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Gallic Acid Provokes DNA Damage and Suppresses DNA Repair Gene Expression in Human Prostate Cancer PC-3 Cells

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ABSTRACT: Our earlier studies have demonstrated that gallic acid (GA) induced cytotoxic effects including induction of apoptosis and DNA damage and inhibited the cell migration and invasion in human cancer cells. However, GA-affected DNA damage and repair gene expressions in human prostate cancer cells are still unclear. In this study, we investigated whether or not GA induces DNA damage and inhibits DNA repair gene expression in a human prostate cancer cell line (PC-3). The results from flow cytometric assay indicated that GA decreased the percentage of viable PC-3 cells in a dose- and time-dependent manner. PC-3 cells after exposure to different doses (50, 100, and 200 μM) of GA and various periods of time (12, 24, and 48 h) led to a longer DNA migration smear (comet tail) occurred based on the single cell gel electrophoresis (Comet assay). These observations indicated that GA-induced DNA damage in PC-3 cells, which also confirm by 4,6-diamidino-2-phenylindole dihydrochloride staining and DNA agarose gel electrophoresis. Alternatively, results from real-time polymerized chain reaction assay also indicated that GA

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inhibited ataxia telangiectasia mutated, ataxia-telangiectasia and Rad3-related, O^6-methylguanine-DNA methyltransferase, DNA-dependent serine/threonine protein kinase, and *p53* mRNA expressions in PC-3 cells. Taken together, the present study showed that GA caused DNA damage and inhibited DNA repair genes as well as both effects may be the critical factors for GA-inhibited growth of PC-3 cells *in vitro*.

Keywords: gallic acid; DNA damage; comet assay; DNA repair; human prostate cancer PC-3 cells

INTRODUCTION

Adenocarcinoma of the prostate, the most frequently diagnosed noncutaneous cancer, is the second leading cause of cancer-related deaths among men in the United States (Wingo et al., 1995; Jemal et al., 2009). In Taiwan, prostate cancer also is the seventh of cancer-related deaths in men and about eight persons per 100,000 die annually from prostate cancer based on 2009 report from the Department of Health, Republic of China (Taiwan) (Liu et al., 2011). So far, the exact molecular mechanisms responsible for the development and progression of prostate cancer in human remain poorly investigated. Currently, there is as yet no effective therapy for human prostate cancer (Grossmann et al., 2001; Nieto et al., 2007). Hence, the understanding of the development in prostate cancer is urgent.

Gallic acid (3,4,5-trihydroxybenzoic acid, GA), a polyhydroxyphenolic compound and a basic unit of tannic acid, is widely distributed in the natural plants (Atkinson et al., 2004; Ng et al., 2004). GA exhibits various biological properties such as antibacterial (Kang et al., 2008), anti-inflammatory (Kim et al., 2006), antiviral (Kaur et al., 2009), antioxidant (Inoue et al., 2000), and anticancer effects (Kawada et al., 2001; Faried et al., 2007; Ji et al., 2009; Kaur et al., 2009; Lo et al., 2010). Much evidence has shown that GA is able to inhibit proliferation of tumor cells in culture by causing apoptosis and/or cell-cycle arrest (Agarwal et al., 2006; Veluri et al., 2006). In our laboratory, we also found that GA induced apoptosis in human lung cancer NCI-H460 cells via a caspase-3 and mitochondrion-dependent pathway and inhibited the *in vivo* tumor growth of NCI-H460 cells in xenograft models (Ji et al., 2009). Also, GA-induced DNA damage of lung cancer cells is examined by Comet assay (Ji et al., 2009). GA triggered apoptotic death in human melanoma A375.S2 cells through caspase-dependent and -independent pathways (Lo et al., 2010). Moreover, GA promoted macrophage phagocytosis in WEHI-3 leukemia mice *in vivo* (Ho et al., 2009). Recently, it was reported that GA induced cell-cycle arrest at G2/M phase through Chk2-mediated phosphorylation of Cdc25C in a bladder transitional carcinoma cell line (Ou et al., 2010), and this might be a DNA damage response as indicated by Ser-139 phosphorylation of histone H2A.X (Ou et al., 2010).

Until now, there is no available information addressing GA-induced DNA damage in human prostate cancer PC-3 cells. Therefore, in the present study, we focused on the *in vitro* GA-induced DNA damage and affected repair gene expression in human prostate cancer PC-3 cells. Results indicated that GA promoted DNA damage and inhibited DNA repair gene expression of PC-3 cells *in vitro*.

