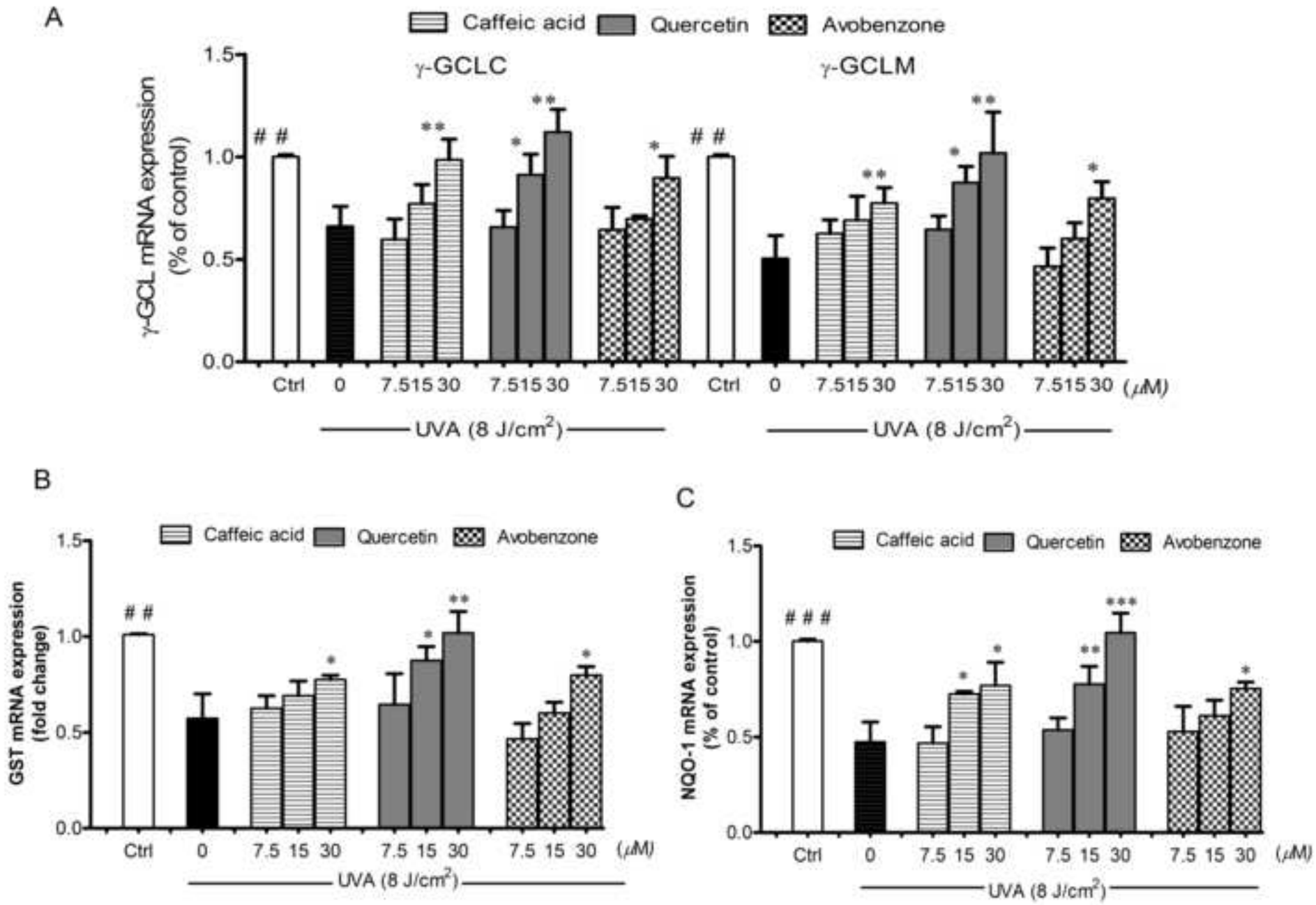

Figure 6

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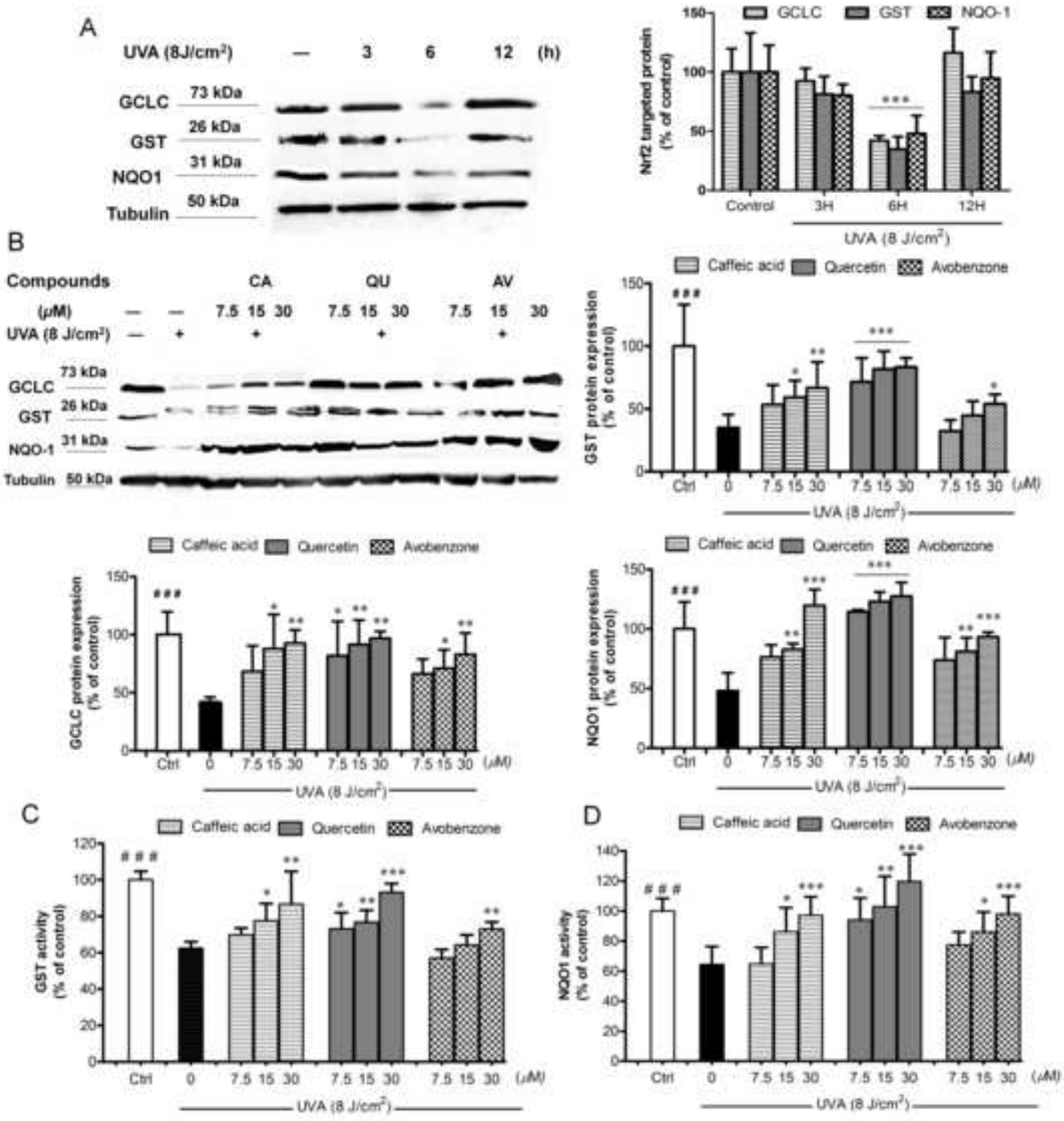

