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Title: Olive oil hydroxytyrosol reduces toxicity evoked by acrylamide in human Caco-2 cells by preventing oxidative stress.

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Abstract: Humans are exposed to dietary acrylamide (AA) during their lifetime, it is therefore necessary to investigate the mechanisms associated with AA-induced toxic effects. Accumulating evidence indicates that oxidative stress contributes to AA cytotoxicity, thus, dietary antioxidants might have a protective role in colonic cells against AA toxicity. We have recently reported that hydroxytyrosol (HTy), a natural antioxidant abundant in olive oil, is able to enhance the cellular antioxidant defence capacity, thereby protecting cells from oxidative stress.
In this study, we evaluate the protective role of HTy on alterations of the redox balance induced by AA in Caco-2 intestinal cells. AA cytotoxicity was counteracted by HTy by powerfully reducing ROS generation, recovering the excited enzyme antioxidant defences and decreasing phospho-Jun Kinase concentration and caspase-3 activity induced by AA. Therefore, AA-induced cytotoxicity and apoptosis are closely related to oxidative stress in Caco-2 cells and the olive oil natural dietary antioxidant HTy was able to contain AA toxicity by improving the redox status of Caco-2 cells and by partly restraining the apoptotic pathway activated by AA.

To: Dr. Marquardt

Editor of Toxicology

Dear Editor,

Please find enclosed the manuscript entitled: Olive oil hydroxytyrosol reduces toxicity evoked by acrylamide in human Caco-2 cells by preventing oxidative stress, by Ildefonso Rodríguez-Ramiro, María Ángeles Martín, Sonia Ramos, Laura Bravo and Luis Goya for submission to *Toxicology*. The results reported in this manuscript have not been submitted to any other journal.

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Manuscript significance

The results of the present study show that acrylamide-induced cytotoxicity and apoptosis are closely related to oxidative stress in Caco-2 cells and the olive oil natural dietary antioxidant HTy is able to decrease acrylamide toxicity by improving the cellular redox status and by partially restraining the apoptotic pathway activated by the toxin.

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Thanking you in advance. We look forward to hearing from you.

Yours sincerely,

Luis Goya

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Highlights

- Acrylamide-induced toxicity is closely related to oxidative stress in Caco-2 cells

- Olive oil natural antioxidant HTy is able to decrease acrylamide-evoked toxicity

- HTy improves cellular redox status and restrains apoptosis activated by acrylamide

Olive oil hydroxytyrosol reduces toxicity evoked by acrylamide in human Caco-2 cells by preventing oxidative stress.

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Abstract

Humans are exposed to dietary acrylamide (AA) during their lifetime, it is therefore necessary to investigate the mechanisms associated with AA-induced toxic effects. Accumulating evidence indicates that oxidative stress contributes to AA cytotoxicity, thus, dietary antioxidants might have a protective role in colonic cells against AA toxicity. We have recently reported that hydroxytyrosol (HTy), a natural antioxidant abundant in olive oil, is able to enhance the cellular antioxidant defence capacity, thereby protecting cells from oxidative stress.

In this study, we evaluate the protective role of HTy on alterations of the redox balance induced by AA in Caco-2 intestinal cells. AA cytotoxicity was counteracted by HTy by powerfully reducing ROS generation, recovering the excited enzyme antioxidant defences and decreasing phospho-Jun Kinase concentration and caspase-3 activity induced by AA. Therefore, AA-induced cytotoxicity and apoptosis are closely related to oxidative stress in Caco-2 cells and the olive oil natural dietary antioxidant HTy was able to contain AA toxicity by improving the redox status of Caco-2 cells and by partly restraining the apoptotic pathway activated by AA.

Keywords: olive oil antioxidants, antioxidant defences, oxidative stress, reactive oxygen species, Jun kinases, colon toxicity.

1. Introduction

The presence of acrylamide (AA) in commonly ingested foods has originated a great concern because humans could be exposed to significant quantities of this toxin during their life-span. Relevant amounts of AA have been identified in heat-treated carbohydrate-rich foods such as fried potatoes, cookies, bread and breakfast cereals (Tareke et al. 2002). Most investigations of AA toxicity have mainly focused on their genotoxic and carcinogenic properties; however, accumulating evidences seem to indicate that AA also possesses cytotoxic properties by affecting the redox status of the cells (Naruszewicz et al. 2009; Park et al. 2010; Parzefal et al. 2008; Yousef et al. 2006; Zhang et al. 2009).

AA is readily absorbed into the intestinal cells and it is usually non-enzymatically and enzymatically conjugated with reduced glutathione (GSH) resulting in a depletion of cellular GSH stores (Lash, 2006; Pernice et al. 2009; Zodl et al. 2007). As occurs under various pathological states (Lash, 2006), the decreased GSH levels evoke the overproduction of reactive oxygen species (ROS) which activates signalling cascades involving members of the mitogen-activated protein kinase (MAPK) family, such as Jun kinases (JNKs), that play a key role in the regulation of many cellular processes including apoptosis (Li et al. 2006; Valko et al. 2007). Studies in intestinal cells have demonstrated that loss of cellular glutathione redox balance is an important player in apoptotic signalling and cell death (Circu et al. 2008; Wang et al. 2000). In line with this, we have recently shown that AA-induced cytotoxicity and apoptosis are closely related to oxidative stress in Caco-2 cells (Rodríguez-Ramiro et al. 2011), a human cell line originating from the gastrointestinal tract that retains many of the morphological and enzymatic features typical of normal human colonocytes. Therefore, depletion of GSH levels favouring cellular

oxidative stress and apoptosis may be suggested as a potential mechanism for AA toxicity in gastrointestinal tract.

The use of natural compounds derived from the diet with antioxidant effects might provide a strategy to reduce AA toxicity. Indeed, we have recently shown the protective effect of cocoa flavanols against an induced AA toxicity in colonic cells Caco-2 (Rodríguez-Ramiro et al. 2011). In line with this, olive oil, the major fat source of Mediterranean diet, is recognized for its antioxidant properties and its positive effects on oxidative stress associated processes (Fitó et al. 2007). The phenolic fraction of virgin olive oil has proved to have antioxidant activity in vitro, scavenging peroxyl radicals (Saija et al. 1998), other free radicals (Gordon et al. 2001) and reactive nitrogen species (De la Puerta et al. 2001), or breaking peroxidative chain reactions and preventing metal ion catalyzed production of reactive oxygen species (Manna et al. 1997; Mateos et al. 2003). The main phenolic compounds in virgin olive oil are secoiridoid derivatives of 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol) (HTy) and of 2-(4-hydroxyphenyl)ethanol or tyrosol, and 2-(3,4-dihydroxyphenyl)ethyl acetate (Mateos et al. 2001). In particular, HTy is considered one of the most abundant and representative olive oil phenols and its protective effect against an oxidative stress-induced cytotoxicity has been systematically demonstrated (Goya et al. 2007; Martín et al. 2010).