MATERIALS AND METHODS

Chemicals and Reagents

GA, dimethyl sulfoxide (DMSO), propidium iodide (PI), trypan blue, sodium chloride (NaCl), Tris–HCl, Na_2EDTA, Triton X-100, and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich Corp (St. Louis, MO). RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine, penicillin–streptomycin, trypsin-EDTA, and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) were obtained from Invitrogen Life Technologies (Carlsbad, CA). Tris/borate/EDTA (TBE) buffer was purchased from Amresco (Solon, OH). High-Capacity cDNA Reverse Transcription Kit and 2X SYBR Green PCR Master Mix was purchased from Applied Biosystems by Life Technologies (Foster City, CA).

Cell Culture

PC-3 human prostate cancer-cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were immediately placed onto 75-cm^2 tissue culture flasks and grown at 37°C under a humidified 5% CO_2 atmosphere with RPMI-1640 medium with 2 mM L-glutamine were adjusted to contain 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Flow Cytometric Assay and a PI Exclusion Method for the Percentage of Viable PC-3 Cells *In Vitro*

Cells (2×10^5 cells/well) maintained in 12-well plates were incubated with GA at final concentrations of 0, 50, 100, and 200 μM, and vehicle (1% DMSO) for 48 h, and exposed to 100 μM of GA for 0, 12, 24, and 48 h. Cells from each treatment were harvested and stained with propidium iodide (PI, 5 μg/mL) and then were analyzed with a flow cytometer (Becton-Dickinson FACSCalibur, San Jose, CA) equipped with an argon ion laser at 488 nm wavelength and calculated by using BD CellQuest Pro software

as previously described (Yeh et al., 2000; Chiang et al., 2011).

Comet Assay for DNA Damage in GA-Treated PC-3 Cells

Cells at a density of 2×10^5 cells/well seeded in 12-well plates were exposed to GA (0, 50, 100, and 200 μM) for 48 h and treated with final concentration of 100 μM GA for 12, 24, and 48 h, or vehicle (1% DMSO) in RPMI 1640 medium grown at 37°C in 5% CO_2 and 95% air. Cells were harvested for the examination of DNA damage by using the Comet assay (single-cell gel electrophoresis) according to the procedures of Chen et al. (2003) and Wang et al. (2006) with slight modifications. Briefly, the glass slides were precoated with 70 μL containing 0.5% (w/v) of normal melting point (NMP) agarose (Sigma-Aldrich Corp.) and 0.5% (w/v) low melting point (LMP) agarose (Sigma-Aldrich Corp.) allowed drying on a flat surface of the slides at room temperature. Subsequently, about 1×10^4 cells per sample from each treatment were gently mixed with 75 μL of 0.5% (w/v) LMP and rapidly layered onto the slides precoated with the mixtures [0.5% (w/v) of NMP agarose and 0.5% (w/v) LMP agarose] before a coverslip was covered at 4°C for 5 min. The coverslip was removed, and cells placed onto a glass slide were immersed in the lysis buffer containing 2.5 M NaCl, 10 mM Tris–HCl, 100 mM Na_2EDTA, and 1% (v/v) Triton X-100 and adjusted to pH 10 with NaOH at 4°C for 1 h. These slides were washed twice with ice-cold deionized water and transferred to an electrophoresis tank with alkalin buffer (300 mM NaOH and 1 mM Na_2EDTA at pH 13) at 4°C for 20 min. Thereafter, the electrophoresis was carried out at 30 V and 300 mA for 20 min, before slides were removed and flooded with neutralization buffer (0.4 M Tris–HCl at pH 7.5) at 4°C for 15 min. Slides were dried in methanol (Sigma-Aldrich Corp.) for 5 min before staining with 50 μL of PI (2.5 μg/mL), and comets were visualized and photographed by using a fluorescence microscope at 200× magnification as previously described (Chen et al., 2009b; Lu et al., 2010). For the quantification of DNA damage, PI-stained DNA tails were quantified by using CometScore software (Tritek Corp, Sumerduck, VA). It is shown that the comet tail that tends to increase rapidly with the levels of damage calculated from the head center. The data from comet tail length were expressed (fold of control) in mean ± SD at least three independent samples as described elsewhere (Chiang et al., 2011; Yu et al., 2011).

