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Glyphosate induces human breast cancer cells growth via estrogen receptors

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ABSTRACT

Glyphosate is an active ingredient of the most widely used herbicide and it is believed to be less toxic than other pesticides. However, several recent studies showed its potential adverse health effects to humans as it may be an endocrine disruptor. This study focuses on the effects of pure glyphosate on estrogen receptors (ERs) mediated transcriptional activity and their expressions. Glyphosate exerted proliferative effects only in human hormone-dependent breast cancer, T47D cells, but not in hormone-independent breast cancer, MDA-MB231 cells, at 10^{-12} to 10^{-6} M in estrogen withdrawal condition. The proliferative concentrations of glyphosate that induced the activation of estrogen response element (ERE) transcription activity were 5-13 fold of control in T47D-KBluc cells and this activation was inhibited by an estrogen antagonist, ICI 182780, indicating that the estrogenic activity of glyphosate was mediated via ERs. Furthermore, glyphosate also altered both ERα and β expression. These results indicated that low and environmentally relevant concentrations of glyphosate possessed estrogenic activity. Glyphosate-based herbicides are widely used for soybean cultivation, and our results also found that there was an additive estrogenic effect between glyphosate and genistein, a phytoestrogen in soybeans. However, these additive effects of glyphosate contamination in soybeans need further animal study.

1. Introduction

Glyphosate, *N*-(phosphonomethyl) glycine, is widely used as an active ingredient of herbicide products to control weeds in cropped and non-cropped fields around the world. In addition, glyphosate formulations have been used extensively in genetically modified glyphosate-resistant plants (Acquavella et al., 2004). The herbicidal activity of glyphosate is rather specific on the targets with the inhibition of the shikimate pathway which only presents in plants and micro-organisms (Solomon et al., 2007). Glyphosate is considered as a non toxic herbicide because of its low LD_{50} (the concentration that caused 50% deaths); >4 g/kg (WHO, 1994). However, the reproductive toxicities of glyphosate have been extensively studied in both animals and human. Up to now, the endocrine disrupting effects of glyphosate were not observed in the *in vivo* but the *in vitro* studies and the epidemiological studies have still conflicted in those findings due to their differences in the experimental designs, methodology and confounding factors (Brake and Evenson, 2004; Dallegrave et al., 2007; Daruich et al., 2001; Mandel et al., 2005; Marc et al., 2004; McDuffie et al., 2001). The synergistic effects of glyphosate and surfactants in its herbicide formulations have been concerned especially the endocrine disrupting activity (Richard et al., 2005). Most studies found that the adjuvants or surfactants in most formulations were more toxic and could enhance the toxic effects of glyphosate (Gasnier et al., 2009; Marc et al., 2004; Walsh et al., 2000). Glyphosate at concentrations used in agriculture (21–42 mM) was found to be toxic to human embryonic and placental cells (Benachour et al., 2007; Richard et al., 2005). Roundup®, a popular formulation could disrupt the synthesis of hormones in the mouse MA-10 Leydig tumor cell line (Benachour et al., 2007; Walsh et al., 2000). Glyphosate has been shown to disrupt the animal cell cycle in urchin eggs based on its surfactant carrying in commercial formulation (Marc et al., 2004). Recently, it was reported that at lower non-toxic concentrations of Roundup® and glyphosate (<1 μg/L), the main endocrine disruption is a testosterone decrease by 35%. Most potential adverse health effects were reported on the commercial glyphosate formulations. The expression of estrogen-regulated genes relating to tumor for-

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mation and tumor growth in hormone dependent human breast cancer MCF-7 cells were reported to be disrupted (Hokanson et al., 2007). Furthermore, synergistic effects between glyphosate and estrogen (17β-estradiol or E2) have been demonstrated. Glyphosate was reported to have a disrupting effect on estrogen receptor alpha (ERα) and beta (ERβ) transcriptional activities in HepG2 cells transiently transfected with ERE-TK-Luciferase and on androgen receptor (AR) in MDA-MB453-kb2 cells (Gasnier et al., 2009). These toxic effects were reported to be more frequent with glyphosate-based herbicides than that with glyphosate alone.

This present study aims to evaluate the estrogenic effects of glyphosate alone at the range of concentrations that has been reported in environmental conditions and exposed human. Estrogenic and/or antiestrogenic effects of glyphosate were investigated and compared with endogeneous estrogen in the estrogen dependent human breast cancer cells T47D. Since glyphosate-based herbicides have been used intensively in soybean cultivation and soybean also contains the phytoestrogen, genistein, the interactive effects of these two compounds were also studied.

2. Materials and methods

2.1. Chemicals and reagents

Glyphosate (>98%) was purchased from AccuStandard (New Haven, CT, USA). 17β-estradiol (E2) was obtained from Sigma–Aldrich (St. Louis, MO, USA). ICI 182780 and genistein were purchased from Tocris Bioscience (Ellisville, MS, USA). All the other reagents and chemicals were of analytical grade and obtained from commercial sources.

2.2. Cell lines and culture conditions

A hormone-dependent human breast cancer, T47D, a stably ERE-luc construct transfected hormone-dependent breast cancer, T47D-KBluc, and a hormone-independent human breast cancer, MDA-MB231, were purchased from the American Type Culture Collection (ATCC) (Rockville, MD, USA). T47D and T47D-KBluc cells were maintained in recommended standard medium of RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (JR Scientific, Woodland, CA, USA), 4.5 g/L D-glucose, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/mL penicillin/streptomycin (P/S) and 8 mg/L insulin. MDA-MB231 cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin/streptomycin (P/S) and 1% non-essential amino acid. All cells were cultured in a humidified incubator with 5% CO_2 and 95% air at 37 °C. Culture medium and supplements were purchased from Gibco-Invitrogen Life Technology (Carlbad, CA, USA).

