The protective effect of luteolin on cytotoxicity and genotoxicity of bisphenol-A-glycidyldimethacrylate in macrophages involved in DNA damage and caspases activation

Chien-Heng Shen[1], Yu-Hsiang Kuan[2], Shiuan-Shinn Lee[3] , Ming-Ling Yang[4], Hsing-Chun Kuo[5]*, Yung-Wei Chiu[6]*

[1]Department of Hepato-Gastroenterology, Chang Gung Memorial Hospital, Chiayi; Graduate Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, Taiwan, ROC
[2]Department of Pharmacology, School of Medicine, Chung Shan Medical University Taichung, Taiwan, ROC; Department of Pharmacy, Chung Shan Medical University Hospital, Taichung, Taiwan, ROC
[3]School of Public Health, Chung Shan Medical University, Taichung, Taiwan, ROC
[4]Department of Anatomy, School of Medicine, Chung Shan Medical University, Taichung, Taiwan, ROC [5]Department of Nursing, Chang Gung University of Science and Technology; Chronic Diseases and Health Promotion Research Center, CGUST, Taiwan., ROC; Research Center for Industry of Human Ecology, Chang Gung University of Science and Technology, Taoyuan, Taiwan, ROC
[6]Emergency Department and hyperbaric oxygen therapy Center, Tungs' Taichung MetroHarbor Hospital, Taichung, Taiwan; Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan, ROC.
guscsi@gmail.com; hboxygen@gmail.com.

Abstract: Bisphenol-A-glycidyldimethacrylate (BisGMA) is one of the most commonly used monomer in biomedical field, especially in dentistry and orthopedics. BisGMA could leach from the thermoset or light-cured biomaterials after polymerization and result in immunological responses. Luteolin is a flavonoid which expresses multiple pharmacological activities, including anti-hypertension, anti-inflammation, anti-allergy, anti-cancer, and antitumor. In this study, we found that luteolin inhibited BisGMA-induced cytotoxicity in a concentration-dependent manner in RAW264.7 macrophages. Furthermore, not only the BisGMA-induced DNA damage and genotoxicity, but also the activation of caspase-3, -8, and -9 were reduced by luteolin in a parallel, concentration-dependent manner. These results indicated luteolin reduced BisGMA-induced cytotoxicity and genotoxicity in RAW264.7 macrophages via DNA damage and its upstream factors which are caspase-3, -8, and -9.
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1. Introduction:

The tissue resident macrophages are differentiated from peripheral monocyte reservoirs of blood, spleen , and bone marrow [1]. Macrophages are professional phagocytes responsible for internalization and elimination of pathogens, development of embryonic tissues, and repairment of wound in a wide range of organisms from invertebrates to vertebrates [2]. Therefore, macrophages play the critical role in both innate and adaptive immunity. Exogenous stimuli such as bacteria, lipopolysaccharide, and bisphenol-A-glycidyldimethacrylate (BisGMA) can lead to macrophages activation. Over-expression of the proinflammatory mediators, such as nitric oxide (NO), reactive oxygen species (ROS), and cytokines generated by activated macrophages results in the damage of peripheral normal cells and tissues. Macrophages activation induces several clinical syndromes, such as oral inflammatory disease, inflammatory bowel disease, and dermatomyositis [3, 4, 5].

BisGMA is originally developed by Bowen for dental composites in 1962 and is one of the most commonly used monomer in the biomedical field, especially in dentistry and orthopedics [6, 7]. However, BisGMA can leach from the thermoset or light-cured biomaterials after polymerization [8]. Its highly lipophilic characteristic the rapid distribution of BisGMA everywhere in the whole animal body once released [9]. BisGMA expresses cytotoxic and genotoxic effects in mammalian cells, such as RAW264.7 maceophages, V79 fibroblasts, human lymphocytes, human dental pulp cells, human gingival fibroblasts, and HaCaT keratinocytes [10, 11, 12, 13, 14, 15, 16]. In previous study, we have demonstrated BisGMA induces cytotoxicity and genotoxicity in RAW264.7 macrophages via DNA damage and caspases activation [10].