DAPI Staining in Apoptotic PC-3 Cells After Exposure to GA

Approximately 2×10^5 PC-3 cells/well onto 12-well plates were exposed to 0, 50, 100, and 200 μM of GA were incubated for 48 h under 5% CO_2 and 95% air at 37°C. Cells in each treatment were individually fixed with 3.7% (v/v) formaldehyde (Sigma-Aldrich Corp.) for 15 min and then stained by 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) dye for determining cell chromatin condensation. All samples were examined and photographed by using fluorescence microscopy as described elsewhere (Chiang et al., 2011; Yu et al., 2011).

DNA Agarose Gel Electrophoresis for Examining the DNA Damage in PC-3 Cells After GA Treatment

Cells (1×10^6 cells/well) in six-well plates were incubated with 0, 50, 100, 150, and 200 μM of GA for 24-h exposure. At the end of incubation, cells were centrifuged, and DNA was collected by using a Genomic DNA Purification kit (Genemark Technology Co., Tainan, Taiwan) as followed according to the manufacturer's protocol. The extracted DNA from each treatment was resuspended with 50 μL TBE buffer (0.045 M Tris, 0.045 M boric acid, 1 mM Na_2EDTA, and pH8.3 at 25°C). Approximately 1 μg/μL (20 μL) of genomic DNA was loaded in each well, and DNA agarose gel electrophoresis was performed using 1.8% agarose (Sigma-Aldrich Corp.). After ethidium bromide (EtBr, Sigma-Aldrich Corp.) staining, the DNA was photographed under UV light as described previously (Lai et al., 2010; Chiang et al., 2011).

Total RNA Extraction and Reverse Transcription Extracted from PC-3 Cells After Incubation with GA

Cells (1×10^6 cells/well) maintained in six-well plates were maintained in RPMI 1640 medium contained with or without 100 μM of GA and then were incubated for 24 h. Cells were washed twice with PBS and trypsinized, and, subsequently, the cell pellets were collected by centrifugation at $1000 \times g$ for 5 min at 4°C. The total RNA from each treatment was extracted by using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) as previously described (Mozaffarieh et al., 2010; Chiang et al., 2011). The RNA purity was measured the ratio of the absorbance at 260 and 280 nm (A260/A280), where a ratio ranging from 1.8 to 2.0 was considered to be pure for further experiment (Mozaffarieh et al., 2010). RNA samples from each treatment were then individually reverse-transcribed for 30 min at 42°C with High Capacity cDNA Reverse Transcription Kit to made first-strand cDNA according to the standard protocol of the supplier (Applied Biosystems, Carlsbad, CA). The product was aliquoted in equal volumes and stored at −20°C for real-time polymerized chain reaction (PCR) analysis.

TABLE I. The DNA sequence was evaluated using the primer express software

Primer Name	Primer Sequence (5′–3′)
Human *ATM*-F	TTTACCTAACTGTGAGCTGTCTCCAT
Human *ATM*-R	ACTTCCGTAAGGCATCGTAACAC
Human *ATR*-F	GGGAATCACGACTCGCTGAA
Human *ATR*-R	CTAGTAGCATAGCTCGACCATGGA
Human *MGMT*-F	CCTGGCTGAATGCCTATTTCC
Human *MGMT*-R	TGTCTGGTGAACGACTCTTGCT
Human *DNA-PK*-F	CCAGCTCTCACGCTCTGATATG
Human *DNA-PK*-R	CAAACGCATGCCCAAAGTC
Human *p53*-F	GGGTTAGTTTACAATCAGCCACATT
Human *p53*-R	GGGCCTTGAAGTTAGAGAAAATTCA
Human *GAPDH*-F	ACACCCACTCCTCCACCTTT
Human *GAPDH*-R	TAGCCAAATTCGTTGTCATACC

ATM, ataxia telangiectasia mutated; ATR, ataxia-telangiectasia and Rad3-related; MGMT, O^6-methylguanine-DNA methyltransferase; DNA-PK, DNA-dependent serine/threonine protein kinase and GAPDH, glyceraldehydes-3-phosphate dehydrogenase. Each assay was conducted at least twice to ensure reproducibility.