2.3. In vitro estrogen receptor activation-reporter assay

In order to study the estrogenicity and/or antiestrogenicity of glyphosate, the T47D-KBluc cell; stably transfected with a triplet ERE (estrogen response element)-promoter-luciferase reporter gene construct, was used in this study (Wilson et al., 2004). To minimize the effect of estrogen in the medium, five days prior to the assay, cells were switched to grow in a non-phenol red RPMI modified medium with a replacement of 10% FBS to 10% dextran-charcoal treated FBS (CSS) (HyClone, South Logan, UT, USA), together with all other supplements except penicillin/streptomycin. One day prior to the assay, cells were seeded at 10^4 cells/100 μL/well in 96-well luminometer plates (Corning Incorporated, Corning, NY, USA) and were allowed to attach overnight. Dosing media was further modified by reduction to 5% CSS. Media was then replaced with 100 μL/well of dosing media in which the final concentration of glyphosate ranged from 10^{-12} to 10^{-6} M. The same range of estradiol (E2) concentrations was used as the positive control agonist for estrogen receptor activation. The dosing media was used as the negative control and wells without cells were used as blank. After 24 h incubation, cells were washed with 100 μL phosphate buffered saline (Sigma–Aldrich, St. Louis, MO, USA) at room temperature, then harvested in 25 μL lysis buffer (Promega, Madison, WI, USA). The luciferase assay was performed by injecting 50 μL of reaction buffer (25 mM glycylglycine, 15 mM $MgCl_2$, 5 mM ATP, 0.1 mg/mL BSA, pH 7.8) and 50 μL of 1 mM D-luciferin (Promega, Madison, WI, USA) by using microplate luminometer (SpectraMax L, Molecular Devices, Sunnyvale, CA, USA) and fluorescent intensity was measured. The luciferase activity was quantified as relative light units (RLU).

2.4. Cell viability MTT assay

Cell growth and viability were tested using the 3-(4,5-dimetylthiazol,2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma–Aldrich, St. Louis, MO, USA) reagent assay. Cells were seeded at 10^4 cells/100 μL/well in 96-well microtiter plates. For the E2 withdrawal condition, cells were cultured in 10% CSS and a non-phenol red RPMI medium containing all supplemented reagents for 4-days before seeding. After 24 h incubation for the attachment, the cells were treated with varying concentrations of E2 or glyphostae ranging from 10^{-12} to 10^{-6} M. In the present of E2 receptor antagonist condition, E2- or glyphosate-treated cells were co-incubation with ICI 182780 (1 and 10 nM). After 24 h incubation period, the medium was removed and 10 μL of MTT [5 mg/mL in phosphate-buffered saline (PBS)] in 90 μL medium was added into each well. Cells were further incubated for 4 h, then the medium was removed and 100 μL dimethyl sulfoxide (Merck, Whitehouse Station, NJ, USA) was added to each well to dissolve the precipitated dye. The optical density was read at 570 nm/650 nm using microplate readers (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA, USA). Cell sensitivity to a chemical was expressed as the % cell viability compared to the control (vehicle treated) cells.

2.5. Western blot analysis

Whole-cell extracts were prepared from cells treated for 6 and 24 h with 10^{-12}, 10^{-9}, and 10^{-7} M glyphosate and non-treated control in two medium conditions, completed medium, and hormone-withdrawal medium by lysis of cold PBS-washed cells in lysis buffer [50 mM Tris–HCl (Sigma, USA), 150 mM NaCl (Sigma, USA), 1% Triton-X (Merck, USA), 1 mM EDTA (Merck, USA), 1 mM sodium orthovanadate (Na_3VO_4, Sigma, USA), 100 mM sodium fluoride (NaF, Sigma, USA), protease cocktail inhibitor (Calbiochem, Germany) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF, Calbiochem, Germany)]. The lysates were then sonicated, incubated on ice for 30 min, and supernatants were collected from centrifugation at 14,000 rpm for 30 min at 4 °C. The lysates were either processed or stored at −80 °C until use. The protein concentration was measured using Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA). Each lysate was aliquot for an equal amount of protein, 30 μg, before mixing with Laemmli loading buffer (62.5 mM Tris–HCl, 25% glycerol, 2% SDS, 0.01% bromophenol red, pH 6.8, containing 5% 2-mercaptoethanol), and then boiled at 95 °C for 5 min. These samples were resolved over 7.5% polyacrylamide-SDS gels using a Mini-PROTEIN II system (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL, USA) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories Hercules, CA). The membrane was blocked with blocking solution (5% non-fat dry milk in 10 mM Tris–HCl, pH 7.6, 160 mM NaCl, and 0.05% Tween-20 (USB Corporation, Cleveland, OH, USA) for 1 h at room temperature. The membrane was probed overnight with primary antibody (ERα 1:1000, ERβ 1:1000 or Beta-actin 1:10,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then washed three times, each for 10 min with Tris-Buffered Saline with 0.05% Tween-20 (TBS-T). HRP-conjugated secondary antibodies (1:3000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added to the membrane for 2 h at room temperature. The membranes were washed three times, each for 10 min with TBS-T. Protein visualization was achieved by using an enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL, USA) and the emitted light was captured on film (Kodak Co., Rochester, NY, USA). The signals on the films were quantified using densitometer (Bio-Rad GS 710 calibrated imaging, Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Cell number counting

T47D Cells were prepared in the E2 withdrawal condition 4 days before assay. Cells were placed into a 24 well culture plate (Corning Incorporated, Corning, NY, USA) with 10^4 cells/mL/well and incubated overnight in CO_2 incubator to allow cells to attach. Media was then replaced with 1 mL of treatment solution and left in CO_2 incubator for 72 h. Cells were washed with 1 mL phosphate buffered saline at room temperature and then 100 μL of a trypsin–EDTA were added to break the cells from their contacts. After trypsinization, most of the cells were removed from contact with the plate, and floated. The cell pellets were resuspended in 900 μL of basal media solution and dissociated cells by aspirating into a 5-mL syringe through a 21G needle and expelling the contents. The experiment was repeated twice and an aliquot of the cells was taken to count the number by using a counter analyzer (Z1 coulter particle counter, Beckman Coulter, Miami, FL, USA).