Luteolin, or 3',4',5,7-tetrahydroxyflavone, is a member of the flavone subclass in flavonoid family. It is widely distributed in vegetables, fruits, and herbs. The strong antioxidative capacity of luteolin is due to its polyphenolic structure. Luteolin also has multiple pharmacological activities, such as anti-hypertension, anti-inflammation, anti-allergy, anti-cancer, and antitumor [17]. In Wistar rats, mutton extract-induced micronuclei and chromosome aberrations were protected by luteolin [18]. In human keratinocytes, luteolin protects against ultraviolet (UV-B) induced human skin damage via activities like DNA damage reduction, antioxidation, and anti-inflammation [19]. Up to now, there is no evidence on effect of luteolin on BisGMA-induced cytotoxicity and genotoxicity in RAW264.7 macrophages. This study aims to investigate whether luteolin has protective effects in BisGMA-induced damage and at the same time attempts to identify the mechanisms involved.

2. Materials and methods:

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from HyClone Laboratories (Logan, UT, USA). 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA) was purchased from Life Technologies (Grand Island, NY, USA). Bisphenol-A glycidyl-dimeth acrylate (BisGMA), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phosphate buffered saline (PBS), and other reagents, unless specifically stated, were purchased from Sigma-Aldrich (St. Louis, MO, USA). CytoTox® 96 nonradioactive assay kit was purchased from Promega (Sunnyvale,CA, USA). Caspase-3, -8, and -9 activity kits were purchased from Enzo Life Sciences (Plymouth Meeting, PA, USA). Luteolin was dissolved and tested at concentrations of 0.01, 0.03, 0.1, and 0.3 μM. BisGMA was dissolved and tested at concentration of 3 μM. The final percentage of DMSO in the reaction was not higher than 0.5% (v/v).

2.2. Cell culture

The mouse macrophage cell line, RAW264.7, was obtained from Bioresource Collection and Research Centre (BCRC 60001; Hsinchu, Taiwan) and cultured in DMEM supplemented with 10 % (v/v) FBS, 100 unit/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5 % CO_2 in a humidified incubator. The cells were seeded in 6-well plates at 2.0×10^6 cells/ml or 24-well plates at 5.0×10^5 cells/ml [20].

2.3. Cytotoxicity assay

Cytotoxictiy was measured by cell membrane integrity, which was in turn by monitoring the release of lactate dehydrogenase (LDH) into the medium. The content of LDH in the medium was detected by CytoTox® 96 nonradioactive assay kit. RAW264.7 cells were treat- ed with indicated concentrations (0, 0.3, 1, 3, and 10 μM) of luteolin for 30 min before stimulation or no stimulation with 3 μM BisGMA for 4 h. The maximal release of LDH was obtained after treating control cells with lysis solution for 4 h at 37°C. Supernatant sample and reconstituted substrate, 50 μL each, were reacted for 30 min at room temperature, then 50 μL of stop solution was added to each well. Absorbance was measured by a microplate reader (Dynatech MR 4000, Boston, MA, USA) at 490 nm [21].

2.4 Micronucleus (MN) assay

RAW264.7 cells were pretreated with indicated concentration of luteolin for 30 min before addition of cytochalasin B (3 mg/mL) and BisGMA (3 μM) for 4 h. After washing with PBS, the cells were further incubated with 3 mg/mL of cytochalasin B for 14 h. Cells were collected by trypsinization, resuspended in 75 mM KCl for 1 min, fixed in cold methanol: acetic acid (3:1), spread on slides, and stained with 3 % Giemsa (pH 6.4) solution. MN was analyzed microscopically in three parallel slides of 1000 binucleated cells/slide per concentration [22].

2.5. Alkaline comet assay

The alkaline comet assay, also called alkaline single cell gel electrophoresis, is a sensitive and rapid technique for the detection of DNA damage at the level of the individual cell. The process of detection was according to our pervious study [23]. After treatment, the cells mixed with 1% low melting point agarose were placed on a microscope slide that had been pre-coated with 1% normal melting point agarose and kept on ice. Then, the slides were immediately submersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1 % Tritron X-100, 200 mM NaOH, 34.1 mM N-Lauroyl-Sarcosine, and 10 % DMSO, pH 10) for 1 h at 4ºC in the dark. After washing with PBS, the slides were placed in a horizontal electrophoresis tank filled with electrophoresis buffer (300 mM NaOH and 1 mM EDTA) for 40 minutes at 4ºC. After electrophoresis, the slides were washed three times with a neutralization buffer (0.4 M Tris-HCl, pH 7.5), dried, stained with ethidium bromide (20 μg/ml), then analyzed by image analysis system Comet v. 3 (Kinetic Imaging Ltd., Liverpool, UK). To quantify DNA damage, the following comet parameters were evaluated: percentage of DNA in tail, which was relative fluorescence intensity of tail, and tail moment, which was calculated as tail length multiplied by percentage of DNA in tail and divided by 100.