Biological actions of phenolic compounds have been commonly related to their free radical scavenging activities, but current evidence strongly support that natural biophenols may also offer an indirect protection by increasing the endogenous antioxidant defence system (Masella et al. 2004; Masella et al, 2005). In fact, we have recently shown an additional mechanism of action of HTy to prevent oxidative stress damage in hepatic cells through the modulation of signalling pathways involved in antioxidant/detoxifying

enzymes regulation (Martín et al. 2010). This double mechanism of action confers HTy a great chemo-protective potential to prevent oxidative stress-associated cell damage. Therefore, the aim of the present study was to investigate whether HTy is able to protect Caco-2 cells against oxidative stress and apoptosis induced by AA.

2. Material and Methods

2.1. Materials and Chemicals

O-phthalaldehyde (OPT), gentamicin, penicillin G, streptomycin, acrylamide and the rest of chemicals for all analysis were purchased from Sigma Chemicals (Madrid, Spain). The fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR). Anti-JNKs, antiphospho-JNK (p-JNKs) and anti-ß-actin were obtained from Cell Signaling Technology (Izasa, Madrid, Spain). Caspase-3 substrate (Ac-DEVD-AMC) was purchased from Pharmingen (San Diego, CA). Materials and chemicals for electrophoresis and the Bradford reagent were from BioRad (Madrid, Spain). Cell culture dishes were from Falcon (Cajal, Madrid, Spain) and cell culture medium and fetal bovine serum (FBS) from Biowhittaker Europe (Lonza, Madrid, Spain).

2.2. Cell culture and AA and hydroxytyrosol treatment

Human Caco-2 cells were grown in a humidified incubator containing 5 % CO_2 and 95 % air at 37 ºC. They were grown in DMEM F-12 medium from Biowhitaker (Lonza, Madrid, Spain), supplemented with 10 % Biowhitaker foetal bovine serum (FBS) and 50 mg/L of each of the following antibiotics: gentamicin, penicillin and streptomycin. Plates were changed to FBS-free medium the day before the assay. HTy was obtained by chemical synthesis from 3, 4-dihydroxyphenylacetic acid by reduction with $LiAlH_4$ and was a kind gift from Dr. José Luis Espartero (Facultad de Farmacia, Universidad de Sevilla, Spain).

The different concentrations of HTy, 5, 10, 20, 40 (and 100 for the viability assay) μM were dissolved in serum-free culture medium and added to the cell plates for 20 h. To evaluate the protective effect of HTy against AA toxicity, concentrations of HTy were diluted in serum-free culture medium and added to the cell plates for 20 h. After that, the medium was discarded and fresh medium containing 5 mM of AA was added for different incubation times; this AA concentration was selected from our previous study (Rodríguez-Ramiro et al. 2011).

2.3. Cytotoxicity assays

Cellular damage induced by AA was evaluated by crystal violet assay (Granado-Serrano et al., 2007) and by lactate dehydrogenase (LDH) leakage (Goya et al. 2007). For the crystal violet assay, Caco-2 cells were seeded at low density (10^4 cells per well) in 96-well plates, grown for 20 h with the different treatments and incubated with crystal violet (0.2% in ethanol) for 20 min. Plates were rinsed with distilled water, allowed to dry, and 1% sodium dodecyl sulfate (SDS) added. The absorbance of each well was measured using a microplate reader at 570 nm (Bio-Tek, Winooski, VT, USA). For LDH assay, the culture medium and the cells scraped in PBS were collected after the different treatments. Cells were first sonicated to ensure breaking down the cell membrane to release the total amount of LDH; then, after centrifugation (1000 *g*, 15 min) to clear up the cell sample, 10 μL were placed into a well of a 96 multiwell for the assay. In the same manner, 10 μL of each culture medium were also deposited into a well of a 96-well multiwell. The LDH leakage percentage was estimated from the ratio between the LDH activity in the culture medium and that of the whole cell content.

2.4. Evaluation of ROS generation

Cellular ROS were quantified by the DCFH assay using a microplate reader as previously described (Goya et al. 2007). For the assay, cells were plated in 24-well multiwells at a rate of 2×10^5 cells per well and changed to FBS-free medium and the different treatments the day before the assay. After that, 5 µM DCFH was added to the wells for 30 min at 37 ºC. Then, cells were washed twice with PBS and 0.5 mL of serum-free medium or serum-free medium with 5 mM AA were added per well. After being oxidized by intracellular oxidants, DCFH will become dichorofluorescein (DCF) and emit fluorescence. ROS generation was evaluated at different times in a fluorescent microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm (Bio-Tek, Winooski, VT, USA).

2.5. Determination of Caspase-3 activity

Apoptosis was evaluated as activation of Caspase-3 (Granado-Serrano et al. 2006). Cells were lysed in a buffer containing 5 mM Tris (pH 8), 20 mM EDTA, and 0.5 % Triton X-100. The reaction mixture contained 20 mM HEPES (pH 7), 10 % glycerol, 2 mM dithiothreitol (DTT), and 30 µg of protein per condition, and 20 µM Ac-DEVDAMC (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) as substrate. Enzymatic activity was determined by measuring fluorescence at an excitation wavelength of 380 nm and an emission wavelength of 440 nm (Bio-Tek, Winooski, VT, USA).

2.6. Determination of GSH concentration

The content of GSH was quantified by the fluorometric assay of Hissin and Hilf (1976). The method takes advantage of the reaction of GSH with OPT at pH 8.0. After the different treatments, the culture medium was removed and cells were detached and homogenized by ultrasound with 5 % trichloroacetic acid containing 2 mM EDTA. Following centrifugation of cells for 30 min at 3000 rpm, 50 µL of the clear supernatant

were transferred to a 96 multiwell plate for the assay. Fluorescence was measured at an excitation wavelength of 340 nm and an emission wavelength of 460 nm. The results of the samples were referred to those of a standard curve of GSH. The precise protocol has been described elsewhere (Goya et al. 2007).

2.7. Determination of glutathione peroxidase (GPx) and glutathione reductase (GR) activities

Cells were collected in PBS and centrifuged at low speed (300 *g*) for 5 min to pellet cells. Cell pellets were resuspended in 20 mM Tris containing 5 mM EDTA and 0.5 mM mercaptoethanol, sonicated and centrifuged at 3000 *g* for 15 min. Enzyme activities were measured in the supernatants. Determination of GPx activity is based on the oxidation of GSH by GPx, using *tert*butyl hydroperoxide as a substrate, coupled to the disappearance of NADPH by GR (Gunzler et al. 1974). GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione (Goldberg et al. 1987). Protein was measured by the Bradford reagent.

2.8. Preparation of cell lysates for Western blotting

To detect the levels of JNKs and p-JNKs, cells were lysed at 4 ºC in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.1% Triton X-100, 200 mM ß-glycerolphosphate, 0.1 mM Na_3VO_4, 2.5 µg/mL leupeptin, 2.5 µg/mL aprotinin and 1 mM phenylmethylsulphonyl fluoride. The supernatants were collected, assayed for protein concentration by using the Bradford reagents, aliquoted and stored at –80 ºC until used for Western blot analyses.

2.9. Protein determination by Western Blotting

Equal amounts of protein (100 µg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) filters (Protein Sequencing Membrane, Millipore).

Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated antirabbit Ig (GE Healthcare, Madrid, Spain). Blots were developed with the ECL system (GE Healthcare). Normalization of Western blot was ensured by ß-actin and bands were quantified by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA).

2.10. Statistics

Statistical analysis of data was as follows: prior to analysis the data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way ANOVA was followed by a Bonferroni test when variances were homogeneous or by the Tamhane test when variances were not homogeneous. The level of significance was $P < 0.05$. A SPSS version 19.0 program has been used.

3.- Results

3.1. Effect of HTy on cell viability, ROS production and antioxidant defences of Caco-2 cells.

Lactate dehydrogenase (LDH) leakage was used as an indicator of cell damage. A range of doses between 5 and 100 μM was tested for 24 h for their potential deleterious effect on Caco-2 cells. Figure 1A shows that no increase in LDH leakage to the culture medium was observed in any condition suggesting that no significant damage was evoked in cell integrity by the presence of HTy in concentrations up to 100 μM for as long as 24 h. Figure 1B shows that cells treated for 4 h with 5-40 μM HTy generated ROS levels that were below those of control unstressed cells. Additionally, treatment of Caco-2 cells with

HTy for 24 h did not alter steady-state GSH concentration or GPx and GR activity (data not shown).

3.2. Protective effect of HTy on cell viability and caspase 3 activity of Caco-2 cells.

Cell viability, as the factual physiological effect in the experiments of protection against AA-induced damage, was evaluated by two different assays, LDH leakage and crystal violet. Both are indicative of cell viability but increase of LDH in culture medium may be considered a marker indicative of necrosis whereas a decrease in crystal violet coloration is a sign of initial damage leading to cell detachment from the culture plate. In our experimental conditions a 16 h treatment with 5 mM AA evoked a great increase in LDH activity in the cell culture medium indicating cell death in Caco-2 cells (figure 2A). In addition, the same treatment evoked a significant reduction in crystal violet absorbance indicating augmented cell damage (figure 2B). In line with these results, a significant increase in caspase-3 activity was observed in Caco-2 cells treated for 8 h with 5 mM AA (figure 2C), indicating that the cytotoxic effect was to some extent due to apoptosis. Pre-treatment for 20 h of Caco-2 cell cultures with HTy significantly reduced cell damage evoked by AA for 16 h (figure 2A) or 24 h (figure 2B). In the same line, pre-treatment of cells with 10-40 μM HTy appreciably moderated the raise in caspase-3 activity induced by 8 h of treatment with 5 mM AA in Caco-2 cells (figure 2C).

3.3. Effect of HTy pre-treatment on GSH concentration and ROS production in Caco-2 cells.

As expected, AA treatment for 2 h induced a decrease of around 50 % in the steady-state concentration of GSH. This impressive decrease in GSH stores could not be prevented by a pre-treatment with HTy (figure 3A). However, figure 3B shows that the significant increase

in ROS production observed in Caco-2 cells treated with 5 mM AA for 4 h was remarkably neutralized when cells were pre-treated with 5-40 μM HTy for 20 h.

3.4. HTy pre-treatment recovers enzyme antioxidant defences to basal status in Caco-2 cells.

Treatment of Caco-2 cells with AA resulted in a time-course increase for 16 h in GPx and GR activity in response to the oxidative challenge (figures 4A and B). The stressful situation induced by AA for 16 h at the antioxidant defence system was efficiently surmounted when cells were pre-treated with all four doses of HTy for 20 h, returning activity of GPx (figure 5A) and GR (figure 5B) more rapidly to a basal condition.

3.5. HTy partly prevents AA-induced JNK activation in Caco-2 cells

Finally, to verify that the improved redox status induced by HTy in Caco-2 cells was correlated with the prevention of AA-induced cell death signalling pathways, we investigated the effect of the treatment with HTy on the protein expression of JNK and its active form p-JNK, which is activated by AA prior to the induction of apoptosis. To this end, Caco-2 cells treated during 20 h with HTy were further exposed to 5 mM of AA for 4 h and then immunoblots were performed using phospho- and non-phospho-antibodies against JNK. Figure 6 shows that cells treated with AA showed a significant three-fold increase in p-JNK. This impressive raise in p-JNK was partly prevented in Caco-2 cells that were pre-treated with all four doses of HTy, indicating that the previous presence of the phenolic compound had a chemo-preventive effect on the AA-induced toxicity and death in these cells.

4. Discussion

We have previously shown that AA-induced oxidative stress leads to cytotoxicity and apoptosis in human intestinal Caco-2 cells (Rodríguez-Ramiro et al. 2011). This is important since ingested AA is rapidly absorbed to a high degree in the human intestine (Zodl et al. 2007) and, therefore, studies assessing AA influence on gastrointestinal cells integrity seem necessary. Interestingly, we have also demonstrated that physiological concentrations of a mixture of cocoa polyphenols, a cocoa polyphenolic extract (CPE), were able to counteract the deleterious effect of AA (Rodríguez-Ramiro et al. 2011). In line with this, in the present study we show the significant protective effect of another natural antioxidant found in olive oil, HTy. Thus, potentiating the synergic effects by a varied intake of these natural antioxidants could be relevant to mitigate AA-associated risks related to oxidative stress.

GSH depletion and ROS overproduction induced by AA strongly contribute to the creation of oxidative stress conditions as previously demonstrated in HepG2 cells (Cao et al. 2008; Zhang et al. 2009) and in human-derived leukocytes in vitro (Naruszewicz et al. 2009). In addition, this redox disturbance precedes activation of caspase-3, considered a very specific and sensitive apoptotic marker (Gómez-Lechón et al. 2002; Li et al. 2006). Although the modes of action underlying this effect have not been clearly defined, one of the potential mechanisms involved could be the activation of signalling pathways implicated in the apoptotic process. Thus, we have recently reported that following GSH depletion at 2 h and ROS hyper-generation at 2-4 h, AA caused a persistent activation of JNKs at 4-6 h that led to an increase in caspase-3 activity at 6-8 h and subsequent Caco-2 cell death (Rodríguez-Ramiro et al. 2011). All together, these results indicate that AA ingestion may constitute a dietary risk factor for intestine injury.

Several natural products have been shown to protect cells against oxidative damage by virtue of their antioxidant properties (Virgili et al. 2008). Accordingly, in a recent study we have shown that a mixture of cocoa polyphenols, namely CPE, effectively suppressed the increase in caspase-3 activity and the cell death induced by AA in Caco-2 cells (Rodríguez-Ramiro et al. 2011). An interesting observation in that study was that the protective effect of CPE against AA cytotoxicity was significantly higher than that of the major phenolic component of the extract, epicatechin (EC), alone. Thus, CPE almost completely blocked the decrease of GSH induced by AA and totally abrogated the subsequently increased ROS generation while these effects were only partially restored with EC. This result suggests that the minor effect exerted by EC could be partially ascribed to the fact that EC mainly acted as scavenger of free radicals or to the lack of synergic effects with other phenolic compounds of the cocoa extract. However, similar to what was reported for other polyphenols and antioxidants (Masella et al. 2005), the mixture of polyphenols (mostly flavanols) constituents of CPE could protect cell constituents not only by neutralising several types of radicals but also by up-regulating antioxidant defences as well as interacting with signalling pathways involved in cell survival (Rodríguez-Ramiro et al. 2011).