2.7. Statistical analysis

Data are presented as the means ± SE. Statistical significance was determined using the Student's *t*-test. A two-tailed *P* value less than 0.05 was evaluated as a statistically significant difference.

3. Results

3.1. Glyphosate induces T47D, hormone dependent breast cancer cell growth

The hormone-dependent T47D and hormone-independent MDA-MB231 cell lines were studied both in completed medium

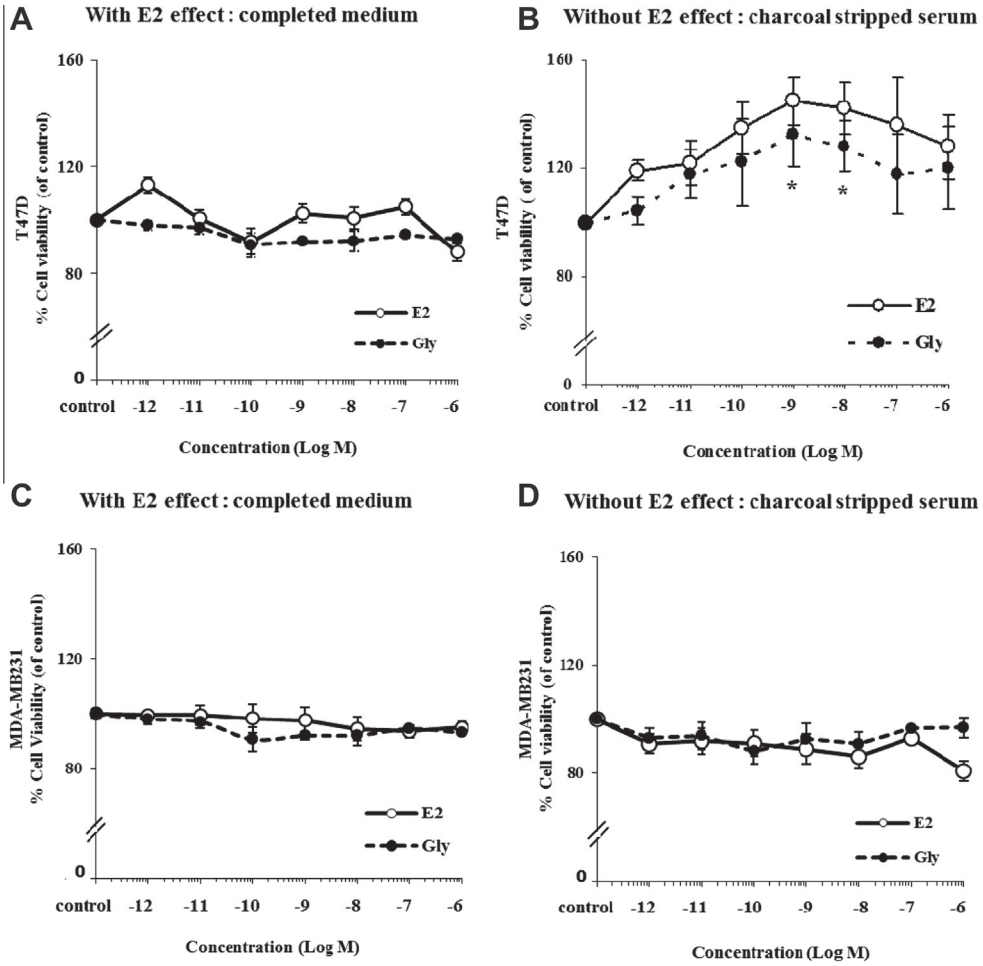

Fig. 1. Concentration-effect relationship of E2 and glyphosate on human breast cancer T47D (A and B) and MDA-MB231 cells (C and D). Cells were treated with varying concentrations ranging from 10^{-12} to 10^{-6} M of E2 and glyphosate. Cell viability was compared between in completed medium (A and C) and in hormone withdrawal medium (B and D) by using MTT assay at 24 h ($n = 3$, $^{*} = p \leqslant 0.05$, significantly different as compared to control).

and estrogen withdrawal medium to differentiate the effects of glyphosate from endogeneous estrogen. E2 at a concentration range from 10^{-12} to 10^{-6} M was used as a positive control. The cell viability was observed by using MTT cell viability assay. The results showed that T47D and MDA-MB231 cells exhibited different patterns of responses to glyphosate (Fig. 1). Glyphosate caused the proliferative effects of T47D approximately 15–30% in the absence of E2 condition (Fig. 1B). This effect was about a half of E2 response which is the most potent agonist in hormone dependent ER-positive breast cancer cell. Meanwhile, glyphosate had no effect on the growth of MDA-MB231 cells both in the absence or presence of E2.