Real-Time PCR for Gene Expressions of ATM, ATR, MGMT, DNA-PK, and p53 in PC-3 Cells After GA Exposure

Quantitative PCR from each sample was carried out for amplifications included by the condition: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C using 1 μL of the cDNA reverse-transcribed as described earlier, 2X SYBR Green PCR Master Mix (Applied Biosystems), and 200 nM forward (F) and reverse (R) primers for each gene as shown in Table I. Finally, each assay was run on an Applied Biosystems 7300 Real-time PCR system in triplicates, and expression fold-changes were derived using the comparative threshold cycles (C_T) method (Heid et al., 1996). Values were shown to normalize the human GAPDH mRNA expression as an endogenous/internal control gene as described elsewhere (Lu et al., 2009; Chiang et al., 2011).

T1

Statistical Analysis

All data were presented as the means (S.D.) and one-way ANOVA followed by Dunnett's test was used to analyze differences between GA-treated and untreated (control) groups. All the statistical analyses were performed $^*P < 0.05$, which was considered significant.

RESULTS

GA Decreased the Percentage of Viable PC-3 Cells

The PC-3 cells were incubated with GA at 0, 50, 100, and 200 μM for 48 h and exposed to 100 μM of GA for 0, 12, 24, and 48 h. The cells were harvested for measuring the percentage of viable PC-3 cells by flow cytometry, and results are shown in Figure 1, which indicated that GA decreased the viability of PC-3 cells, and these effects are in a dose- and time-dependent manner [Fig. 1(A,B)].

F1

GA-Triggered DNA Damage in PC-3 Cells Was Examined by Comet Assay

It is shown that GA induced cytotoxic effects (decrease the percentage of viable cells) on PC-3 cells (Fig. 1) to confirm GA whether or not affects DNA damage in PC-3 cells. Thus, the comet assay was selected for determining the DNA damage in examined PC-3 cells. The results are shown in Figure 2(A,B), which indicated that GA-induced DNA damage of PC-3 cells in a dose-dependent effect [Fig. 2(A,B)]. The higher concentrations (100–200 μM) of GA led to a longer

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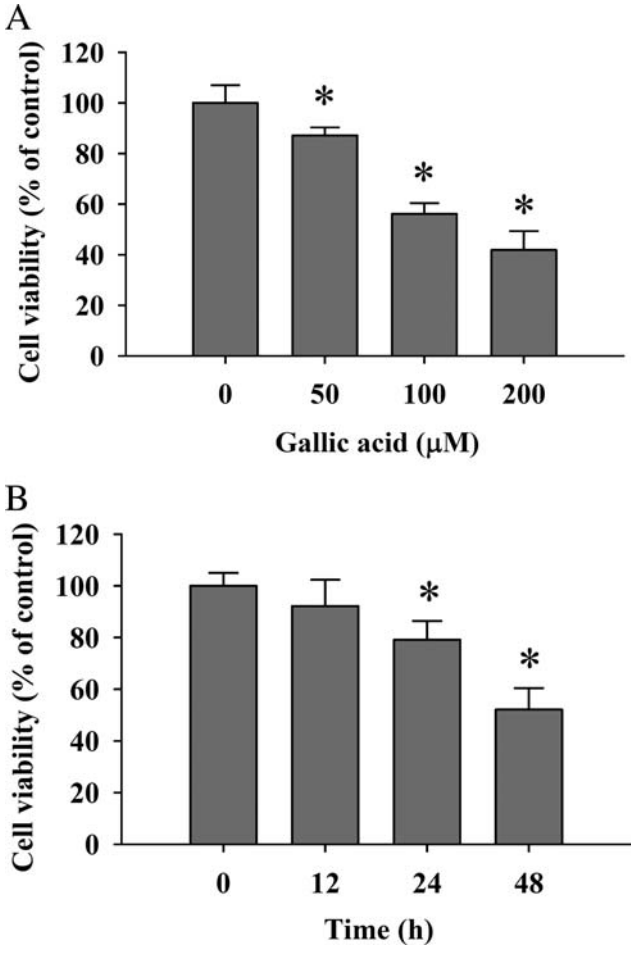

Fig. 1. GA decreased the percentage of viable PC-3 cells. Cells (2×10^5 cells/well) seeded in 12-well plates were incubated with GA at final concentrations of 0, 50, 100, and 200 μM and vehicle (1% DMSO) for 48 h (A). Cells were treated with 100 μM of GA for 0, 12, 24, and 48 h (B). Cells from each treatment were stained with PI (5 μg/mL) and analyzed by flow cytometry as described in the Materials and Methods section. $^*P < 0.05$ was considered significant.