2.6. Detection of caspases activity

Activities of caspase-3, -8, and -9 were measured

by caspase-3, -8, and -9 fluorometric assay kits. The process of detection was according to pervious study [24]. After treatment, the cells were lysed by lysis buffer and 100 μg of protein was resuspended in the reaction buffer containing caspase-3, -8, and -9 fluorogenic substrates, which are DEVD-AFC, IETD-AFC, and LEHD-AFC respectively. The samples were incubated for 4 h at 37°C, and data were collected using a fluorescence microplate reader (Molecular Devices, CA, USA) at excitation/emission wavelengths of 400/505 nm.

2.7. Statistical Analysis

At least four independent experiments were performed as indicated in the figure legends. All data were expressed as mean ± standard deviation (SD). Statistical analyses were performed using ANOVA followed by the Bonferroni's t-test for multi-group comparisons test; $p < 0.05$ was considered significant for each test.

3. Results:

3.1. Effects of luteolin on BisGMA-induced cytotoxicity

The cytotoxicity on RAW264.7 cells was significantly induced by treating with BisGMA at 3 μM for 4 h ($p < 0.05$). The effect was significantly prevented by luteolin in a concentration-dependent manner started at 1 μM ($p < 0.05$) (Fig 1). These results indicated luteolin has protective effect on BisGMA-induced cytotoxicity.

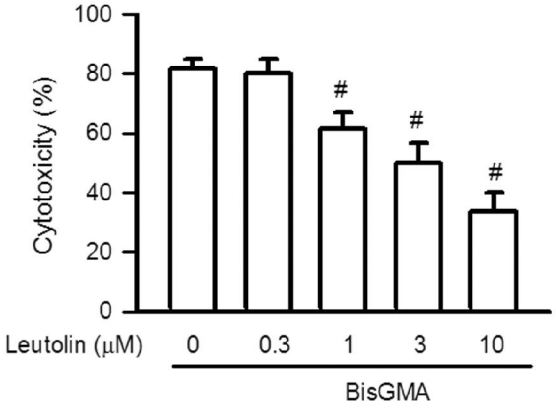

Fig. 1. Effect of luteolin on BisGMA-induced cytotoxicity in RAW264.7 macrophages. The cytotoxicity was measured by LDH colorimetric assay. Cells were incubated with vehicle or 0-10 μM luteolin for 30 min at 37°C before stimulation with 3 μM BisGMA for 4 h. Value is expressed as a percentage of the vehicle treated control cells. Results are expressed as means ± SD (n = 3). #$p < 0.05$ is considered signifiant.ompares with the BisGMA values (first column).

3.2. Effects of luteolin on BisGMA-induced DNA damage

The formation of MN was significantly induced by BisGMA at 3 μM for 4 h as compared with control group (Fig 2). Pretreatment with luteolin for 30 min reduced the BisGMA induced MN generation significantly in a concentration-dependent manner started at 3 μM ($p < 0.05$) (Fig 2). In addition, we also found BisGMA-induced DNA injury via comet assay. Images of cells with increasing levels of DNA damage from comet assay are shown in Fig. 3A. According to the data obtained, tail moment (Fig. 3B) and percentage of DNA in tail (Fig. 3C) were significantly increased by BisGMA as compared with control ($p < 0.05$). Pretreatment with luteolin for 30 min reduced the BisGMA induced tail moment and percentage of DNA in tail significantly in a concentration-dependent manner started at 3 μM ($p < 0.05$).