3.2. The proliferative effect of glyphosate is mediated via estrogen receptors

Due to the fact that the proliferative effect of glyphosate occurred only in T47D cells in the absence of E2 condition, it was hypothesized that ER signaling may be involved in the glyphosate-induced proliferative effect. T47D cells were further studied using pure ER antagonist, ICI 182780, to inhibit the estrogen receptor mediated response. The effective concentration (1 nM) of ICI 182780 was added to varying concentrations of glyphosate and E2 to observe its antagonistic activity. The results showed that ICI 182780 at 1 nM mitigated the proliferative effects of both glyphosate and E2. Furthermore, higher concentration of ICI 182780 (10 nM) completely inhibited the growth promoting effects of glyphosate (Fig. 2). These results suggest that glyphosate may produce the proliferative effect via ER.

3.3. Glyphosate induces ERE-transcription activity via estrogen receptors

We further investigated the estrogenic effect of glyphosate on ERE-transcription activity. T47D-KBluc cells, which stably transfected with a triplet ERE (estrogen response element)-promoter-luciferase reporter gene construct, were treated with the proliferative concentrations of glyphosate. The results showed that glyphosate at a concentration range from 10^{-12} to 10^{-6} M induced ERE activation 5–13-fold of the control and these effects were less than about a half of that induced by E2 (Fig. 3A). Furthermore, glyphosate co-incubation with a pure ER antagonist, ICI 182780 exhibited the significant reduction in responses. Indeed, ICI 182780 at the concentration of 10 nM completely inhibited ERE transcriptional activity of glyphosate (Fig. 3A). These results correlated with the earlier growth promotion study, confirming that glyphoste at low concentrations (10^{-12} to 10^{-6} M) produce proliferative effects in hormone dependent breast cancer cells via ER.

Since glyphosate could induce cell proliferation and ERE activation via ER, next we investigated the potential effects of glyphosate on endogenous E2 signaling. Cells were co-incubated with glyphosate and E2. The results revealed that glyphosate suppressed

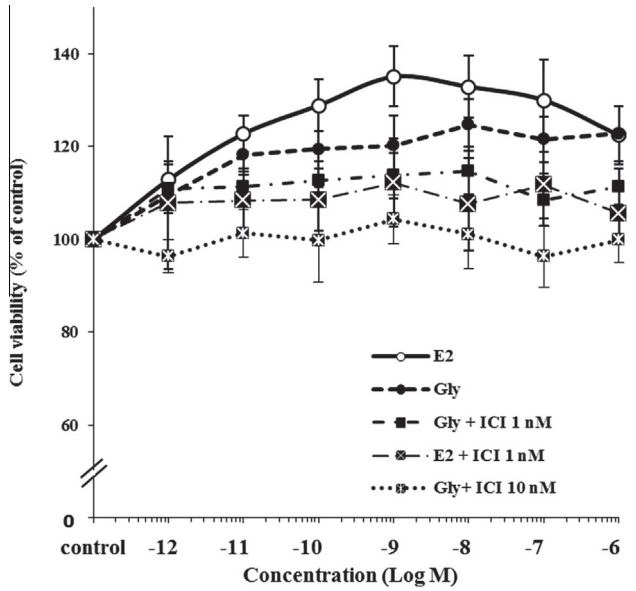

Fig. 2. Proliferative effects of E2 and glyphosate on human breast cancer T47D cells. T47D cells were treated with varying concentrations of E2 and glyphosate ranging from 10^{-12} to 10^{-6} M and co-incubation with ICI 182780 (1 or 10 nM). Cells were cultured in hormone withdrawal medium for 5 days prior treatments. The cell viability was detected by MTT assay at 24 h. Each point was plotted from the mean value of three independent experiments ± SE as shown in the graph ($^{*}p \leqslant 0.05$ significantly different as compared to glyphosate alone).

the E2-induced ERE activation (Fig. 3B). This result suggests that in the presence of endogenous agonist (E2), glyphosate behaves as an antagonist.

3.4. Glyphosate modulates the expression of ERα and ERβ in human breast cancer cells

We demonstrated that the induction of ERE transcription activity by glyphosate was mediated via ERs. Next, the expressions of proteins that involved in the classical ERs including ERα and ERβ, were studied by using Western blot technique. The results demonstrated that glyphosate altered the levels of ERα and ERβ proteins (Fig. 4A–D). At 6 h of exposure, glyphosate increased the levels of both ERα and ERβ in a concentration-dependent manner while at 24 h of exposure, only ERα showed a significant induction at the highest glyphosate concentration (10^{-7} M) compared to the control group. In addition, ERβ protein levels were not changed in glyphosate-treated group when compared to the control group after 24 h of exposure. This result suggests that glyphosate alters the expression of both ERα and ERβ in human breast cancer cells.

3.5. Interactive effects of glyphosate and phytoestrogen genistein

3.5.1. Genistein induces T47D cell proliferation and ERE activation

The phytoestrogen, genistein, is a major isoflavone found in soybeans. Genistein has a structure similar to E2 and displays estrogenic activity through ER signaling pathways (Seo et al., 2006). The results showed that genistein at a concentration range 10^{-9} to 10^{-4} M produced the concentration dependent proliferative effects (104–170% of the control), with the significant effect starting from 10^{-8} M. In addition, we also found that genistein at the highest tested concentration (10^{-3} M) had the inhibitory effect (Fig. 5A). The results were similar to previously described by Wang and his colleagues that genistein stimulated growth of MCF-7 cells at concentrations 10^{-8} to 10^{-6} M while higher concentrations (>10^{-5} M) of genistein inhibited cell growth (Wang et al., 1996).