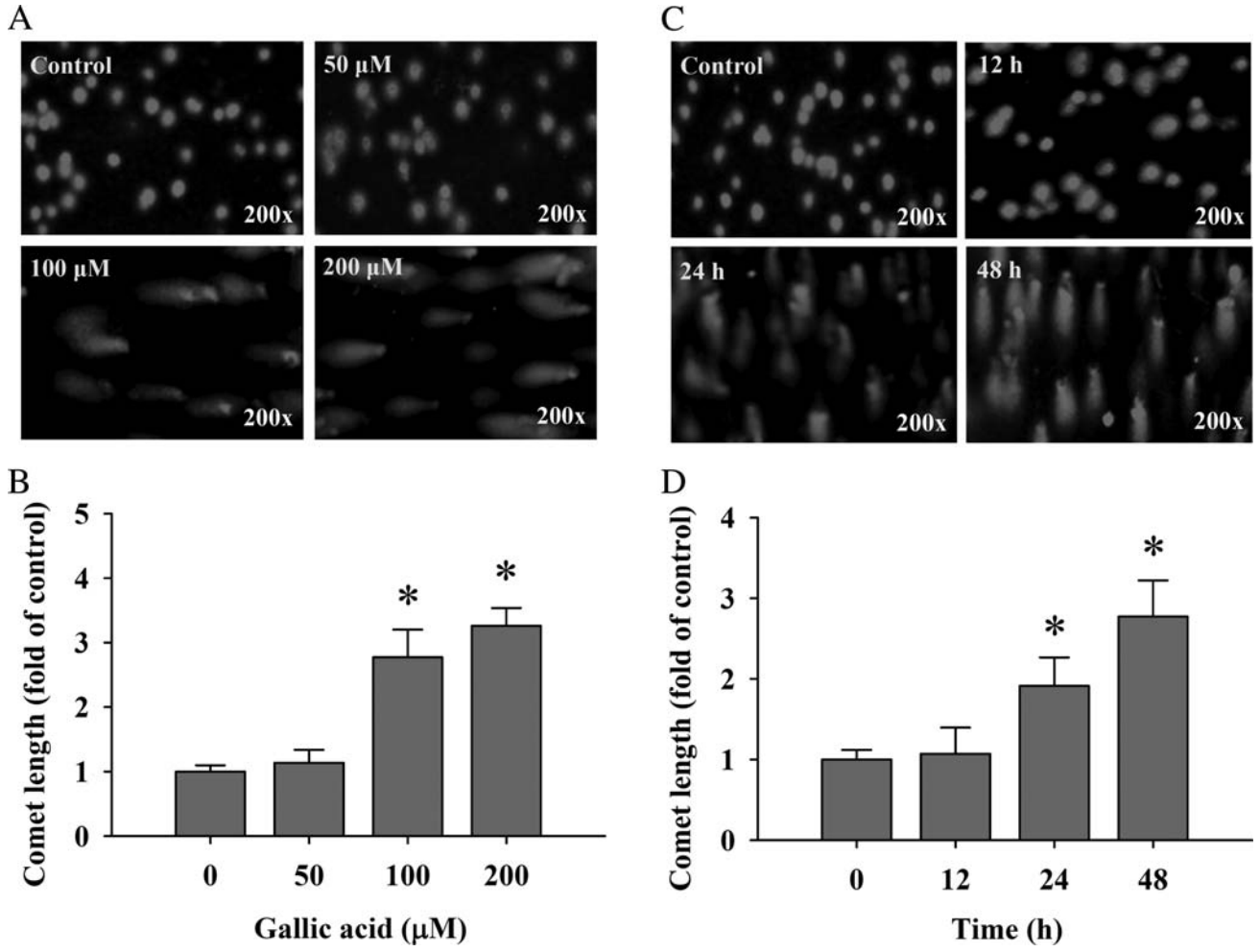

Fig. 2. GA-induced DNA damage in PC-3 cells was examined by Comet assay. Cells (2×10^5 cells/well) were incubated with 0, 50, 100, and 200 μM of GA for 48 h and exposure to 100 μM of GA for 12, 24, and 48 h in PC-3 cells. DNA damage was determined by Comet assay as described in the Materials and Methods section. Representative images of Comet assay for dose-dependent effect (A) and quantification for comet length (fold of control) (B); representative pictures of Comet assay for a time-dependent response (C) and quantification (D). *$P < 0.05$ shows a significant difference between control and treated cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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DNA comet tail (migration smear). Figure 2(B) indicates that more DNA was damaged in PC-3 cells when compared with the control sample. Alternatively, GA also increased DNA damage in PC-3 cells after exposure to 100 μM of GA for various intervals of time (12–48 h), and there is a time-dependent response as can be seen in Figure 2(C,D).