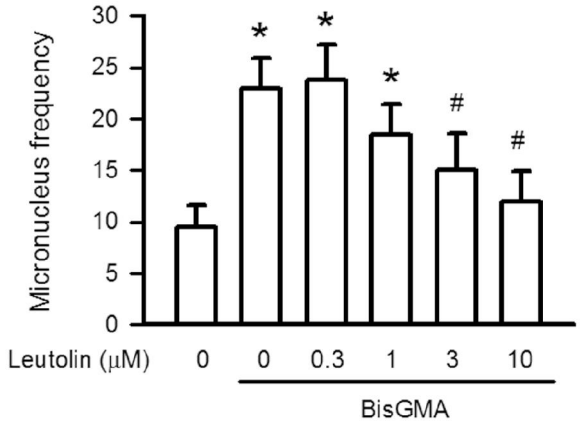

Fig. 2. Effect of luteolin on BisGMA-induced DNA damage via MN assay. Value is expressed as a percentage of the vehicle treated control cells. Results are expressed as means ± SD (n = 3). *$p < 0.05$ is considered significant compares with control values (first column). #$p < 0.05$ compares with and the BisGMA values (secondary column).

3.3. Effects of luteolin on BisGMA-induced caspase-3, -8, and -9 activities

Fig 4 has shown BisGMA significantly stimulated caspase-3, -8, and -9 activities as compare with control ($p < 0.05$). Pretreatment with luteolin for 30 min reduced the BisGMA induced caspase-3, -8, and -9 activities (Fig. 4), and significantly started at 3 μM ($p < 0.05$).

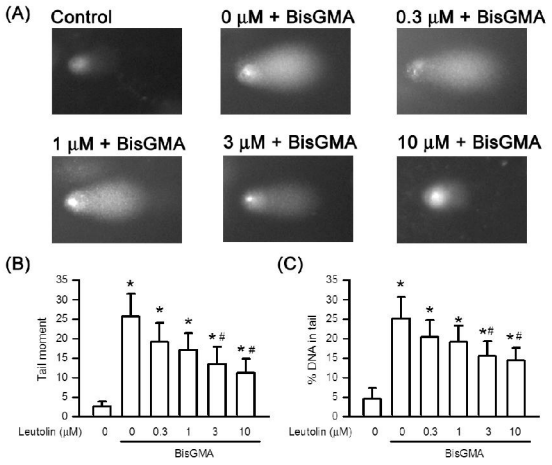

Fig. 3. Effect of luteolin on BisGMA-induced DNA damage via comet assay. (A) Gel electrophoresis of luteolin treated RAW264.7 cells at 0-10 μM for 30 min at 37°C before stimulation with 3 μM BisGMA for 4 h. (B) and (C) are quantifications of tail moment and percentage of DNA in tail respectively; data are expressed as means ± SD (n= 3). *p < 0.05 is considered significant compares with control values (first column). #p < 0.05 compares with and the BisGMA values (secondary column).

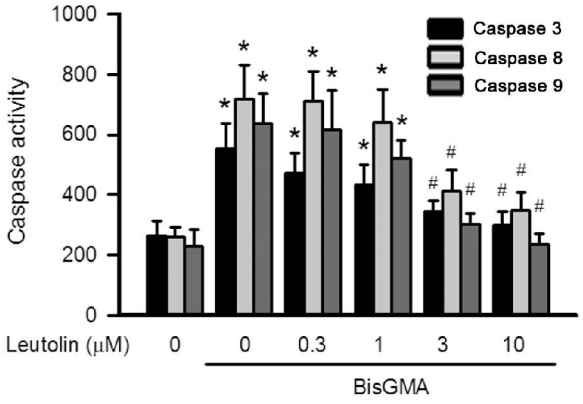

Fig. 4. Effects of luteolin on BisGMA-induced caspase-3, -8, and -9 activities. Value is expressed as a percentage of the vehicle treated control cells. Results are expressed as means ± SD (n = 3). *p < 0.05 is considered significant compares with control values (first column). #p < 0.05 compares with and the BisGMA values (secondary column).