Interestingly, the moderate effect of EC reported in the study described above can be comparable to that of HTy reported in the present study, that is, a negligible GSH recovery but a significant ROS quenching that led to an incomplete decrease of caspase-3 activity and a partial protection of cell viability. Therefore, as in the case of EC, the effect of HTy in AA-treated Caco-2 cells could be predominantly attributed to the capacity of the olive oil phenolic as free radical scavenger. In agreement with this, similar results concerning redox status and cell viability have recently been reported in HepG2 cells

treated with HTy and submitted to AA (Zhang et al. 2009). However, we have recently shown an additional mechanism of action of HTy to prevent oxidative stress damage induced by *tert*butyl hydroperoxide in liver-derived HepG2 cells through the modulation of signalling pathways involved in antioxidant/detoxifying enzymes regulation (Martin et al. 2010). But the present results suggest that this additional protective mechanism of HTy is either more efficient in hepatic cells or only competent in conditions of short term/acute oxidative stress, such as cells challenged with a strong and rapid pro-oxidant such as *tert*butyl hydroperoxide, and not functional in situations of long term/chronic cell damage, such as in cells submitted to AA.

In our previous study in Caco-2 cells treated with cocoa flavanoles and then challenged to AA, we had shown that AA-induced activation of JNKs at 4 h was significantly blocked by CPE treatment but not by EC (Rodríguez-Ramiro et al. 2011). Based on this result, it was suggested that the modulation of GSH levels through the enhancement of cellular defences by CPE could inhibit the AA-induced intracellular signals leading to apoptosis and increase cell survival. However, neither EC, in the previous study in Caco-2, nor HTy, in the present study with the same cells, were able to efficiently recover the AA-decreased GSH values and entirely reduce the AA-induced JNKs and caspase-3 activity, considerably reducing their chemo-protective capacity. Our studies, together with those of other authors, point to unremitting cell damage by the continual toxicity of the challenge with AA, perhaps due both, to a high conjugation with GSH resulting in a dire depletion of cellular GSH stores, along with its specific genotoxic effect (Zhang et al. 2009). Only the synergic effect of a mixture of flavonoids or the induction of antioxidant/detoxificant defences, as was accomplished with CPE, are able to fully counteract the intense damage induced by AA.

It is worth noting that the concentrations of AA applied to the cell cultures in the present study were to some extent high; however, other authors (Naruszewicz et al. 2009) and ourselves (Rodríguez-Ramiro et al. 2011) have shown that smaller doses of AA can act as toxicant when administered in long-term exposure. It can not be ruled out that daily exposure to a lower dose of AA may bring about a risk factor of toxicity in human. Therefore, efforts designed both to reduce the AA content of processed foods and to mitigate adverse manifestation of AA after consumption are necessary.

5.1. Conclusion.

In summary, our present study provides evidence that AA cytotoxicity was partially counteracted by the dietary phenol HTy which prevented ROS overproduction but could not efficiently recover GSH depletion and inhibit the apoptotic pathway. The results underline the important role of dietary antioxidants such as olive oil HTy as well as the synergic effects of a varied intake of plant bioactive compounds for nutritional prevention of certain pathological states linked to oxidative stress.

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5. References

Cao, J., Liu, Y., Jia, L., Jiang, L-P., Geng, Ch., Yao, X-F., Kong, Y., Jiang, B-N., Zhong, L., 2008. Curcumin attenuates acrylamide-induced cytotoxicity and genotoxicity in HepG2 cells by ROS Scavenging. J. Agric. Food Chem. 56, 12059-63.

Circu, M.L., Rodriguez, C., Maloney, R., Moyer, M.P., Aw, T.Y., 2008. Contribution of mitochondrial GSH transport to matrix GSH status and colonic epithelial cell apoptosis. Free Rad. Bio. Med. 44, 768–778.

De la Puerta, R., Martínez-Domínguez, M.E., Ruiz-Gutierrez, V., Flavill, J.A., Hoult, J.R., 2001. Effects of virgin olive oil phenolics on scavenging of reactive nitrogen species and upon nitrergic neurotransmission. Life Sci. 69, 1213-22.

Fitó, M., de la Torre, R., Covas, M.I., 2007. Olive oil and oxidative stress. Mol. Nutr. Food Res. 51, 1215 – 1224.

Goldberg, D.M., Spooner, R.J., 1987. In: Bergmeyer, H.V. (Ed.), Glutathione reductase. Methods of enzymatic analysis, Weinheim, Verlag-Chemie. pp. 258-265

Gomez-Lechon, M.J., O'Connor, E., Castell, J.V., Jover, R,. 2002. Sensitive markers used to identify compounds that trigger apoptosis in cultured hepatocytes. Toxicol. Sci. 65, 299–308.

Gordon, M.H., Paiva-Martins, F., Almeida M., 2001. Antioxidant activity of hydroxytyrosol acetate compared with that of other olive oil polyphenols. J. Agric. Food Chem. 49, 2480-2485.

Goya, L., Mateos, R., Bravo, L., 2007. Effect of the olive oil phenol hydroxytyrosol on human hepatoma HepG2 cells. Protection against oxidative stress induced by tert-butylhydroperoxide. Eur. J. Nutr. 46, 70–78.

Granado-Serrano, A.B., Martin, M.A., Bravo, L., Goya, L., Ramos, S., 2006. Quercetin induces apoptosis via caspase activation, regulation of Bcl-2, and inhibition of PI-3-kinase/Akt and ERK pathways in a human hepatoma cell line (HepG2). J. Nutr. 136, 2715–2721.

Granado-Serrano, A.B., Martín, M.A., Izquierdo-Pulido, M., Goya, L., Bravo, L., Ramos, S., 2007. Molecular mechanisms of (-)-epicatechin and chlorogenic acid on the regulation of the apoptotic and survival/proliferation pathways in a human hepatoma cell line. J. Agric. Food Chem. 55, 2020-2027.

Gunzler, W.A., Kremers, H., Flohe, L., 1974. An improved coupled test procedure for glutathione peroxidase. Klin. Chem. Klin. Biochem. 12, 444-448.

Hissin, P.J., Hilf, R., 1976. A fluorometric method for determination of oxidised and reduced glutathione in tissues. Anal. Biochem. 74, 214-226.

Lash, L.H., 2006. Mitochondrial glutathione transport: physiological, pathological and toxicological implications. Chem. Biol. Interact. 163, 54-67.

Li, S., Cui, N., Zhang, C., Zhao, S., Yu, S., Xie, K., 2006. Effect of subchronic exposure to acrylamide induced on the expression of bcl-2, bax and caspase-3 in the rat nervous system. Toxicology 217, 46–53.

Manna, C., Galletti, P., Cucciolla, V., Moltedo, O., Leone, A., Zappia, V., 1997. The protective effect of the olive oil polyphenols (3,4-dihydroxyphenyl)-ethanol counteracts reactive oxygen metabolite-induced cytotoxicity in Caco-2 cells. J. Nutr. 127, 286-292.