GA Induced DNA Condensation and Fragmentation in PC-3 Cells

Results from DAPI-staining analysis indicated that GA increased DNA condensation and break in PC-3 cells [Fig. 3(A,B)], and this effect is a dose-dependent response. To confirm the induction of DNA damage and condensation in GA-treated PC-3 cells, we also used DNA agarose gel electrophoresis for examining the DNA fragmentation. DNA was isolated from each treatment of PC-3 cells for 24 h, and then DNA fragments were determined by DNA agarose gel electrophoresis. Results shown in Figure 3(C) indicated that DNA damage, condensation, and fragments were carried out in GA-treated PC-3 cells [Fig. 3(C)]. The higher doses of GA (100–150 μM) led to more DNA damage and fragments in PC-3 cells than that of low dose (50 μM) incubation in the examined cells.

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GA Influenced DNA Damage and Repair Genes Expression on PC-3 Cells by Real-Time PCR

Results from comet assay, DAPI staining, and DNA agarose gel electrophoresis have shown that GA induced

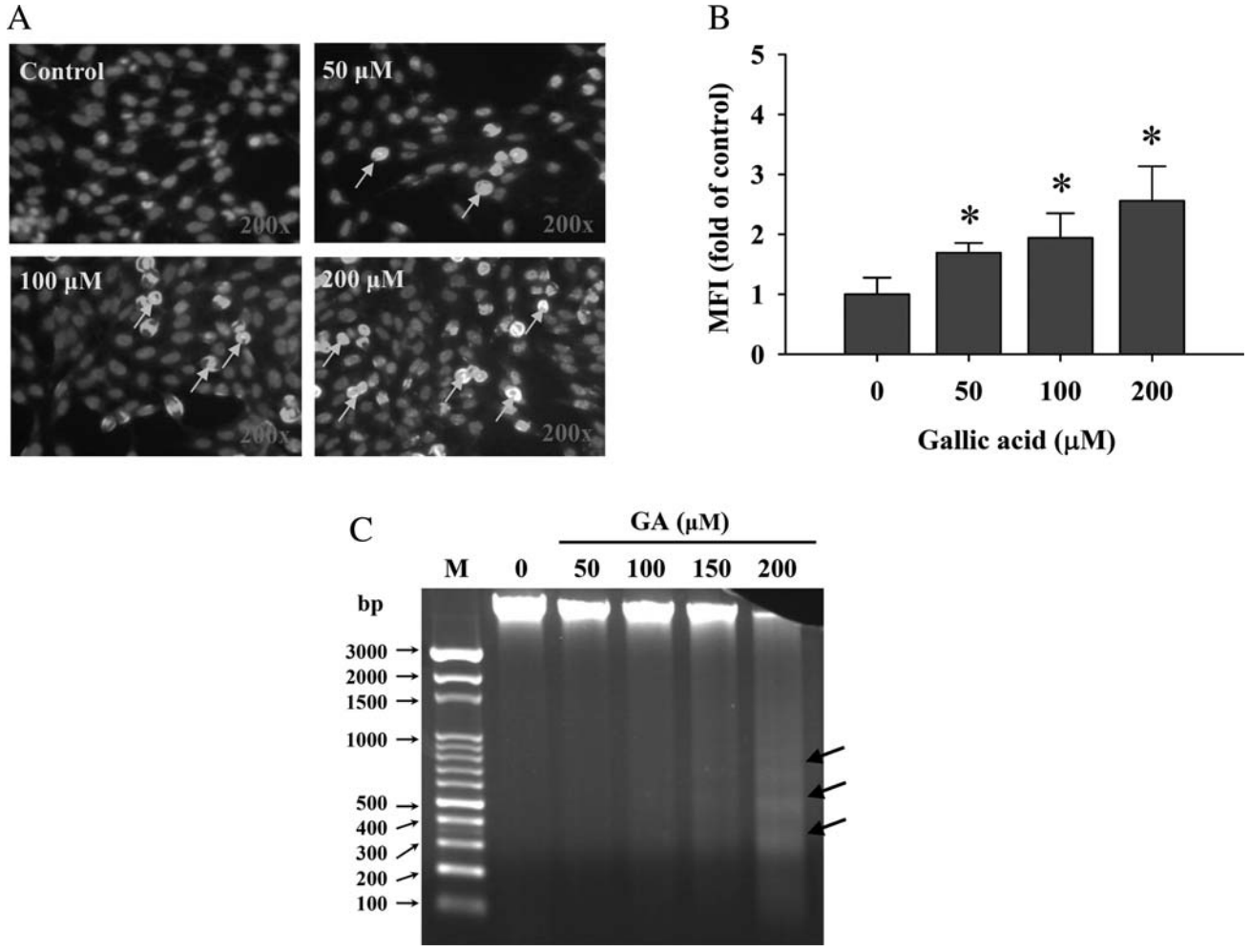

Fig. 3. GA-induced DNA condensation and damage in PC-3 cells was examined by DAPI staining and DNA agarose gel electrophoresis. Cells were incubated with 0, 50, 100, and 200 μM of GA for 48 h and then were harvested for DAPI staining (A) and quantification (B). The arrow shows the DNA condensation in PC-3 cells. MFI means mean fluorescence intensity. Cells were exposed to 0, 50, 100, 150, and 200 μM of GA for 24 h, and DNA were isolated from each treatment for gel electrophoresis (C) as described in the Materials and Methods section. M, marker. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DNA damage, condensation, and fragments in PC-3 cells. To investigate whether or not GA affects DNA damage and repair genes expressions, PC-3 cells were treated without (untreated control) or with 100 μM GA for 24 h. Thereafter, total RNA was isolated from each treatment, and the associated genes expressions were examined by real-time PCR. The results shown in Figure 4 revealed F4 that genes expression-associated