Article

Protection of Polyphenols Against Glyco-Oxidative Stress: Involvement of Glyoxalase Pathway

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Abstract: Chronic high glucose (HG) exposure increases methylglyoxal (MG)-derived AGEs and is involved in the onset of pathological conditions, such as diabetes, atherosclerosis and chronic-degenerative diseases. Under physiologic condition the harmful effects of MG are contrasted by glyoxalase system that is involved in the detoxification of Reactive Carbonyl Species (RCS) and maintain the homeostasis of the redox environment of the cell. Polyphenols are the most abundant antioxidants in the diet and present various health benefits. The study aimed at investigating the role of polyphenols extracted from an apple high in polyphenols (Calville White Winter), on glyco-oxidative stress induced by chronic HG-exposure. Intestinal Caco-2 cells were treated in physiological glucose condition (25mM) as a control and in HG condition (50mM) with or without apple extract for one week. Our data demonstrated that HG-treatment triggers glyco-oxidation stress with a significantly increase in ROS, lipid peroxidation, AGEs and Glyoxalase I (GlxI) activity with a significant decrease in total antioxidant intracellular defense. Treatment with polyphenols under HG condition restores to the control levels GlxI activity, decreases Glyoxalase II (GlxII) in relation to the control and induces a drop of glyco-oxidative damage. This paper seeks to highlight the roles of polyphenols in glyco-oxidative stress.

Keywords: Methylglyoxal; Glyoxalase system; AGEs; glutathione; hyperglycemia

1. Introduction

Glyoxalase system is an important enzymatic system involved in the detoxification of reactive carbonyl species (RCS) such as glyoxal (GO) methylglyoxal (MG) and 3-deoxyglucosone (3-DG). Glyoxal, methylglyoxal, and other physiological α-oxoaldehydes are formed by the lipid peroxidation, glycation, and degradation of glycolytic intermediates. The effects of MG, the most reactive metabolite, have been widely studied. [1,2] MGO rate depends on the organism, tissue, cell metabolism and physiological conditions; accumulation of MG to toxic level inhibits cell growth and induces cell death [3-5]. In fact, an abnormal accumulation of RCS triggers dicarbonyl stress resulting in an increase of irreversible adducts on proteins, known as advanced glycation end-products (AGEs), and DNA modification. MG can react also with other biomolecules such as nucleotides and basic phospholipids, thus yielding AGEs [6,7]. The increase in RCS modifications contribute to cell and tissue dysfunction and is involved in ageing and in the molecular mechanisms of various chronic disease such as dyslipidemia, obesity and vascular complications of diabetes [8,9]. Several studies have shown that the harmful effects of MG are contrasted by glyoxalase system [10,11]. Glyoxalase system includes two consecutive enzymes: glyoxalase I (GlxI) and glyoxalase II (GlxII). D-Lactate is

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a final product and glutathione is used as cofactor [12]. Glyoxalase I (EC 4.4.1.5), a lactoylglutathione lyase, catalyzes the isomerization of the hemithioacetal formed non-enzymatically from MG and reduced glutathione (GSH) into S-D-lactoylglutathione. Glyoxalase II (EC 3.1.2.6), hydroxyacylglutathione hydrolase, catalyzes the hydrolysis of S-D-lactoylglutathione to D-lactic acid and regenerates the GSH molecule consumed in the first reaction [13] (Figure 1).