Martín, M.A., Ramos, S., Granado-Serrano, A.B., Rodríguez-Ramiro, I., Trujillo, M., Bravo, L., Goya, L., 2010. Hydroxytyrosol induces antioxidant/detoxificant enzymes activity and Nrf2 translocation via ERKs and PI3K/AKT pathways in HepG2 cells. Mol. Nutr. Food Res. 54, 956-966.

Masella, R., Varı, R., D'Archivio, M., Di Benedetto, R., Matarrese, P., Malorni, W., Scazzocchio, B., Giavannini, C., 2004. Extra Virgin Olive Oil Biophenols Inhibit Cell-Mediated Oxidation of LDL by Increasing the mRNA Transcription of Glutathione-Related Enzymes. J. Nutr. 134, 785-91.

Masella, R.T., Di Benedetto, R., Varı, R., Filesi, C., Giovannini, C., 2005. Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. J. Nutr. Biochem. 16, 577–586.

Mateos, R., Espartero, J.L., Trujillo, M., Ríos, J.J., León-Camacho, M., Alcudia, F., Cert, A., 2001. Determination of phenols, flavones, and lignans in virgin olive oils by solid-phase extraction and high-performance liquid chromatography with diode array ultraviolet detection. J. Agric. Food. Chem. 49, 2185-2192.

Mateos, R., Domínguez, M.M., Espartero, J.L., Cert, A., 2003. Antioxidant effect of phenolic compounds, α-tocopherol, and other minor components in virgin olive oil. J. Agric. Food Chem. 51, 7170-7175.

Naruszewicz, M., Zapolska-Downar, D., Kosmider, A., Nowicka, G., Kozłowska-Wojciechowska, M., Vikstrom, A.S., Tornqvist, M., 2009. Chronic intake of potato chips in humans increases the production of reactive oxygen radicals by leukocytes and increases plasma C-reactive protein: a pilot study. Am. J. Clin. Nutr. 89, 773-7.

Park, H., Kim, M-S., Kim, S.J., Park, M., Kong, K.H., Kim, H.S., Kwack, S.J., Kang, T.S., Kim, S.H., Kim, H.S., Lee, J., 2010. Acrylamide induces cell death in neural progenitor cells and impairs hippocampal neurogenesis. Toxicol. Lett. 193, 86–93.

Parzefal, W., 2008. Minireview on the toxicity of dietary acrylamide. Food Chem. Toxicol. 46, 1360–1364.

Pernice, R., Hauder, J., Koehler, P., Vitaglione, P., Fogliano, V., Somoza, V., 2009. Effect of sulforaphane on glutathione-adduct formation and on

glutathione_S_transferase-dependent detoxification of acrylamide in Caco-2 cells. Mol. Nutr. Food Res. 53, 1540–1550.

Rodríguez-Ramiro, I., Ramos, S., Bravo, L., Goya, L., Martín, M.A., 2011. Procyanidin B2 and a cocoa polyphenolic extract inhibit acrylamide-induced apoptosis in human Caco-2 cells by preventing oxidative stress and activation of JNK pathway. J. Nutritional Biochem. DOI: 10.1016/j.jnutbio.2010.10.005.

Saija, A., Trombetta, D., Tomaino, A., Lo Cascio, R., Princi, P., Uccella, N., Bonina, F., Castelli, F., 1998. In vitro evaluation of the antioxidant activity and biomembrane interaction of the plant phenols oleuropein and hydroxytyrosol. Int. J. Pharm. 166, 123-133.

Tareke, E., Rydberg, P., Karlsson, P., Eriksson, M., Tornqvist, M., 2002. Analysis of acrylamide, a carcinogen formed in heated food-stuffs. J. Agric. Food. Chem. 50, 4998–5006.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39, 44-84.

Virgili, F., Marino, M., 2008. Regulation of cellular signals from nutritional molecules: a specific role for phytochemicals, beyond antioxidant activity. Free Rad. Biol. Med. 45, 1205–1216.

Wang, T.G., Gotoh, Y., Jennings, M.H., Rhoads, C.A., Aw, T.Y., 2000. Lipid hydroperoxide-induced apoptosis in human colonic CaCo-2 cells is associated with an early loss of cellular redox balance. FASEB J. 14, 1567–1576.

Yousef, M.I., El-Demerdash, F.M., 2006. Acrylamide-induced oxidative stress and biochemical perturbations in rats. Toxicology 219, 133–141.

Zhang, X., Cao, J., Jiang, L., Geng, Ch., Zhong, L., 2009. Protective effect of hydroxytyrosol against acrylamide-induced cytotoxicity and DNA damage in HepG2 cells. Mutat. Res. 664, 64–68.

Zödl, B., Schmid, D., Wassler, G., Gundacker, C., Leibetseder, V., Thalhammer, T., Ekmekcioglu, C., 2007. Intestinal transport and metabolism of acrylamide. Toxicology 232, 99–108.

Figure captions

Figure 1.- Effect of HTy on cell viability and ROS generation. Caco-2 cells were treated for 24 h with the noted concentrations of HTy and cell viability was determined as relative percent of viable cells by crystal violet assay (A) and intracellular ROS production was evaluated at 4 h (B). Data represent means ± SD of 10-12 samples per condition. Different letters denote statistically significant differences, $P < 0.05$

Figure 2.- Protective effect of HTy on AA-induced cell toxicity and caspase-3 activity. Caco-2 cells were treated for 20 h with the noted concentrations of HTy and further

incubated for 16 h (A), 24 h (B) and 8 h (C) with 5 mM AA and LDH leakage (A), crystal violet (B) and caspase-3 activity (C) were assayed. Data represent means ± SD of 6-8 samples per condition. Different letters denote statistically significant differences, $P < 0.05$

Figure 3.- Protective effect of HTy on AA-induced GSH depletion and ROS overproduction. Caco-2 cells were treated with the indicated concentrations of HTy for 20 h and then with 5 mM AA for either 2 h to determine GSH levels (A) or 4 h to evaluate ROS production (B). Values are means ± SD of 10-12 different samples per condition. Different letters denote statistically significant differences, $P < 0.05$.

Figure 4.- Effect of AA on GPx and GR activity. Caco-2 cells exposed to 5 mM AA for the indicated times, were collected and assayed for GPx and GR. Values are means ± SD of 4 different samples per condition. Means without a common letter differ, $P < 0.05$.

Figure 5.- Protective effect of HTy on AA-induced GPx and GR activity. Caco-2 cells were treated with the noted concentrations of HTy for 20 h and then exposed to 5 mM AA for 16 h; then cells were collected and assayed for GPx and GR. Values are means ± SD of 6-8 different samples per condition. Means without a common letter differ, $P < 0.05$.