Involvement of oxidative DNA damage and alteration of antioxidant defense system in patients with basal cell carcinoma: a case-control study

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Running title: A case-control study on redox status in basal cell carcinoma

Keywords: oxidative stress, basal cell carcinoma (BCC), oxidative DNA damage, antioxidant defense

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Disclosure of Potential Conflicts of Interest: No potential conflicts of interest were disclosed.

Total number of figures: 3

Total number of tables: 3

Abstract

Background: Oxidative damage has been suggested to play a role in the pathogenesis of basal cell carcinoma (BCC).

Methods: This study illustrated an involvement of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage, and changes in antioxidant defenses including catalase (CAT), glutathione peroxidase (GPx), NAD(P)H: quinone oxidoreductase 1 (NQO1), superoxide dismutase (SOD) activities and glutathione (GSH) in controls and BCC patients pre-surgery and 1-month post-surgery. 8-OHdG levels as well as protein and mRNA expressions of DNA repair enzyme hOGG1 and antioxidant defenses (CAT, glutamate-cysteine ligase (GCL), GPx, Nrf2, MnSOD) in skin tissues of control and BCC subjects were also determined.

Results: This study observed an induction in urinary 8-OHdG levels, increased 8-OHdG expression and reduced hOGG1 protein and mRNA in BCC tissues, decreased antioxidant enzyme activities but elevated SOD activities and GSH levels in BCC patients compared to controls and reduction of all antioxidant proteins and genes studied in BCC tissues. Furthermore, CAT, GPx and NQO1 activities in BCC patients were increased at 1 month and and 8-OHdG levels in BCC patients declined at 6 months post-operation compared with preoperative levels.

Conclusions: We reported for the first time that BCC patients were associated with oxidative DNA damage, defect in hOGG1 and depletion of antioxidant defenses. Surgical removal of BCC tissues correlated with improved oxidative damage.

Impact: Our case-control study identifies that oxidative DNA damage and changes in redox status were associated with BCC and may serve as oxidative stress biomarkers for prediction of BCC development and progression.

Introduction

Basal cell carcinoma (BCC) is the most common non-melanoma skin cancers (NMSC) worldwide, in particular, in fair-skinned population and its incidence has been rising over the past several years (1-3). Although BCC can be generally diagnosed and treated by surgical excision, it can be destructive and has a significance impact on patients' quality of life as the tumor removal can cause morbidity including functional disability and disfigurement. Therefore, it is of significance to understand pathogenesis of BCC in order to develop effective strategies for its detection and prevention. Photo-damage by chronic exposure to ultraviolet radiation (UVR) has been suggested to play a role in the pathogenesis of BCC, which originates from keratinocytes of the epidermal basal layer (4, 5).

Excessive reactive oxygen species (ROS) generated by UVR have been shown to contribute to malignant transformation of keratinocytes into cancerous cells including BCC probably through oxidative DNA damage, defects in DNA repair and interference with cellular signaling (6, 7). The modification of oxidative DNA base at guanine, 8-hydroxy-2'-deoxyguanosine (8-OHdG) pairs between adenine and cytosine during DNA replication, resulting in GC-TA mutations has been found to be associated with development of BCC (8). 8-OHdG has also been recognized to be the most abundant and potentially mutagenic if not substantially repaired and has thus been developed as a sensitive and stable biomarker for evaluating the degree of oxidative DNA damage. Case-control studies have previously reported that increased urinary 8-OHdG levels were detected in patients with metastatic head and neck cancer, breast cancer and lung cancer (9, 10). Furthermore, impaired DNA repair capacity was suggested to associate with enhanced susceptibility to cancer and deficiency in DNA repair enzyme, human 8-oxoguanine DNA N-glycosylase 1 (hOGG1), a key enzyme responsible for the base excision repair of 8-OHdG, may also be involved in carcinogenesis (11).

It has been proposed that promotion of antioxidant defenses including catalase (CAT), glutathione peroxidase (GPx), NAD(P)H: quinone oxidoreductase 1 (NQO1), a crucial detoxifying enzyme and superoxide dismutase (SOD) that can cope with excessive ROS formation in human body and redox regulation of nuclear factor-erythroid-2-related factor 2 (Nrf2) transcription factor may be useful to prevent ROS-mediated neoplastic transformation of various tissues including the skin (12, 13). A link between increased oxidative DNA damage/decreased antioxidant defense capacity and cancer has been proposed and oxidative/antioxidant defense status has thus been intensively investigated as promising biomarkers in cancer (14-16). However, relationship between the oxidative DNA damage/antioxidant defense parameters and BCC and whether surgical removal of tumors affects the redox status in BCC patients have not yet been reported. Here, we conducted a case-control study to investigate whether BCC was associated with the oxidative DNA damage, hOGG1 levels and antioxidant defense status and whether tumor removal affected the redox status of patients with BCC compared to control subjects with non-malignant skin diseases.

Materials and Methods

Study Population

This case-control study involving 37 Thai subjects (mean age, 66 years; range, 39-87 years, 16 males and 21 females) was approved by the ethics committee of the Siriraj Institutional Review Board (SIRB), Faculty of Medicine Siriraj Hospital, Mahidol University, and written informed consent was obtained by all participants. Case group comprising 17 patients newly diagnosed with BCC (mean age, 67 years; range, 41–87 years, 5 males and 12 females) and control group comprising 20 patients with non-malignant skin diseases (mean age, 66 years; range, 39-82 years, 11 males and 9 females) were recruited from the outpatient clinic of the Department of Dermatology, Faculty of Medicine, Siriraj Hospital from 2011 to 2014 and every diagnosis was confirmed by a pathologist. Twenty control subjects diagnosed with non-malignant skin diseases were patients with 7 dermatitis (5 males and 2 females), 2 fibroepithelial polyp (2 males), 1 melanocytic nevus (1 female), 4 normal skin (4 females), 6 seborrheic keratosis (4 males and 2 females).