4. Discussion

BisGMA is a monomer for resin composites commonly used in polymeric dental materials, e.g. restorative composites, adhesives, and prophylactic sealants [6, 7]. BisGMA-based resin displayed much better thermal and mechanical properties, lower cure shrinkage, higher modulus, and lower volatility than resins form by other smaller monomers, such as triethyleneglycol dimethacrylate (TEGDMA), 2-hydroxyethyl methacrylate (HEMA), and glycerol dimethacrylate (GDMA) [25, 26]. Recently, the toxicity of BisGMA monomer has raised concern. BisGMA can be released from resin polymerized incompletely and may injure the peripheral normal tissues [8]. Our laboratory has purposed BisGMA induced cytotoxicity in RAW264.7 macrophages in a concentration and time-dependent manner [10]. The evidences gathered from previous study points out that 81.6% of cytotoxicity was induced by treating with BisGMA at 3 μM for 4 h, however, luteolin pretreatment reduced BisGMA-induced cytotoxicity significantly. Our results concerning the protective effect of luteolin are similar to those obtained by other researchers studied on different stimulators with various cell lines, for example, on 6-hydroxy- dopamine-stimulated pheochromocytoma cells and pyrogallol-stimulated endothelial cells [27, 28]. These evidences demonstrated that luteolin has a protective effect on BisGMA treated macrophages.

The change in the DNA structure due to damage is varitable, such as the break in the DNA strand, the missing of the DNA base pair, and the chemically changed DNA base [29]. In general, DNA damage is due to the environmental genotoxins, including biphenyls, alkylating compounds, polycyclic aromatic hydrocarbons, heterocyclic amines, ultraviolet light, and radiation. DNA damage is the major cause of cell death via genotoxicity [30]. BisGMA is able to induce DNA damage and genotoxicity in RAW264.7 macrophages, V79 fibroblasts, human lymphocytes, and human gingival fibroblasts which had been demonstrated through comet and MN assays [10, 31, 11, 13, 32], our data also confirmed these findings. However, dietary supplemented with luteolin can reduce DNA damage causes by ultraviolet light or Fenton-induced 8-hydroxy-2'-deoxyguanosine formation in calf thymus [33]. In Caco-2 cells, luteolin shows the protective effect against hydrogen peroxide-induced DNA damage [34]. Ultraviolet-B light-induced DNA damage is effectively attenuated by luteolin [19]. At present, we found luteolin reduced BisGMA induced DNA damage in RAW264.7 macrophages in a concentration-dependent manner.

These results indicated luteolin has a protective effect on BisGMA induced cytotoxicity and genotoxicity. Caspase-3 was found to play a striking role in the DNA damage [35]. Caspases are cysteine proteases that specifically cleave target proteins at sites next to aspartic acid residues. The dimer of the catalytic domain, which is cleaved into a large and a small

subunits that interact with each other, exists in the full activation of caspases [36]. In apoptosis, the activation of caspase-3 is dependent on two activated cascades, involved both intrinsic and extrinsic pathways. The upstream factors of intrinsic and extrinsic pathways are caspases-9 and -8, respectively [37]. In RAW264.7 macrophages, the activation of caspases-3, -8, and -9 are induced by BisGMA [10]. In this study, we also purposed caspases-3, -8, and -9 are activated by BisGMA. In previous study, luteolin significantly reduced the expression of active caspase-3 in cisplatin-induced kidney damage [38]. Luteolin suppressed the caspase-8 activation in pyrogallol-triggered endothelial cells [28]. At present, we found luteolin reduced BisGMA induced activation of caspases-3, -8, and -9 in a concentration-dependent manner. These results indicated luteolin exerts a protective effect on BisGMA induced DNA damage by decreasing the activation of caspase-3, -8, and -9.

In conclusion, BisGMA caused cell death was inhibited by luteolin in a concentration-dependent manner in RAW264.7 macrophages which was demonstrated by LDH assay. In addition, luteolin reduced the DNA damage in a parallel, concentration-dependent manner which were detected by MN assay and comet assay. Luteolin also reduced the BisGMA-induced caspase-3, -8, and -9 activation in a parallel, concentration-dependent manner. These results indicated luteolin reduced cytotoxicity and genotoxicity in RAW264.7 macrophages via DNA damage, and its upstream factors which are caspase-3, -8 , and -9.

Corresponding Authors:
Hsing-Chun Kuo
Institute of Nursing and Department of Nursing, Chang Gung Institute of Technology, Chia-Yi Campus, Taiwan. Telephone: +886-5-3628800, Fax: 886-5-3628866; E-mail address: guscsi@gmail.com;
Yung-Wei Chiu
Emergency Department and hyperbaric oxygen therapy Center, Tungs' Taichung MetroHarbor Hospital, Taichung, Taiwan; Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan. E-mail address: hboxygen@gmail.com.

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