Figure 6.- Protective effect of HTy on AA-induced JNK activity. Caco-2 cells were treated with the noted concentrations of HTy for 20 h and then submitted to 5 mM AA for 4 h. Cells were then subjected to Western blot analysis using phospho and total specific antibodies to JNKs. Representative Western blot (A) and percent values (B) of p-JNKs/JNKs of 5 different experiments relative to the control conditions (means ± SD) are

shown. Normalization of Western blots was ensured by ß-actin. Means without a common letter differ, $P < 0.05$

Figure 1
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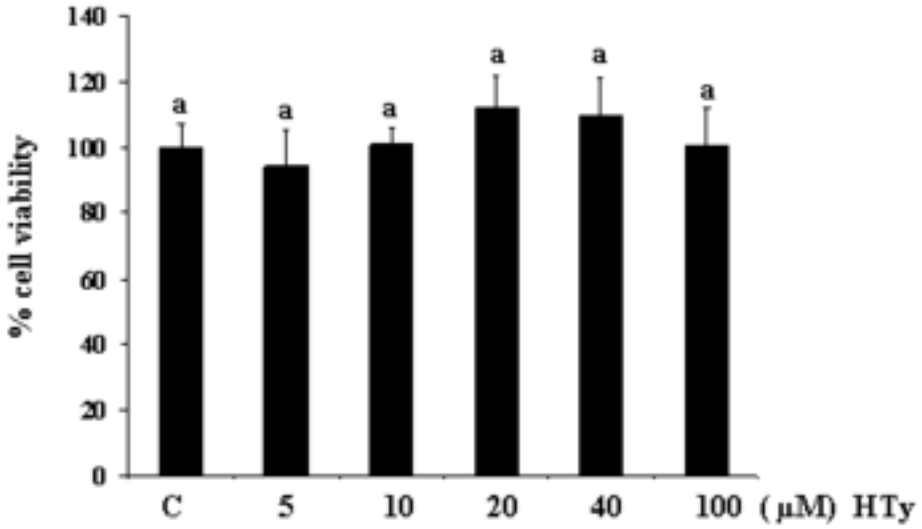

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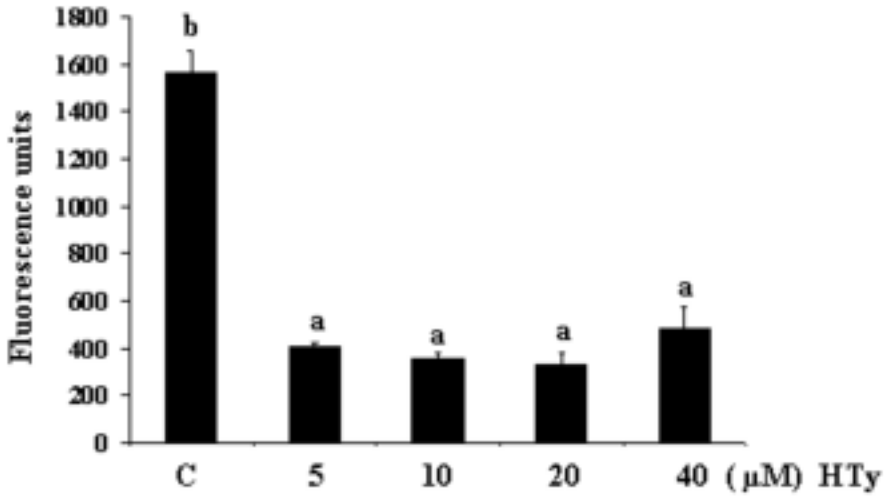

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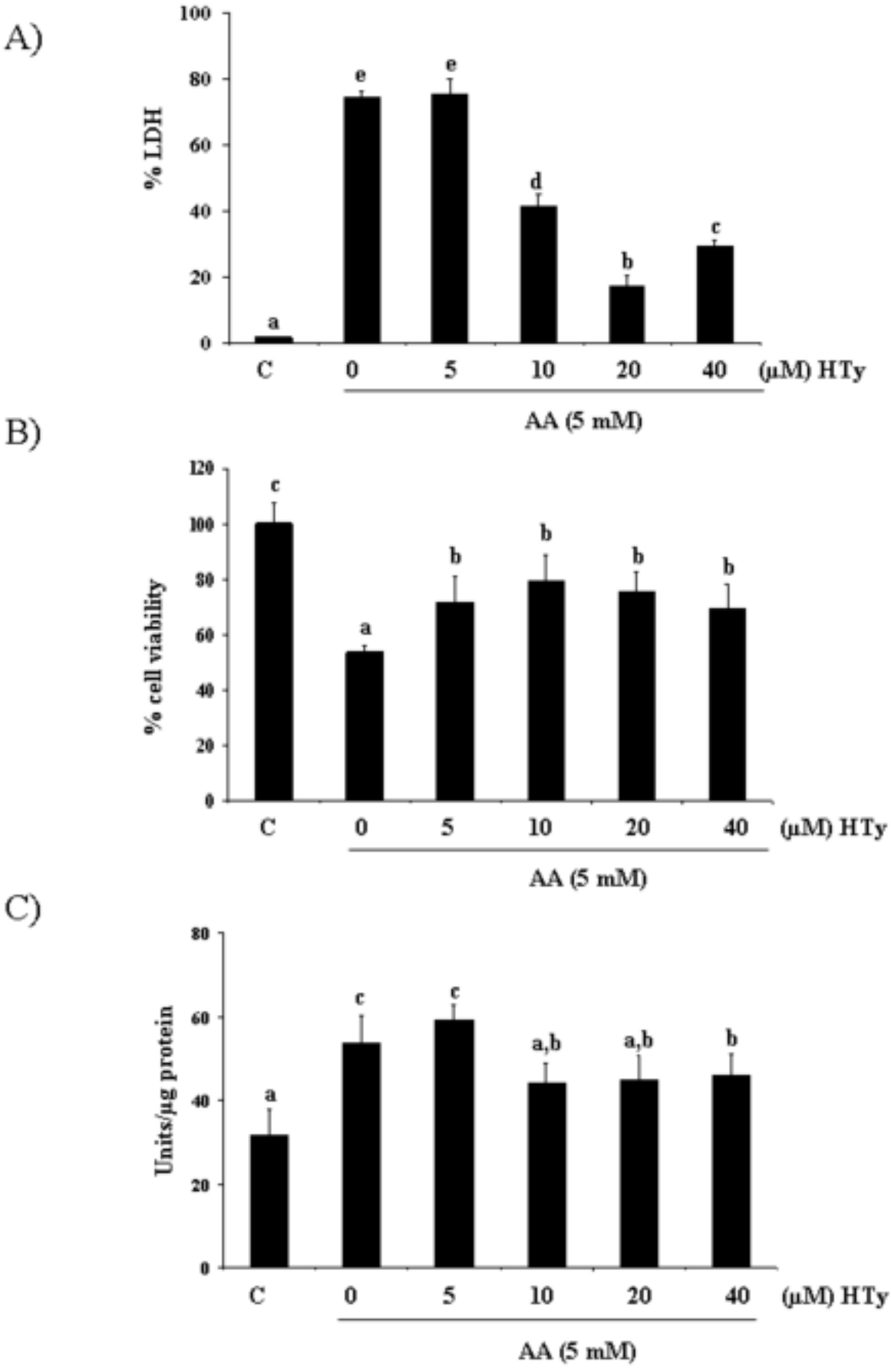