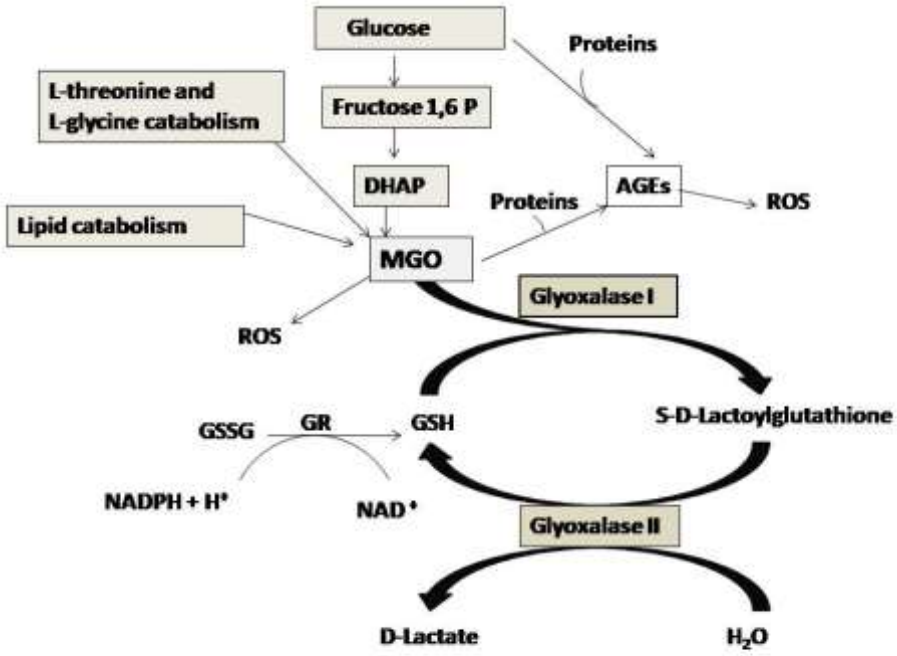

Figure 1. Methylglyoxal (MG) formation from intermediates of glucose, protein and fat metabolism, and its degradation by the Glyoxalase System. Glyoxalase I (Glx-I) converts hemithioacetal formed from GSH and methylglyoxal (MG) into S-D-Lactoylglutathione (SLG) which is hydrolyzed by Glyoxalase II (Glx-II) to D-lactic acid and GSH. Abbreviations: AGEs, advanced glycation end products; DHAP, dihydroxacetone phosphate; ROS, reactive oxygen species; GSH, reduced glutathione; GR, glutathione reductase.

Metabolic dysfunction involved in an increase of MGO, and consequently AGEs formation often also increases oxidative stress through an increase of Reactive Oxygen Species (ROS) formation [14]. Dicarbonyl stress may be exacerbated by oxidative stress. A decrease in GSH levels in oxidative stress directly impairs the metabolism of MG and glyoxal, leading to their accumulation [15]. Hyperglycemia associated with diabetes is among metabolic dysfunction driving increased formation of MGO and reactive oxygen species (ROS) as shown in different cell models. For instance, incubation of erythrocytes with high concentrations of glucose in vitro increases the flux of methylglyoxal metabolized to D-lactic acid via the glyoxalase pathway. The increase was proportional to glucose concentrations ranging from 5 to 100 mM and it has been proposed that glyoxalase pathway during periodic hyperglycemia may be a biochemical factor involved in the development of chronic clinical complications associated with diabetes mellitus [16,17]. More recent studies have shown that glyoxalase system has a physio-pathological role in other chronic diseases [8]. Indeed, was shown that glyoxalase I reduced dicarbonyl and oxidative stress and prevented age-related endothelial dysfunction, a major contributor to cardiovascular disease [18]. Dicarbonyl stress is a contributing mediator of obesity and vascular complications of diabetes. Moreover, dicarbonyl stress is involved also in cerebrovascular diseases and neurological disorders [19,20]. Therefore, a growing interest is devoted to molecules able to decrease dicarbonyl stress targeting the glyoxalase system. Our recent study has shown that high glucose concentrations triggers an increase of levels of

intracellular ROS, lipid peroxidation and formation of fluorescent AGEs and GA-modified proteins in intestinal cells [21]. In this study we investigated the effect of high glucose chronic exposure on glyco-oxidation and glyoxalase system in intestinal cells. Therefore, activities of glyoxalase I, glyoxalase II and GSH levels were evaluated in Caco2 cells. Furthermore, in the same cell model, we studied the effect of apple polyphenols. The interest of the study is supported by literature data which demonstrate the ability of some polyphenols to modulate glyoxalase activity, oxidative and dicarbornyl stress as shown in different experimental models [22-25].

2. Materials and Methods

2.1. *Reagent*

All cell culture reagents were obtained by Euroclone (Euroclone, Italy). All chemical reagents and rabbit polyclonal β-actin (A2066), and goat polyclonal anti-AGE (AB9890) antibodies were obtained by Sigma Aldrich (Sigma, St Louis, MO, USA). Carboxy-H2DCFDA (C400) was supplied by Invitrogen (Invitrogen, Carlsbad, CA, USA). Human colon epithelial cells Caco-2 (ATCC®HTB-37™) were purchased from the American Type Culture Collection (Rockville, MD, USA).