Data Collection

Data were collected on demographics, clinical characteristics and lifestyle as shown in Table 1. All subjects underwent surgical intervention and the collection of blood, urine and skin tissue samples was done on surgery day prior to operation. At 1 month after surgery, blood samples were collected for evaluation of antioxidant defense parameters and the urinary 8-OHdG levels of BCC patients were determined at both 1 and 6 months after surgery.

Blood samples processing

Blood samples were collected in sodium fluoride tube, EDTA tube and lithium heparin tube for clinical chemistry and antioxidant assays. Whole blood was processed

according to standard protocols and centrifuged at 1,000 X g for 10 minutes at 4 °C and plasma samples were stored at -80 °C until testing.

Urine samples processing

Fresh urine samples were collected in urine container tube for clinical chemistry assays and for oxidative DNA damage assay. Urine samples were processed according to standard protocols and centrifuged at 3,000 X g for 10 minutes at 4 °C, and the supernatants were collected and stored at -80 °C until testing.

Tissue samples processing

The skin tissue samples were obtained from lesions of BCC and non-malignant skin diseases. Tissue samples were divided into 2 parts; the first part was fixed in formalin solution and then embedded in paraffin block for immunohistochemistry and the second part was fixed in liquid nitrogen for real-time RT-PCR until testing.

Determination of 8-OHdG levels in urine by ELISA

Urinary 8-OHdG level was quantified using a competitive enzyme immunoassay following the kit protocol (STA-320, Cell Biolabs, San Diego, CA). Briefly, urine samples or 8-OHdG standards were incubated with an 8-OHdG/BSA conjugate preabsorbed enzyme immunoassay plate and an anti-8-OHdG monoclonal antibody was then added, followed by a secondary reaction with a horseradish peroxidase-conjugated antibody. 8-OHdG levels in the urine of each subject were adjusted by urinary creatinine level and were measured as ng/g creatinine.

Determination of antioxidant defense status in plasma

Catalase (CAT) activity was determined following the kit protocol from Cayman chemical (Ann Arbor, MI). The assay was based on the reaction of methanol and the enzyme in the presence of an optimal concentration of H_2O_2. The formaldehyde produced was

measured colorimetrically at 540 nm using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. CAT activity was expressed in unit/mg protein.

Glutathione peroxidase (GPx) activity assay was performed following manufacturer's instruction (Trevigen, Gaithersburg, MD). GPx activity was coupled to glutathione reductase (GR), which catalyzed NADPH-mediated reduction of GSSG back to GSH as previously described (17). The rate of NADPH oxidation by H_2O_2 was monitored at 340 nm and GPx activity was expressed in unit/mg protein.

NQO1 activity was evaluated spectrophotometrically as previously described (18) using 2,6-dichloroindophenol (DCPIP) as a substrate. The assay was based on the activities for NAD(P)H-dependent reduction of DCPIP at 600 nm and the reaction was specifically inhibited by dicumarol. The NQO1 activity was thus measured as the dicumarol inhibitable reduction in absorbance at 600 nm and was expressed as nmole DCPIP reduced/min/mg protein.

Assay for measurement of total superoxide dismutase (SOD) activity was modified following the method of Johns *et al*. (19, 20). and the kit protocol from Cayman chemical (Ann Arbor, MI). Superoxide anions ($O_2^{\bullet-}$) were generated by a xanthine/xanthine oxidase (XOD) system and were detected using 3'-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzenesul-fonic acid (XTT) as chromogen. The SOD activity was determined by a decrease in XTT reaction rate at 470 nm as a result of superoxide produced by xanthine/XOD system. The SOD activity was calculated using the following equation: [(0.953) X ((Std.conc. 0 – blank)/(Sample – blank)) - 0.097) X Dilution factor]. Total SOD activity was expressed in unit/mg protein, where one unit of activity was the amount of protein required for a 50% decrease in the rate of XTT reduction.

GSH assay was carried out by glutathione reductase:DTNB enzymatic recycling method following the kit protocol from Sigma-Aldrich (MO, US). Determination of GSH

levels involves GSH oxidation by the sulfhydryl reagent DTNB (5,5′-dithio-bis-2-(nitrobenzoic acid) to produce the yellow TNB (5′-thio-2-nitrobenzoic acid) measured at 412 nm. The glutathione disulfide (GSSG) formed can be recycled to GSH by glutathione reductase in the presence of NADPH. The rate of TNB production is directly proportional to this recycling reaction in turn directly proportional to the concentration of GSH. The GSH level was expressed in nmol/mg protein.

Determination of protein content in plasma by Bradford assay

Protein concentration was measured using the Bio-Rad Protein Assay Kit (Bio-Rad, Munich, Germany) and bovine serum albumin (BSA) was used as protein standard.

Immunohistochemical (IHC) determination of oxidative DNA damage, antioxidant enzyme and hOGG1 expression in skin tissues

Paraffin-embedded tissues were sectioned (2 μm thickness) and placed on Super-FrostPlus glass slides fixed at 60 °C overnight. The sections were deparaffinized in xylene and rehydrated in ethanol series, then incubated in citrate Buffer, pH 6, (DaKo). Slides were incubated with 3% hydrogen peroxide and then with 2% BSA. Slides were incubated with primary antibodies against 8-OHdG (ab48508; Abcam, Cambridge, UK) (1:50), hOGG1 (NB100-106, Novus Biologicals, USA) (1:100), CAT (ab125688, Abcam, Cambridge, UK) (1:200), GCLC (ab53179; Abcam, Cambridge, UK) (1:100), GPx (ab108429, Abcam, Cambridge, UK) (1:50) and Nrf2 (ab31163, Abcam, Cambridge, UK) (1:50). The slides were then incubated with Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse for 30 min. Peroxidase activity was visualized with 3, 3-diamino-benzidine tetrahydrochloride (DAB) as the substrate and hematoxylin. All tissues were stained with hematoxylin-eosin (H&E) for detection of cell structure.

IHC staining of all samples was evaluated visually and scored by a pathologist twice in different days. If the IHC staining evaluated in duplicate gave different IHC scores, visual

interpretation of the IHC staining would be repeated. The semi-quantitative analysis of the stained sections was carried out by light-microscopy according to the immunoreactive score (IRS) by Kaemmerer *et al*. (21). The level of antibody staining was evaluated by IRS and calculated by multiplying the scores of staining intensity by the percentage of positive cells. Based on the IRS, antibody staining pattern was defined as IRS – classification score.