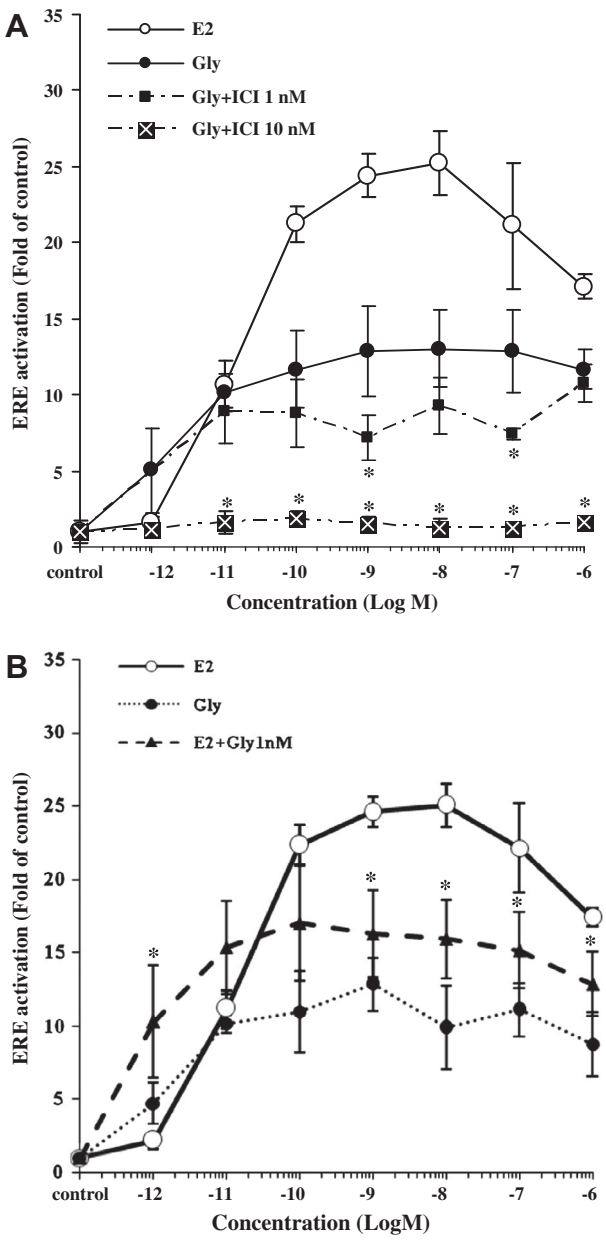

Fig. 3. The effects of 17β-estradiol (E2), glyphosate, and glyphosate co-incubation with ICI 182780 on ERE transcription activity in T47D-KBluc cells (A). Cells were cultured in E2 withdrawal medium for 5 days before the treatment in each experiment. ICI 182780 at the concentrations 1 and 10 nM were used. The experiment was observed at 24 h treatment ($n = 3$, $^{*} = p \leqslant 0.05$, significantly different as compared to glyphosate alone). Glyphosate at 1 nM suppressed to the E2 effects along varying concentrations (B) ($n = 4$, $^{*}p \leqslant 0.05$ significantly different as compared to glyphosate alone).

Genistein also demonstrated the ability to stimulate ERE-gene transcription activity at the concentration range used in the cell viability study (Fig. 5B). Genistein at the concentrations of 10^{-11} to 10^{-6} M exhibited concentration dependent ERE-activation which was approximately 5–25-fold of control.

3.5.2. The additive effects of genistein on glyphosate-induced ERE activation

Glyphosate is a herbicide extensively used in soybean plantations. Therefore, glyphosate has the potential to contaminate soybean products. Thus, it is interesting to evaluate whether there is an additive or synergistic effect of both compounds on the growth