with DNA damage and repair including ataxia telangiectasia mutated (*ATM*), *ATR*, methylguanine DNA methyltransferase (*MGMT*), DNA-dependent serine/threonine protein kinase (*DNA-PK*), and *p53* mRNA from PC-3 cells after 24-h treatment of GA were suppressed (Fig. 4) when compared with the untreated control group.

DISCUSSION

It is well documented that agents induced DNA damage, which is associated with cytotoxic effects including cell death and inhibition of cell growth (Kondo et al., 2010; Lou et al., 2010; Namdar et al., 2010). Previous studies have shown that GA induced cytotoxic effects in many types of human cancer cell lines (Kawada et al., 2001; Agarwal et al., 2006; Veluri et al., 2006; Faried et al., 2007; Ji et al., 2009; Kaur et al., 2009). However, regarding the effects of GA on DNA damage and repair gene expression in human prostate cancer cells is not well investigated. Thus, in the present study, we found that a dose- and time-dependent increase in DNA damage was observed in PC-3

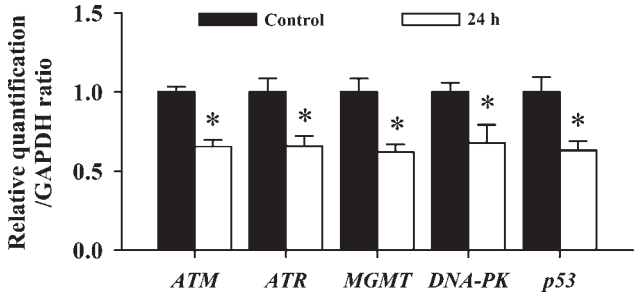

Fig. 4. GA-affected DNA damage and -inhibited repair genes expression in PC-3 cells was determined by real-time PCR. The total RNA was extracted from the PC-3 cells after treatment without (control) and with 100 μM GA for 24 h, and RNA samples were reverse-transcribed cDNA then for real-time PCR as described in the Materials and Methods section. The ratios of *ATM, ATR, MGMT, DNA-PK*, and *p53* mRNA/GAPDH are shown in panel. Data represent mean $\pm$ SD of three experiments. *$P < 0.05$ is considered significant when compared with the control sample.

cells after exposure to various concentrations of GA (Fig. 2), which was associated with a loss of cell viability (Fig. 1). These results and conclusions showed a significant increase in the tail moment of the comets of PC-3 cells from comet assay (single-cell gel electrophoresis) (Fig. 2). The longer of comet tail meant that the higher DNA damage and DNA condensation and fragments from DAPI staining [Fig. 3(A,B)] and DNA agarose gel electrophoresis [Fig. 3(C)], respectively, in GA-treated PC-3 cells.