Quantitative real-time reverse transcription-polymerase chain reaction: determination of hOGG1, catalase, GCLC, GCLM, GPx, Nrf2, CuSOD, MnSOD

Improm-II™ reverse transcriptase (Promega, Medison, USA) was used to synthesize cDNA from total RNA following the manufacturer's protocol. Sequences for PCR primer sets of genes studied were designed using the Primer Express version 3.0 software (Applied Biosystems, USA). Sequences of PCR primer (in 5′-3′ direction) were as follows: hOGG1 sense (product sizes = 164), TGGAAGAACAGGGCGGGCTA, and antisense, ATGGACATCCACGGGCACAG; CAT sense (product sizes = 148), CCTTCGACCCAA GCAACATG, and antisense, CGAGCACGGTAGGGACAGTTC; GCLC sense (product sizes = 160 bp), GCTGTCTTGCAGGGAATGTT, and antisense, ACACACCTTCCTTCCCATTG; GCLM sense (product sizes = 200 bp), TTGGAGTTGCACAGCTGGATT, and antisense, TGGTTTTACCTGTGCCCACTG; GPx sense (product sizes = 94 bp), ACGATGTTGCCTGGAACTTT, and antisense, TCGATGTCAATGGTCTGGAA; Nrf2 sense (product sizes = 161 bp), TTCTGTTGCTCA GGTAGCCCCTCA, and antisense, GTTTGGCTTCTGGACTTGG; CuSOD sense (product size = 109 bp), TGCTGGTTTGCGTCGTAGTC, and antisense, ACGCACACGGCCTTCGT; MnSOD sense (product size = 141 bp), TGGCCAAGGGAGATGTTACAG, and antisense, CTTCCAGCAACTCCCCTTTG; GAPDH sense (product size = 150 bp), CCTCCAAAATCAAGTGGGGCGATG, antisense, CGAACATGGGGGCATCAGCAGA. Real-time PCR was performed using FastStart

universal SYBR Green Master with ROX (Roche diagnostic, USA) and mRNA expression was quantified by real-time PCR using ABI prism 7300 Real Time PCR System (Applied Biosystems, USA). Melt curve analysis was performed to verify specificity of the amplified product. mRNA expression was normalized to the expression of GAPDH gene. The mean Ct of each gene in each sample was compared with the mean Ct from GAPDH determinations from the same cDNA sample in order to assess mRNA expression. Ct values were then used to calculate fold change in gene expression.

Statistical Analysis

Descriptive statistics were reported as frequencies and percentage. Categorical variables were analyzed by the chi-square test and performed with SPSS 18.0 software (SPSS, Chicago, IL). Data were expressed as means ± standard deviation (SD). The results were subjected to statistical analysis using Prism 5.0 (GraphPad Software, La Jolla, CA, USA). The Shapiro-Wilk test was employed to test the normal distribution. The statistical significance between nonparametric variables was analyzed by Mann Whitney U-test or Kruskal-Wallis test and between parametric variables by unpaired student's t-test or one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. P-values less than 0.05 were considered statistically significant. To investigate systematically similarity of biochemical profiles, we performed the principal component analysis using JMP Pro. Data were then ploted using custom MATLAB code.

Results

Demographic, lifestyle and clinical characteristics of the study subjects

Table 1 showed that there were no differences in all characteristics between BCC patients and control subjects, indicating that demographics and clinical characteristics of the study subjects did not affect all parameters of oxidative DNA damage and antioxidant defense status studied.

The urinary 8-OHdG levels and antioxidant defense status in BCC and control subjects.

The urinary 8-OHdG levels and all antioxidant defense parameters studied in BCC and control subjects were shown in Table 2. Oxidative DNA damage was significantly higher in BCC patients compared to control subjects (Fig. 1). After surgery, urinary 8-OHdG levels of BCC patients were insignificantly altered at 1 month, although they were substantially reduced at 6 months compared to preoperative levels (Fig. 1A). In addition, while preoperative values of plasma CAT, GPx and NQO1 activities were observed to be lower in BCC patients compared to control (Fig. 1B-D), plasma total SOD activities and GSH levels were substantially higher in BCC patients compared to control (Fig. 1E-F). In comparison between preoperative and postoperative levels of plasma antioxidant defense status in BCC patients, CAT, GPx and NQO1 activities were increased at 1 month postoperatively, although total SOD activities and GSH levels remained unchanged.

In control subjects, urinary 8-OHdG levels and all antioxidant defense parameters studied were not significantly different between pre- and post-surgery.

We then used the principal component analysis to systematically investigate similarity of DNA damage and antioxidant defense parameters from different groups of patients and to identify co-variation among these parameters (Fig. 1G). Although the first and second principal components explain only 38.3% and 18.1% of the observed variance, we can confirm similar parameter profile between pre-surgery and 1-month post-surgery in the

control group. Patients with BCC, however, demonstrate distinct antioxidant defense parameters between pre- and 1-month post-surgery. Interestingly, the parameters from BCC group at 1-month post-surgery begin to look more similar to the control group, although roughly half of the patients in this group still exhibit unique profiles. This finding implies the inter-patient heterogeneity of antioxidant defense response.

IHC determination of oxidative DNA damage, antioxidant enzyme and hOGG1 expressions in skin tissues of BCC and control subjects

The IHC staining was classified as negative, weak positive, mild positive and strong positive and the data were presented as average IRS scores. H&E staining identified structures of skin sections of case and control subjects as shown in Fig. 2A-C. Table 3 demonstrated that expressions of nuclear 8-OHdG (Fig. 2D,E,G) were higher and hOGG1 protein (Fig. 2H,I,K) in nucleus and cytoplasm were lower in BCC tissues than those in the epidermis of control subjects. Additionally, protein expressions of CAT (Fig. 2L,M,O), GCLC (Fig. 2P,Q,S), GPx, (Fig. 2T,U,W), Nrf2 (Fig. 2X,Y,AA) and MnSOD (Fig. 2AB,AC,AE) were observed to be lower in BCC tissues compared to the epidermis of control subjects. Furthermore, the intra-subject comparison of BCC and adjacent epidermis demonstrated a higher expression of 8-OHdG (Fig. 2E,F,G) and a lower expression of hOGG1 (Fig. 2I,J,K), CAT (Fig. 2M,N,O), GCLC (Fig. 2Q,R,S), GPx (Fig. 2U,V,W), Nrf2 (Fig. 2Y,Z,AA) and MnSOD (Fig. 2AC,AD,AE) in BCC lesions compared to adjacent normal skin. In consistent with our studies for plasma antioxidant defense status showing higher total SOD activities and GSH levels in BCC patients compared with control subjects, protein expressions of GCLC and MnSOD were higher in non-neoplastic tissues of BCC patients compared to both BCC tissues and the epidermis of control subjects.