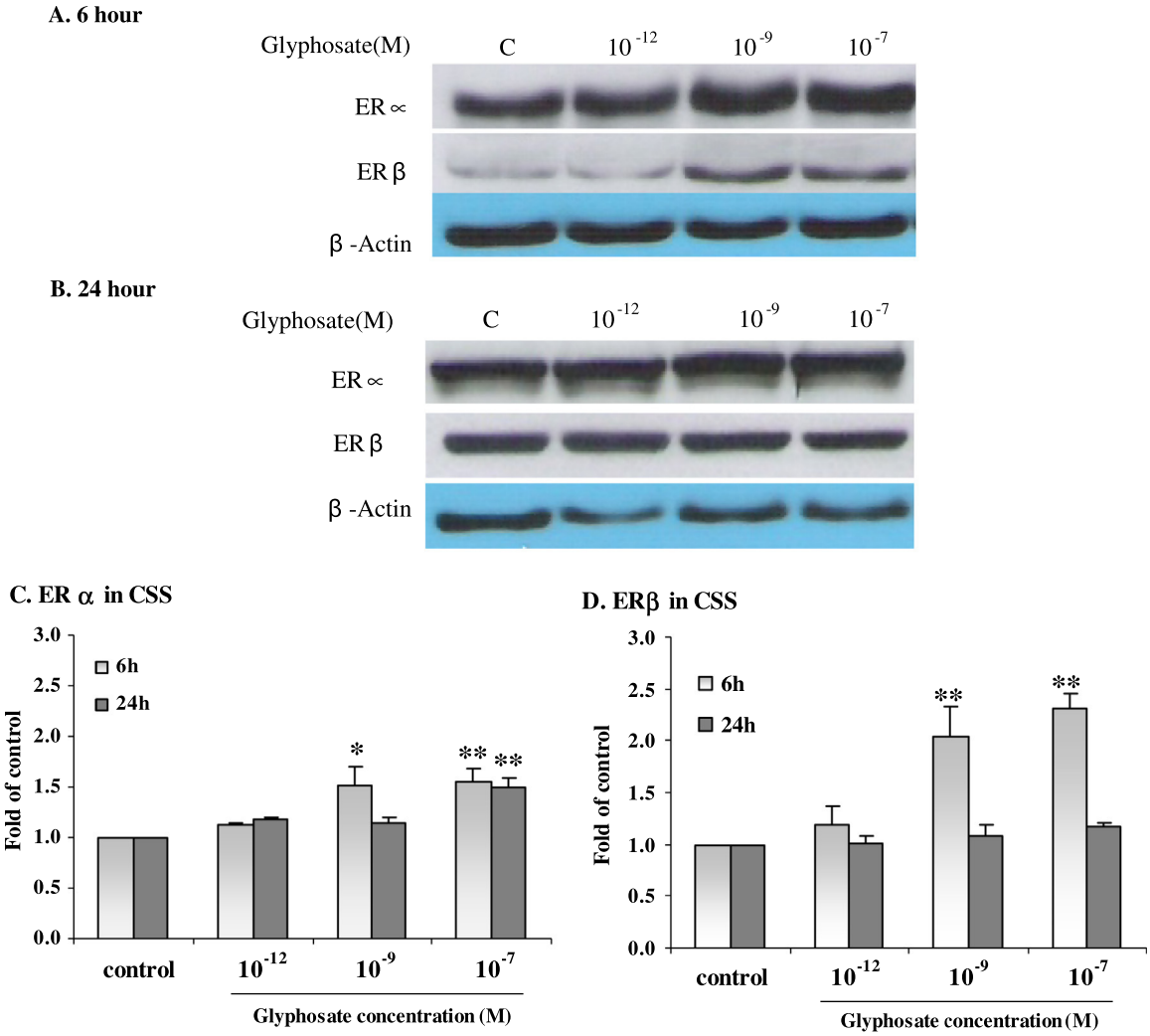

Fig. 4. The effects of glyphosate on ERα and β expression. E2-withdrawal T47D cells were used. (A) 6 h and (B) 24 h incubation time showed specific band of ERα, ERβ (66 kDa) and β-actin (44 kDa), a representative sample from one experiment. Optical densities of specific band ERα and ERβ were determined from western blot and each band was normalized to the β-actin band. The normalized mean of three replications ± SE optical density values are shown in the histogram of ERα (C) and ERβ (D) with $^{*} = p \leqslant 0.05$, $^{**} = p \leqslant 0.01$ significantly different as compared to control.

of cancer cells. The selection of interactive concentrations between glyphosate and genistein were based on the significant effects on the induction of ERE activity of each compound. The concentration ranges of glyphosate and genistein inducing ERE activity more than 10-fold of control included 10^{-11} to 10^{-9} M and 10^{-7} to 10^{-5} M, respectively. Actually, the concentration of glyphosate residue in soybeans should be lower than of genistein. As the information of glyphosate residues in soybeans were found in the range of 0.1–5.6 μg/g (Arregui et al., 2004; Sharma, 2009) while genistein concentrations were in the range of 0.01–1.2 mg/g (Morton et al., 1999; Murphy et al., 1999; Nakajima et al., 2005). We used this information to set the interaction model of two compounds as possible as in a real situation. The interactive levels used in this study correspond to the possible levels of glyphosate and genistein in human. Setchell and Cassidy showed that consumption of 50 mg/day of isoflavones in an adult can give rise to plasma concentrations of genistein ranging from 2.0×10^{-7} to 3.2×10^{-6} M (Setchell and Cassidy, 1999), while glyphosate concentration in human body could be 1.8×10^{-8} to 1.4×10^{-6} M (Acquavella et al., 2004) or less than 5.9×10^{-10} M (Jauhiainena et al., 1991). According to these data, we had set the interaction model of these two compounds as genistein ranging from 10^{-7} to 10^{-5} M and glyphosate ranging from 10^{-11} to 10^{-9}. The interactive effects of glyphosate and genistein were studied by varying concentrations with fixed ratio of both compounds as shown in Fig. 6A. The results showed the significant enhancing of ERE activation in the combination of 10^{-10} M glyphosate with 10^{-6} M genistein and 10^{-9} M glyphosate with 10^{-5} M genistein.

3.5.3. The additive effects of glyphosate on genistein-induced cell proliferation

To further investigate the interactive effect on cell growth of T47D cells, glyphosate and genistein at concentrations of 10^{-9} and 10^{-7} M, respectively, were combined in E2-withdrawal condition for 72 h incubation time and cell numbers were counted as % of control (Fig. 6B). This selected concentration was considered based on the equal effects of glyphosate and genistein on cell proliferation which was about 140% of the control. The results revealed that genistein at 10^{-7} M significantly enhanced the cell growth effect of 10^{-9} M of glyphosate up to 169% of control.