Numerous studies have been demonstrated that comet assay is a high sensitive technique for DNA damage examination (Pool-Zobel et al., 1994; Ashby et al., 1995). Furthermore, Comet assay can be used as a measurement for trend-break formation during the process of excision repair of DNA may also cause DNA migration (Tice et al., 1990). It is well known that some of the major characteristic of apoptosis are DNA condensation (Chiang et al., 2011) and DNA ladder (DNA fragmentation) of nuclei (Bakshi et al., 2010; Ramachandran et al., 2011). Herein, our results from DAPI staining and DNA agarose gel electrophoresis demonstrated that GA induced DNA condensation and fragmentation in PC-3 cells (Fig. 3). It was reported that GA induction of apoptosis also part through the reactive oxygen species (ROS) production in mammalian cells (Chen et al., 2009a; You and Park, 2011). Thus, GA-induced DNA damage might be mediated through the production of ROS in PC-3 cells. Apparently, further studies are needed to establish the role of the interaction of GA with DNA in carcinogenesis.

It was also reported that in mammalian cells, the DNA damage can be reduced by DNA repair through eliminating DNA lesions (Moeller et al., 2010; Kryston et al., 2011). These ROS, produced either directly by tumors or indirectly via inflammatory responses, can cause DNA damage in healthy neighboring cells as well as distant sites (Kryston et al., 2011). Agents may cause DNA damage by an indirect pathway through promoting oxidative stress and inflammatory responses through dysfunction of mitochondria or inflammasomes (Kryston et al., 2011; Nabeshi et al., 2011). It was reported that ROS interacted with the biological molecules and disrupt the normal synthesis and repair of DNA, and this disruption is primarily associated with inhibition/inactivation of antioxidant key proteins as well as DNA repair enzymes induced by ROS-damage to these biomolecules (Gillard et al., 2004; Eiberger et al., 2008).

In response to genotoxic agents, cells remained homeostatic via activation of signaling pathways that turn on specific gene expressions; one of crucial guardian of genomic integrity is the checkpoint kinase ATM (Cuadrado et al., 2006). ATM and ATR are two master checkpoint kinases activated by double-stranded DNA breaks (DSBs) (Lavin, 2007). Our results showed that GA inhibited DNA repair genes expression including *ATM, ATR, DNA-PK, MGMT*, and *p53* in PC-3 cells (Fig. 4). In response to DSBs, the ATM kinase phosphorylates and regulates a cascade of downstream effectors such as checkpoint kinase Chk2 and other components of the DNA repair pathways and the cell-cycle check points, in order to minimize the risk of genetic damage (Lavin, 2007; Matsuoka et al., 2007). DNA-PK plays a critical role in DNA damage repair (Mi et al., 2009). The O^6-MGMT reduces cytotoxicity of therapeutic or environmental alkylating agents (Jesien-Lewandowicz et al., 2009).

In conclusion, GA induced DNA damage in PC-3 cells and then followed by inhibiting DNA repair-associated gene expressions including *ATM, ATR, MGMT, DNA-PK*, and *p53* and thereafter led to DNA damage maintain as the proposed model can be shown in Figure 5. F5

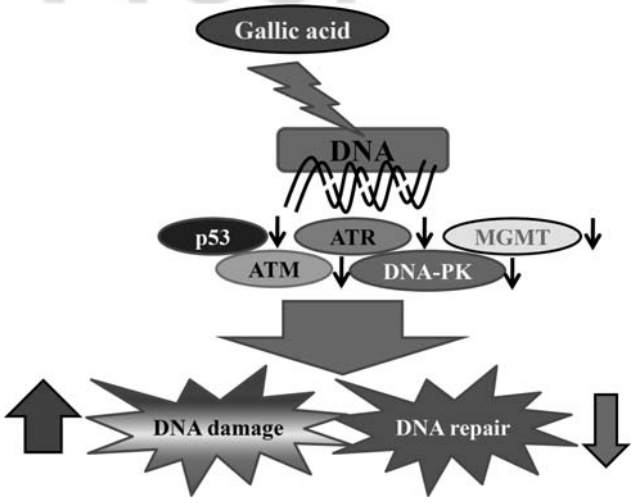

Fig. 5. The possible flow chart for GA-inhibited gene expression of DNA damage and repair in human prostate cancer PC-3 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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