In comparison of non-cancerous skin lesions and normal skin of control subjects, there were no significant differences in expressions of all parameters studied (data not shown).

mRNA expression of hOGG1 and antioxidant defense system in skin tissues of BCC and control subjects

In agreement with protein expression data, Fig. 3 demonstrated that mRNA expressions of hOGG1 (0.8 ± 0.1-fold decrease, $p < 0.001$), CAT (0.87 ± 0.03-fold decrease, $p < 0.001$), GCLC (0.7 ± 0.1-fold decrease, $p < 0.001$), GCLM (0.3 ± 0.2-fold decrease, $p < 0.001$), GPx (0.8 ± 0.1-fold decrease, $p < 0.001$), Nrf2 (0.6 ± 0.1-fold decrease, $p < 0.001$), CuSOD (0.5 ± 0.2-fold decrease, $p < 0.001$) and MnSOD (0.7 ± 0.1-fold decrease, $p < 0.001$) were substantially lower in BCC tissues than in skin tissues of control subjects.

Discussion

An involvement of oxidative damage in the pathogenesis of BCC has been widely discussed since several studies have shown possible mechanisms through which excessive ROS generation and antioxidant defense impairment may play a role in malignant transformation to NMSC or keratinocytic cancer including BCC (6, 22). Potential mechanisms of carcinogenesis may involve oxidative DNA damage accountable for genomic mutations because attacks on DNA by ROS result in considerable DNA lesions including strand breaks and DNA base oxidation products, in particular 8-OHdG, considered the most potentially mutagenic (23). hOGG1 is highly specific for the removal and repair of 8-OHdG from oxidatively damaged DNA. Increased 8-OHdG formation and/or loss of hOGG1's expression and function were reported to play a role in the development and progression of skin cancers including BCC (24-26). In this study, we conducted a case-control study of BCC risk in association with urinary oxidative DNA damage (8-OHdG) levels as well as expressions of 8-OHdG as well as hOGG1 protein and mRNA in the skin tissues. Our observations indicated elevation of preoperative 8-OHdG levels in urine and its expression in BCC tissues in comparison with epidermis of control subjects. At 1 month after operation, there were no significant changes in urinary 8-OHdG levels in BCC patients compared with preoperative levels, although the postoperative 8-OHdG levels in BCC patients were substantially reduced at 6 months. Hence, it is possible that greater urinary 8-OHdG in BCC patients than in control subjects could be attributed to oxidative stress in neoplastic cells. We also observed decreased protein and mRNA expressions of hOGG1 in BCC tissues in comparison with epidermis of control subjects. Furthermore, the intra-subject comparison of non-lesional and lesional BCC skin demonstrated a higher expression of 8-OHdG and a lower expression of hOGG1 in BCC tissues compared with the adjacent epidermis. Our results are consistent with those from previous case-control studies suggesting a contribution of elevated

oxidative DNA damage and impaired hOGG1 to the development of various cancers including breast, pancreatic, gastric and lung cancers (9, 27-29). Hence, augmented levels of 8-OHdG could be related to a defect in hOGG1 expression in BCC patients that may be deficient in the repair of 8-OHdG, although the mechanism by which hOGG1 impairment contributes to the development of BCC needs further investigation.

A disturbance in redox homeostasis probably contributed to development of multiple tumors including NMSC can be attributed to not only increased oxidative DNA damage but also impaired antioxidant defense capacity (6, 30). Major antioxidant defenses in the human body include GSH and enzymatic antioxidants including GPx and CAT, which neutralize H_2O_2, NQO1, which catalyzes detoxification of various electrophilic toxicants and oxidants and SOD, which dismutates the superoxide radical ($O_2^{\bullet-}$) to H_2O_2. Thus, preoperative and postoperative levels of plasma GSH levels and antioxidant enzyme (CAT, GPx, NQO1 and SOD) activities as well as expressions of related proteins (GCLC, CAT, GPx and MnSOD) and Nrf2, a well-known transcription factor that regulates expression of detoxifying enzyme genes, and levels of the corresponding genes in skin lesions were determined in BCC patients compared with control subjects. Our results indicated that while decreases in CAT, GPx and NQO1 activities in BCC patients and in protein expressions of CAT, GCLC, GPx, Nrf2 and MnSOD in BCC tissues compared to epidermis of control subjects were observed, plasma SOD activities and GSH levels were higher in BCC patients than in control subjects. In correlation with IHC findings showing diminished expressions of all antioxidant proteins in BCC tissues, mRNA levels of the corresponding genes including CAT, GCLC, GCLM, NQO1, Nrf2, CuSOD and MnSOD were also reduced in BCC tissues compared to epidermis of control subjects.

While CAT, GPx and NQO1 activities markedly declined in BCC patients compared to control subjects, elevation of SOD activities and GSH levels was observed in association

with upregulated protein expressions of GCLC, a rate-limiting enzyme in GSH synthesis, and MnSOD in the adjacent non-neoplastic tissues of BCC patients. In agreement with previous studies, lower activities of GPx and CAT as well as higher activities of SOD were observed in patients with oesophageal, gastric and cololectal cancers compared with control subjects (31, 32) and low NQO1 activity was found to be linked to increased risk of acute leukemia (33). NQO1 polymorphism, which can cause reduction of its activity, was reported to affect susceptibility to lung, bladder and colorectal cancers that could either increase or decrease cancer risks associated with ethnicity and exposure to carcinogens (34). Differential expression of antioxidant proteins was found in several cancers. Reduction of CAT and GPx protein expressions was demonstrated in neoplastic tissues of patients with esophageal, colorectal and lung cancers, although both downregulation and overexpression of MnSOD protein in lung and esophageal cancers, respectively, were reported (35-37). In addition, this study demonstrated decreased Nrf2 expression in BCC tissues compared to both adjacent non-neoplastic tissues of BCC patient and non-cancerous tissues of control subjects that was in agreement with previous reports showing down-regulation of Nrf2 and its target genes including NQO1 at mRNA and protein levels during malignant transformation and upregulation of antioxidant mRNA including CAT, GCLC, GCLM, GPx and NQO1 genes by Nrf2 overexpression was able to delay tumor growth (38). Nevertheless, the role of Nrf2 in cancer is complex as it can play a dual role in both cancer prevention and promotion depending on cellular environment (39).

Enhancement of plasma SOD activities and GSH levels in correlation to increased protein expressions of MnSOD and GCLC in the adjacent non-neoplastic tissues of BCC patients may be due to an adaptive response of normal skin cells to persistent elevation of oxidative stress and damage in cancer patients. It has been suggested that upregulation of antioxidant defenses including GSH and MnSOD may serve as the defense mechanisms for

cell survival against stress and inflammatory insults, which can take place during cancer initiation and progression (40). Further investigations are needed to clarify whether an impaired antioxidant system favors ROS accumulation leading to cancer initiation or the antioxidant system may be either downregulated or upregulated as a consequence of alterations in cellular homeostasis and anti-oxidative metabolism in tumor growth and progression. Case-control studies of genetic polymorphisms in DNA repair and antioxidant enzymes in association with BCC risk with a large number of subjects should also be done in future studies.