4. Discussion

The present study provides a better understanding of possible mechanisms underlying glyphosate toxicity in a hormone dependent human breast cancer cell. Concentrations of glyphosate tested in this study that exhibited estrogenic activity and interfered with

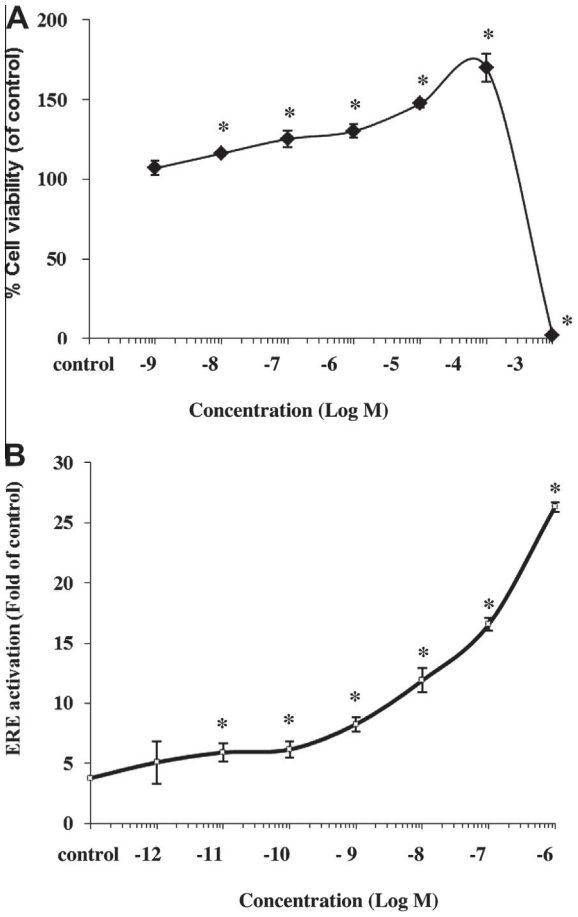

Fig. 5. Cytotoxicity of genistein on cell growth and ERE activation in T47D cells. T47D cells were investigated in E2-withdrawal media within 24 h treatment time. Genistein exhibited the stimulation of cell growth in the range of 10^{-8} to 10^{-4} M as access by MTT assay (A) and the ERE activation was increased in the range of 10^{-11} to 10^{-6} M (B) ($n = 3$, $^{*}p \leqslant 0.05$, significantly different as compared to control).

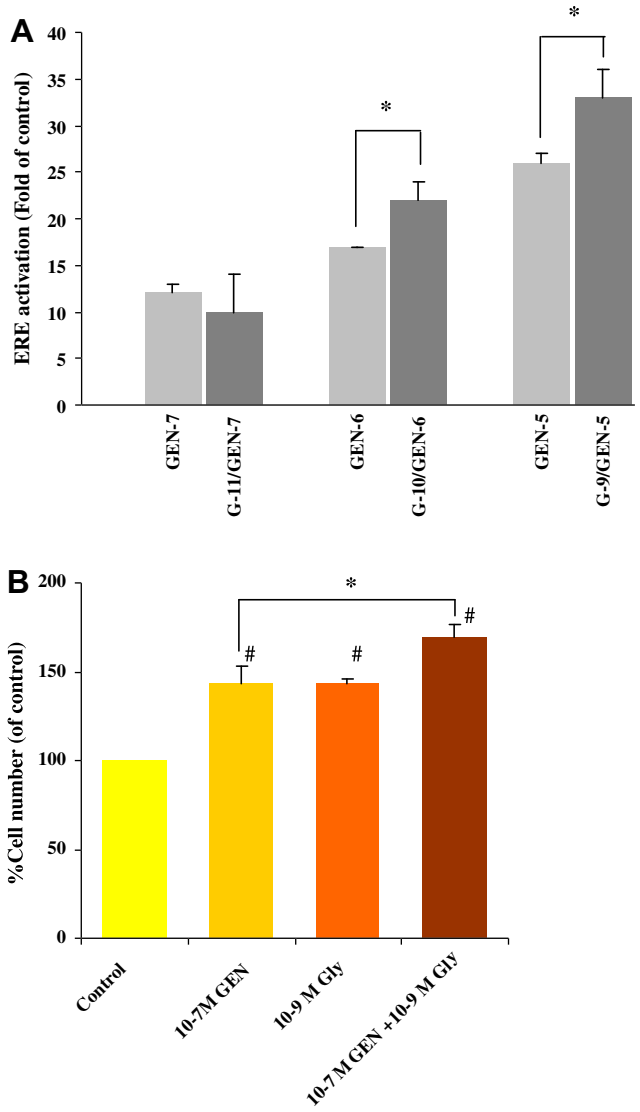

Fig. 6. Effect of co-incubation of glyphosate and genistein. (A) ERE-gene transcription activity of T47D-KBluc cells in co-incubation of glyphosate (Gly) and genistein (Gen). The effects were compared between varying concentration of genistein alone or in combination with glyphosate (Gly 10^{-11} + Gen 10^{-7} M, Gly 10^{-10} + Gen 10^{-6} M and Gly 10^{-9} + Gen 10^{-5} M). Three replications and SE were shown in graph ($^{*}p \leqslant 0.05$, significantly different as compared to compound alone). (B) The effect of glyphosate and genistein on cell growth. Cell number of T47D cells was counted after 3 days incubation with each compound alone or co-incubation treatments ($n = 3$, $p \leqslant 0.05$, *significantly different as compared to compound alone, # significantly different as compared to control).

normal estrogen signaling were relevant to the range of concentrations that has been reported in environmental conditions and exposed human. The detectable concentrations in human urines have been reported to be in the range of <0.1–233 ppb (<5.9×10^{-10} to 1.4×10^{-6} M) with the highest estimated systemic dose of 0.004 mg/kg (Acquavella et al., 2004; Jauhiainena et al., 1991).