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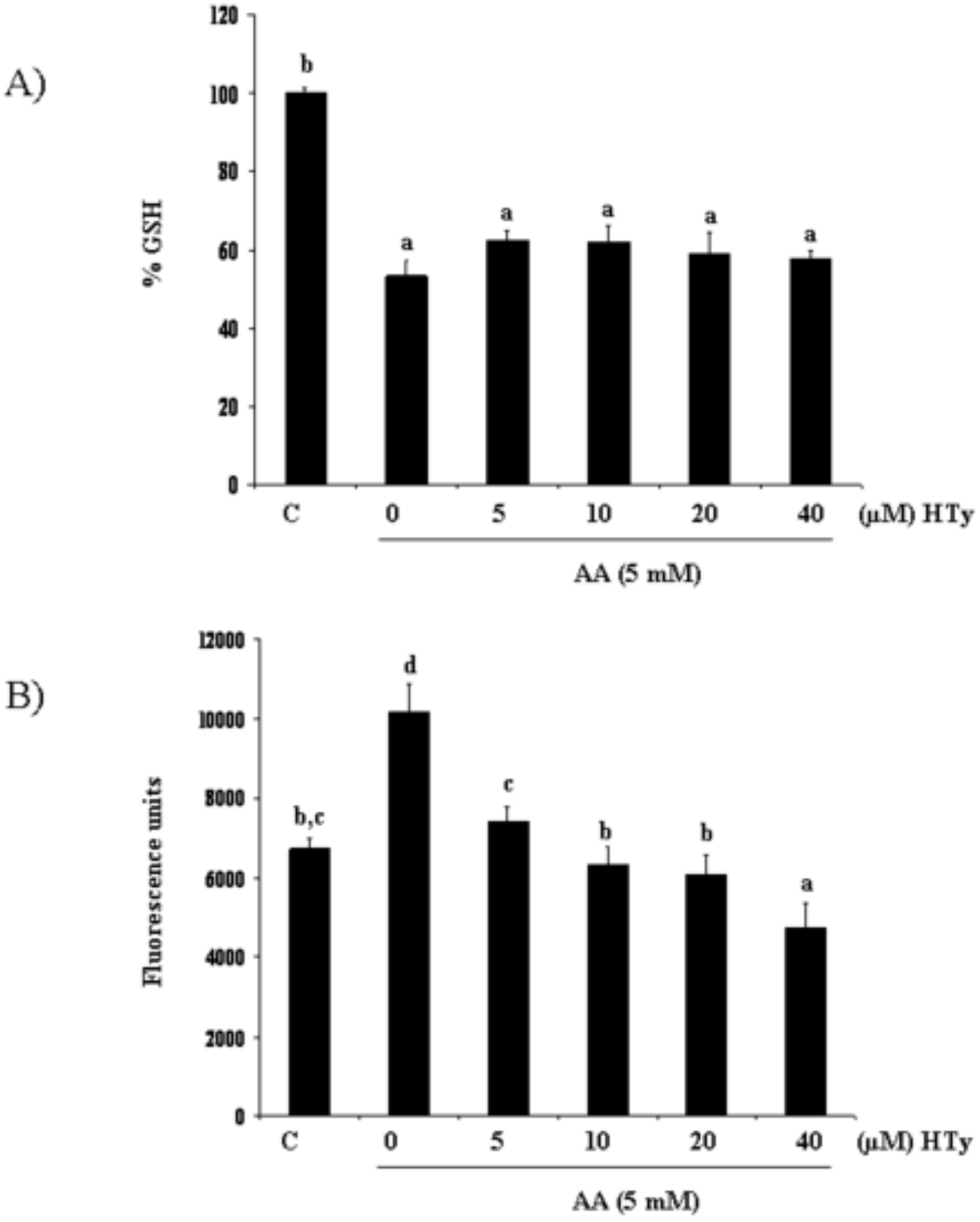

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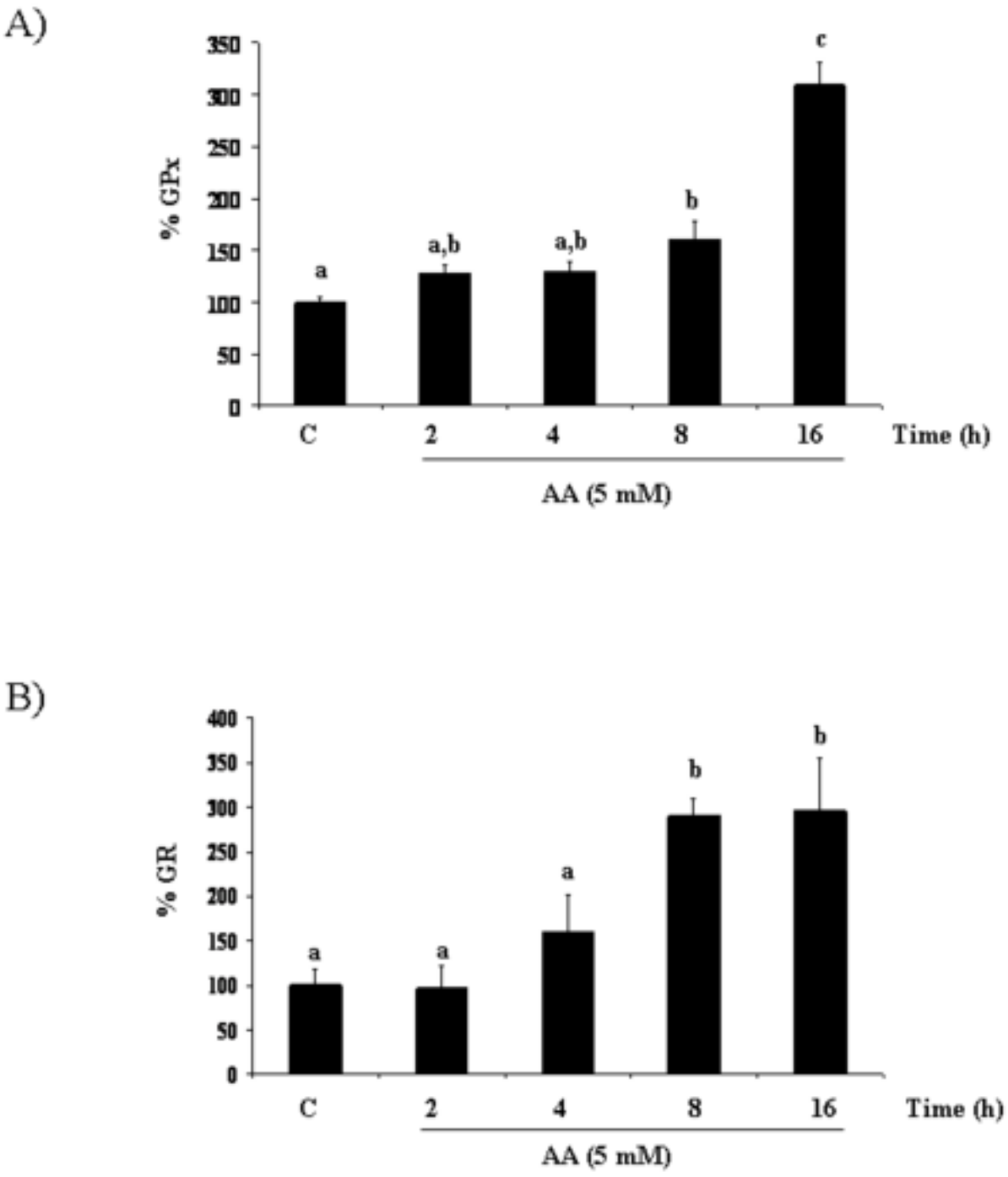