This study showed that patients with BCC, a locally invasive malignant skin cancer, could exhibit systemic disturbance in redox status. Acute exposure of mice skin to UVA and UVB irradiation was also reported to affect markers of oxidative damage and antioxidant defenses in plasma and non-skin tissues including erythrocytes and liver (41). Furthermore, we demonstrated that surgical intervention could influence oxidative DNA damage and antioxidant defense status only in case patients with BCC but not control subjects with non-malignant skin diseases as tumor removal was observed to be related to improvement of redox status by reducing 8-OHdG levels at 6 months postoperatively and enhancing plasma antioxidant defenses including CAT, GPx and NQO1 activities at 1 month postoperatively.

In conclusion**,** patients with BCC may be under oxidative stress associated with induction of oxidative DNA damage, defects in DNA repair hOGG1 at protein and mRNA levels and reduction of plasma CAT, GPx and NQO1 activities and of all antioxidant proteins and genes studied in the BCC tissues. Surgical removal of BCC tissues correlated with improved redox status. An elevation of plasma total SOD activities and GSH levels as well as protein expressions of MnSOD and GCLC in non-neoplastic tissues of BCC patients may indicate an adaptive response to oxidative stress. Whether oxidative DNA damage and

antioxidant defense parameters can serve as biomarkers of oxidative stress to predict development and progression of BCC needs further studies.

Conflicts of interest

No conflicts of interest were declared in relation to this article.

Acknowledgments

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Table

Table 1. Demographics and clinical characteristics of controls subjects and BCC patients.

Characteristics	Control (n=20)	Cases (n=17)	*p*-value
Demographic			
Age (years)	65.65 ± 11.24	66.82 ± 12.71	0.714[b]
Gender, n (%)			
Male	11(55.00)	5(29.40)	0.117[a]
Female	9(45.00)	12(70.60)	
BMI (kg/ m^2)	23.62 ± 2.53	22.98 ± 3.63	0.217[b]
Clinical characteristics			
Glucose (mg/dl)	96.90 ± 13.00	101.60 ±15.46	0.317[c]
BUN (mg/dl)	13.78 ± 4.06	14.32 ± 5.59	0.891[b]
Creatinine (mg/dl)	1.06 ± 0.25	1.05 ± 0.28	0.949[c]
Cholesterol (mg/dl)	191.20 ± 38.70	201.8 ± 37.52	0.407[c]
Triglyceride (mg/dl)	135.20 ± 66.10	109.8 ± 47.55	0.156[b]
HDL-Chol (mg/dl)	59.55 ± 20.09	63.59 ± 18.95	0.402[b]
LDL-Cal (mg/dl)	104.60 ± 33.28	116.2 ± 34.08	0.110[b]
AST (U/L)	28.30 ± 12.76	26.82 ± 14.81	0.359[b]
ALT (U/L)	21.50 ± 9.90	21.29 ± 13.91	0.593[b]
eGFR (mL/min/1.73 m^2)	66.55 ± 20.65	65.24 ± 20.77	0.849[c]
CBC			
Hemoglobin (g/dl)	13.37 ± 1.87	12.84 ± 1.38	0.200[b]
Rbc count (X 10^6/ul)	4.65 ± 0.65	4.49 ± 0.53	0.404[c]
Wbc count (X 10^3/ul)	7.05 ± 1.76	7.63 ± 1.63	0.195[b]
Lifestyle			
Smoking, n (%)			
Non-Smoker	14(70.0)	14(82.5)	1.265[a]
Ex-Smoker	5(25.0)	3(16.7)	
Current Smoker	1(5.0)	0(0)	
Drinking alcohol, n (%)			
Non-Drinker	15(75.0)	14(82.4)	0.940[a]
Ex-Drinker	4(20.0)	3(17.6)	
Current Drinker	1(5.0)	0(0)	

Abbreviations: BMI = Body Mass Index, BUN = Blood Urea Nitrogen, HDL-Chol = High Density Lipoprotein-Cholesterol, LDL-Cal = Calculated Low Density Lipoprotein Cholesterol, AST = Aspartate aminotransferase, ALT = Alanine aminotransferase, eGFR = estimated glomerular filtration rate, CBC = Complete Blood Count, RBC = Red Blood Cell, Wbc = White blood cell. Data are presented as the mean ± SD. The statistical significance of differences in categorical variables data was evaluated by chi-square test (a), nonparametric variables was analysed by the Mann-Whitney U test (b) and parametric variables by unpaired student's t-test (c).

Table 2. Comparison of urinary oxidative DNA damage levels and plasma antioxidant defense status between control subjects and BCC patients.

Parameters	Controls (n = 20)		Cases (n = 17)		
	Before surgery	1 month after surgery	Before surgery	1 month after surgery	6 months after surgery
8-OHdG (ng/g creatinine)	63.68 ± 15.57	75.36 ± 17.97	105.79 ± 30.52***	102.91 ± 27.33***	66.86±20.19$^{\#\#\#}$
CAT (unit/mg protein)	4.21 ± 0.77	3.82 ± 0.63	2.62 ± 0.45***	4.01 ± 1.15$^{\#\#\#}$	
GPx (unit/mg protein)	0.73 ± 0.20	0.73 ± 0.20	0.45 ± 0.14***	0.73 ± 0.11$^{\#\#\#}$	
NQO1 (μmole DCPIP reduced/min/mg protein)	888.33 ± 198.64	968.97 ± 165.71	710.71 ± 134.46**	1009.64 ± 306.05$^{\#\#\#}$	
Total SOD (unit/mg protein)	0.02 ± 0.01	0.02 ± 0.00	0.03 ± 0.01***	0.04 ± 0.01***	
GSH (μmol/mg protein)	161.29 ± 42.35	164.40 ± 40.27	223.43 ± 42.29***	206.69 ± 51.88*	

Results were expressed as mean ± standard deviation (SD).

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control, before surgery.

$^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to case, before surgery.

Table 3. Expressions of oxidative DNA damage, DNA repair enzyme and antioxidant proteins in skin tissues of control subjects and BCC patients by IRS score.

Parameters	Controls (n = 20)	Cases (n = 17)	
	Epidermis	BCC	Adjacent epidermis
8-OHdG	2.35 ± 0.67	2.88 ± 0.33*	2.13 ± 0.74$^{\#}$
hOGG1	2.60 ± 0.50	1.76 ± 0.44***	2.59 ± 0.51$^{\#\#\#}$
CAT	2.10 ± 0.64	1.00 ± 0.35***	2.00 ± 0.54$^{\#\#\#}$
GCLC	2.30 ± 0.47	1.56 ± 0.62*	2.88 ± 0.33$^{*,\#\#\#}$
GPx	2.05 ± 0.39	1.06 ± 0.24***	1.73 ± 0.46$^{\#\#\#}$
Nrf2	2.45 ± 0.61	1.53 ± 0.51***	2.31 ± 0.48$^{\#\#}$
MnSOD	2.00 ± 0.56	1.24 ± 0.66*	2.66 ± 0.49$^{*,\#\#\#}$

Results were expressed as mean ± standard deviation (SD).