In this study, we found that glyphosate at a log interval concentration ranging from 10^{-12} to 10^{-6} M increased the cell proliferation of a hormone dependent breast cancer T47D cell while this effect was not observed in a hormone independent breast cancer MDA-MB231 cell. The ERE luciferase assay also supported that glyphosate behaved as a xenoestrogen to induce ERE activation because these responses can be blocked by ICI 182780, an ER antagonist. Although the ERs binding of glyphosate is still unknown, the ability of glyphosate to stimulate the ERE-gene transcription activity and up-regulation of ERα protein expression suggests that glyphosate may exert the stimulatory effects via the ER-dependent mechanism. As is known, ERs can bind with a wide variety of compounds with typical structures of two hydroxyl groups separated by a rigid hydrophobic linker region and, in addition, the effective ligands possess a phenolic hydroxyl group (Ascenzi et al., 2006). Although glyphosate structure does not totally match, its responses observed in this study supported the contention that it acted like ligand binding. This unknown interaction may occur in a polar pocket at ligand binding site of ERs. Due to the hydrophilic property of glyphosate, it may access via an active phosphate group. This may affect the conformation of other domains that respond to recruit other coregulators that differ from normal ER ligands. Furthermore, glyphosate also altered the levels of ER protein expression both ERα and ERβ in T47D cell at 6 h. The increased ratio of ERα/ERβ protein in the late stage, 24 h, corresponded to the observed proliferative effect of glyphosate. These results supported the finding about the regulatory role of ERβ in T47D cells (Sotoca et al., 2008). They also demonstrated that the effects of estrogen like compounds on T47D cell proliferation were dependent on the actual ERα/ERβ ratio in these cells (Sotoca et al., 2008; Speirs and Walker, 2007). Glyphosate showed a different expression profile of ERα from those estrogenic stimulation induced by E2 or genistein as previously described by Seo and coworker. They showed that E2 and genistein down-regulated

ERα and enhanced ERE gene expression on MCF-7 cells (Seo et al., 2006). Different cell lines have different sensitivity to estrogen, in addition, natural estrogen and estrogen-mimicking chemicals also exert a differential regulatory effect on ERα and ERβ (Cappelletti et al., 2003). This present study revealed that glyphosate treatment induced both ERs. However, patterns of ERα and ERβ induction by glyphosate were different. Glyphosate induced rapid activation of ERβ while activation of ERα was slower but prolonged. We hypothesized that glyphosate may behave like weak xenoestrogen which can activate both subtypes of ER but with a different time course.

On the other hand, our finding contradicts a recent study by Gasnier and his colleagues (Gasnier et al., 2009) who found the inhibition of the transcription activities of ERα and ERβ in HepG2 cells by Roundup® formulation, but it was not significant with pure glyphosate. This discrepancy may be due to cell types and experimental conditions. In their study, the HepG2 cells which transiently transfected with ERE-TK-Luciferase may lack some contents making it different from E2 targeted cells like breast cancer cells (Gasnier et al., 2009). Moreover, the concentrations of glyphosate in their experiments were higher than in the present study ($>10^{-5}$ M). Most of the studies used glyphosate-based formulation while a few studies used pure glyphosate. Furthermore, the used concentrations were not environmentally relevant (Williams et al., 2012). Another study showed the non-estrogenic effect of glyphosate at 10^{-5} to 10^{-4} M in MCF-7 cells (Lin and Garry, 2000), concentration ranges which cannot be compared to our study. However, the low concentration ranges should be taken into account due to many substances including pesticides and natural nutrients exerting their effects at relatively low concentrations from pico molar to micro molar (Miodini et al., 1999; Pink and Jordan, 1996; Safe and Papineni, 2006). The present study used pure glyphosate substance at log intervals from 10^{-12} to 10^{-6} M. These concentrations are in a crucial range which correlated to the potential biological levels at part per trillion (ppt) to part per billion (ppb) which have been reported in epidemiological studies (Acquavella et al., 2004; Lavy et al., 1992; Mandel et al., 2005). In this present *in vitro* study, we showed an estrogenicity of pure glyphosate. However, further *in vivo* study using an animal model such as a xenograft mouse model for breast cancer will confirm the present *in vitro* results and provide more physiological relevant evidence.

In addition, a single agent or chemical may exhibit a weak biological activity while mixture of compounds found environmentally could produce more noticeable effect by acting synergistically (Singleton and Khan, 2003). In fact, it has been reported that the concentrations of glyphosate in the environmental compartment and food chain are further increased due to high technology of transgenic crops and fruits demonstrating high degree of tolerance to the high levels of this compound (Solomon et al., 2007). Glyphosate-resistant soy is a popular genetically modified crop which is now becoming normal agricultural practice, thus glyphosate has higher possibility of getting into living organisms via the food chain through its application (Acquavella et al., 2004; Mandel et al., 2005). It is well known that soybean contains the phytoestrogen, genistein. Genistein acts as a weak agonist in breast tumor cells *in vitro*, it competes with E2 for binding to ERα protein, and induces activity of estrogen-responsive reporter gene constructs in the presence of ERα protein (Rajah et al., 2009). Thus, it should be of concern whether the contaminated glyphosate in soybean can interact with genistein causing alterations in their effects on the cellular system. In the present study, we showed that glyphosate had an additive effect with genistein in *in vitro* testing model. This finding should raise concern about the existence of more than one xenoestrogen such as phytoestrogen and contaminants in plant derived food which may be beneficial or harmful depending on the hormonal and pathological status of consumers. This study implied that the additive effect of glyphosate and genistein in postmenopausal woman may induce cancer cell growth. In this present *in vitro* study, we showed an estrogenicity of pure glyphosate.

In summary, we found that glyphosate exhibited a weaker estrogenic activity than estradiol. Furthermore, this study demonstrated the additive estrogenic effects of glyphosate and genistein which implied that the use of glyphosate-contaminated soybean products as dietary supplements may pose a risk of breast cancer because of their potential additive estrogenicity.

Conflict of Interest

Authors declare that there are no conflicts of interest.

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