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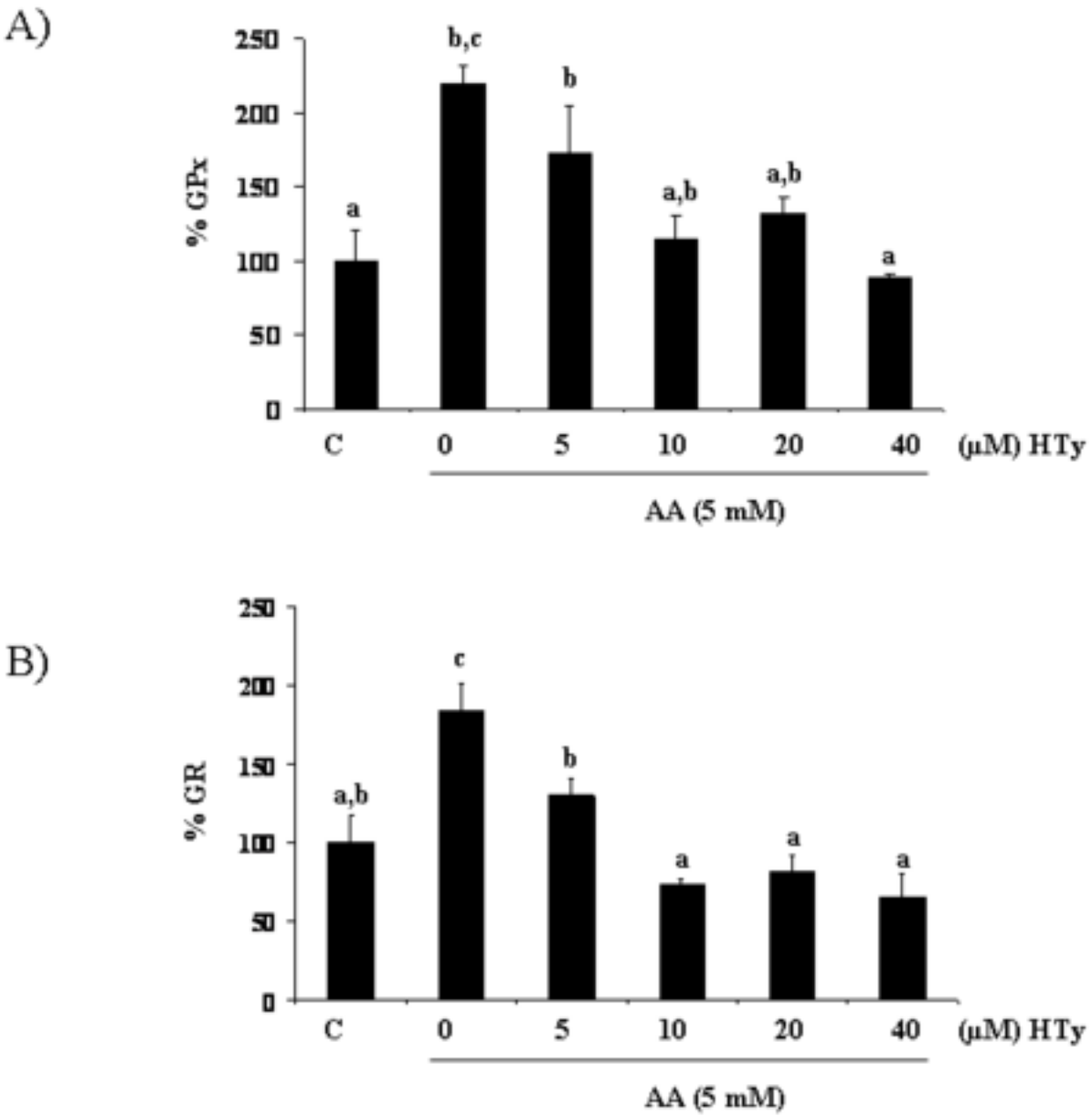

Figure 6

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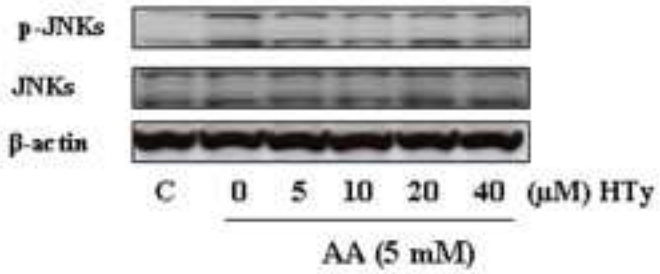
Figure 6

A)



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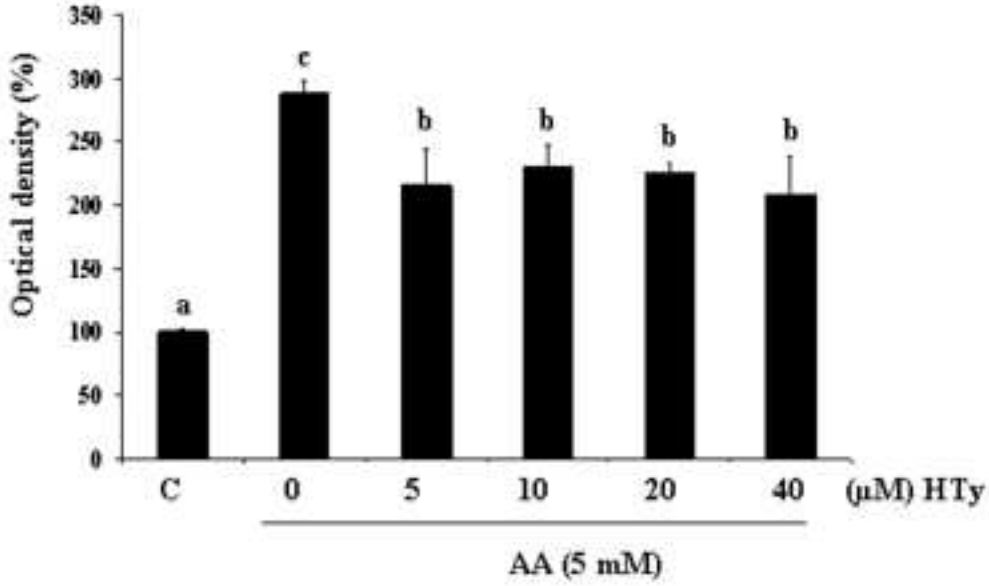

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