$^{*}P < 0.05$, $^{***}P < 0.001$ compared to epidermis of control.

$^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to BCC lesion.

Figure legends

Figure 1. The urinary 8-OHdG levels (A) and plasma antioxidant defense status [CAT (B), GPx (C), NQO1 (D) and total SOD (E) activities and GSH levels (F)] in control subjects and BCC patients before and after surgery. Values given are mean ± SD. The statistical significance of differences between the control and case was evaluated by one-way ANOVA followed by Tukey's *post hoc* test. * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$ compared to preoperative values in control subjects prior to surgery, $^{###}P < 0.001$ compared to preoperative values in BCC patients prior to surgery. Principal component analysis was performed to systematically investigate similarity of DNA damage and antioxidant defense parameters from different groups of patients (G). Factor analysis is overlaid on top of the patient scores, both for pre-surgery (case: triangle; control: square) and 1-month post-surgery (case: circle; control: plus). The dashed and dotted ovals delineate the approximated distribution of each group with 95% confidence intervals.

Figure 2. The H&E staining (A-C) and IHC staining for oxidative DNA damage, 8-OHdG (D-G), DNA repair enzyme, hOGG1 (H-K), and antioxidant proteins, CAT (L-O), GCLC (P-S), GPx (T-W), Nrf2 (X-AA) and MnSOD (AB-AE), in control subjects and tumor and non-tumor lesions of BCC patients. Values given are mean ± SD. The statistical significance of differences between the control and case was evaluated by nonparametric variables with Kruskal-Wallis test followed by Dunnett's *post hoc* test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to control, $^{#}P < 0.05$, $^{##}P < 0.01$, $^{###}P < 0.001$ compared to tumor lesions of BCC patients.

Figure 3. DNA repair and antioxidant gene expression in non-malignant skin tissues of control subjects and BCC tissues. Gene expression was evaluated by real-time PCR with the $2^{-\Delta\Delta Ct}$ method. The data are presented as the fold change in gene expression normalized to GAPDH. Values given are mean ± SD. The statistical significance of differences between the

control and case was evaluated by nonparametric variables with Mann Whitney U-test. ***$P < 0.001$ compared to control.

Figure 1

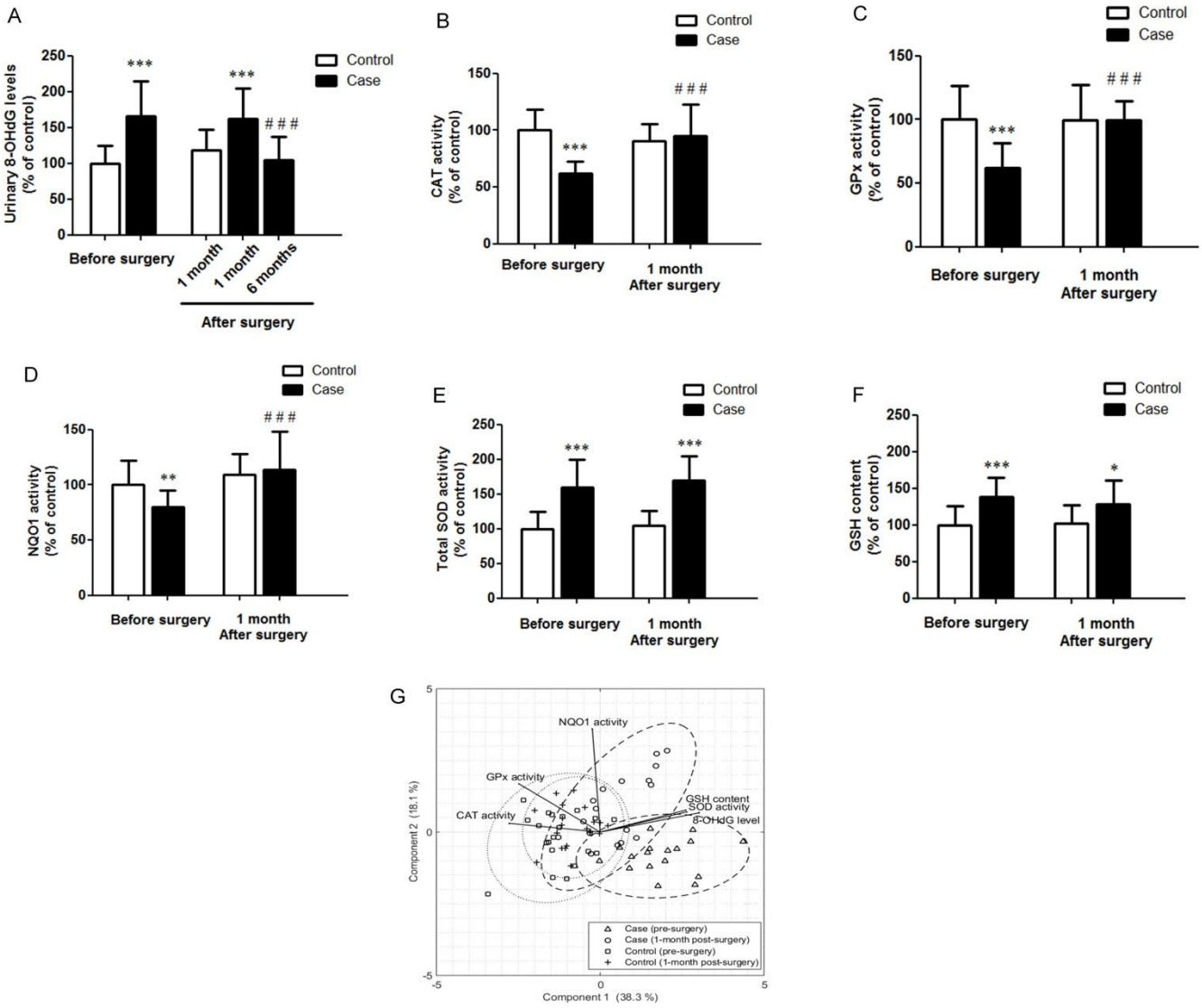

A
Urinary 8-OHdG levels (% of control)
Control
Case
***
***
###
Before surgery
1 month
1 month
6 months
After surgery
B
CAT activity (% of control)
Control
Case
***
###
Before surgery
1 month
After surgery
C
GPx activity (% of control)
Control
Case
***
###
Before surgery
1 month
After surgery
D
NQO1 activity (% of control)
Control
Case
**
###
Before surgery
1 month
After surgery
E
Total SOD activity (% of control)
Control
Case
***
***
Before surgery
1 month
After surgery
F
GSH content (% of control)
Control
Case
***
*
Before surgery
1 month
After surgery
G
NQO1 activity
GPx activity
CAT activity
GSH content
SOD activity
8-OHdG level
Component 2 (18.1 %)
Component 1 (38.3 %)
Case (pre-surgery)
Case (1-month post-surgery)
Control (pre-surgery)
Control (1-month post-surgery)

Figure 2

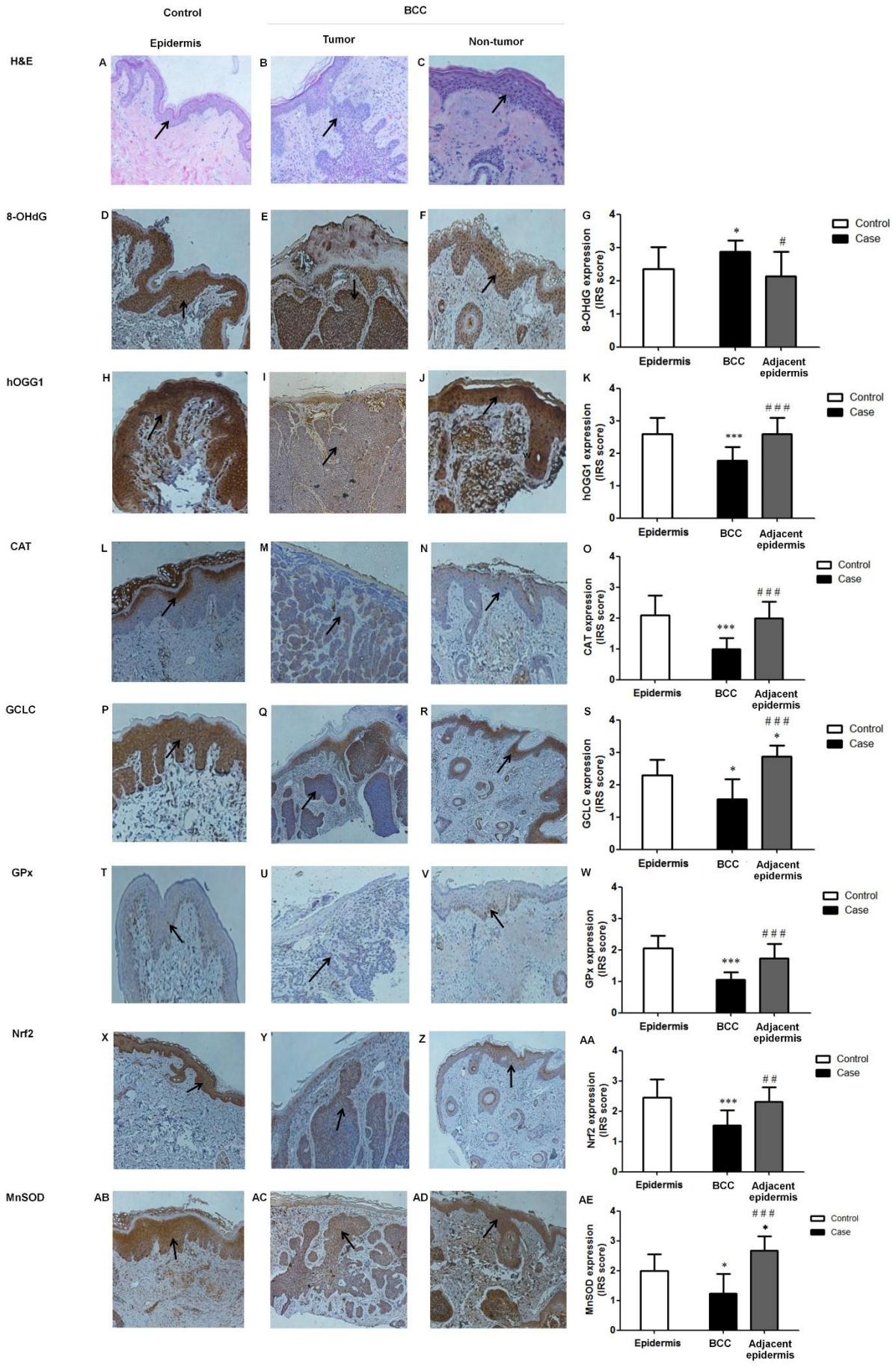
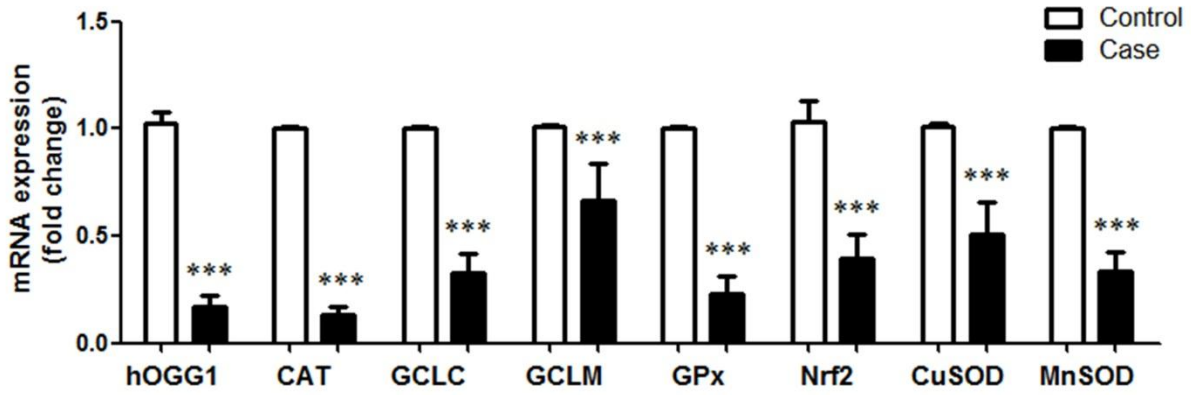

Control
BCC
Epidermis
Tumor
Non-tumor
H&E
A
B
C
8-OHdG
D
E
F
G
8-OHdG expression (IRS score)
Control
Case
Epidermis
BCC
Adjacent epidermis
hOGG1
H
I
J
K
hOGG1 expression (IRS score)
CAT
L
M
N
O
CAT expression (IRS score)
GCLC
P
Q
R
S
GCLC expression (IRS score)
GPx
T
U
V
W
GPx expression (IRS score)
Nrf2
X
Y
Z
AA
Nrf2 expression (IRS score)
MnSOD
AB
AC
AD
AE
MnSOD expression (IRS score)

Figure 3


Control
Case
mRNA expression (fold change)
1.5
1.0
0.5
0.0
***
hOGG1
CAT
GCLC
GCLM
GPx
Nrf2
CuSOD
MnSOD