2.2. *Polyphenolic extract*

In this study Calville White Winter (W.W.) apples were used. These apples included in the Regional Repertory of Agro biodiversity of Marche (Italy) managed by Agency for Agrofood Sector Services of the Marche Region (ASSAM). Quantification and characterization of polyphenolic compound of this apple was previously analyzed (table 1) [26]. For the extract apples were carefully cut into slices, the pits were removed, and samples were freeze dried for 4 days in a Heto Dry Winner 685 (Denmark) lyophilize. Freeze dried samples were ground to powder using a laboratory mill and then stored at −20°C until analyzed. Lyophilized apples (2 g) were treated with 25 mL of 80% methanol and 0.1% formic acid for 30 min at room temperature to extract phenolic compounds. To remove polar non-phenolic compounds such as sugars and organic acids, CHROMABOND® PA C18 cartridges (Carlo Erba Reagents s.r.l, Italy) have been used. After the aqueous sample is passed through preconditioned C18 cartridges, the cartridges are washed with acidified water to remove sugar, organic acids and other water-soluble constituents. The polyphenols are then eluted with absolute methanol. For the cells treatment, methanol was eliminated by rotavapor and the extract was lyophilized and resuspended in sterile phosphate buffer saline (PBS).

Table 1. **Polyphenolic compounds in Calville W.W. apple.** Data are expressed as mean value (mg/100 g fresh weight) (n = 5).

Classes of polyphenols	Polyphenolic compound	mg/100 g FW Calville W.W.
FLAVANOLS	procyanidin B1	5.78
	procyanidin B2	229.68
	procyanidin trimer	88.74
	procyanidin tetramer	11.87
	procyanidin pentamer	5.25
	±catechin	0.88
	epicatechin	2.37
FLAVONES	Luteolin-glycoside	0.010
FLAVONOLS	rutin+hyperin	3.130
	isoquercitrin	0.018
	reynoutrin	0.016
	guajaverin	0.011
	avicularin	0.027
HYDROXYCINNAMIC ACIDS	chlorogenic acid	0.53
DIHYDROCHALCONES	phloretin-2-O-xyloglucoside	1.75
	phloridzin	5.55
ANTHOCYANINS	cyanidin-3-O-galactoside	5.15
TOTAL POLYPHENOLS		361± 11

2.1. *Cell model and apple polyphenols treatment*

CaCo-2 cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine,100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mM nonessential amminoacids at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2. Cell viability was estimated by examining the ability to exclude 0.1% Trypan Blue in 0.9% NaCl. Cells were treated with high glucose (HG) concentrations (50 mM) in the absence or in the presence of 250 μg/mL of Calville White Winter (W.W.) extract (150 μg polyphenols/mL; 0,8mmol/L) for 1 week [26]. Cells cultured in normal medium (25mM glucose concentration) were used as control. Medium was replaced two time per week.

2.3. *Total protein extraction and quantification*

Cells were trypsinized and centrifuged at 1200g for 10 minutes. Pellets were washed twice in phosphate-buffered saline (PBS). The extracts were obtained by resuspending cellular pellet with extraction buffer containing sodium-phosphate buffer pH 6.8, protease inhibitors (2.08 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1.6mM aprotinin, 0.08mM bestatin, 0.03mM E-64, 0.04mM leupeptin, 0.3 mM pepstatin A) and 0.5% NP40 detergent. After 30 min incubation on ice, cell lysates were centrifuged at 12,000 x g for 15 min, at 4 °C. Supernatants were recovered and total protein concentration was determined by the Bradford protein assay.

2.4. *Advanced glycation end products (AGEs) level*

Levels of fluorescent AGEs were detected in 100 μg of total protein extracts by evaluating intrinsic fluorescence of AGEs (340 nm/420 nm as excitation and emission wavelengths) (Synergy microplate reader, BioTek Instruments, Inc.). Results were expressed by fluorescence intensity per mg cell proteins. [27] For detection of total glycolaldehyde (GA)-modified proteins western blot analysis was performed. Cells extracts containing 50 μg protein were subjected to 12.5% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. After regular blocking and washing, the membranes were incubated overnight with goat polyclonal anti-AGE (#AB9890 Merk Millipore, Burlington, Massachusetts, USA) at 4 °C. β-actin (A2066 Sigma-Aldrich, St. Louis, MO, USA) has been used as loading control. After this incubation, the membranes were washed for three times with Tris-buffered saline containing 0.1% Tween 20 (TBST) and then incubated with specific HRP-labeled secondary antibodies (A50-101P Dako, Santa Clara, USA) for 1.30 h. Protein bands were developed by the enhanced SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA). The chemiluminescent signal was acquired using ChemiDoc XRS+ System (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed by using the Image J software (Version 1.50i, National Institute of Health, Bethesda, MD, USA).

2.5. *Intracellular ROS levels*

Intracellular ROS levels were detected by flow cytometry using H_2DCFDA (C400) as probe. Cells were trypsinized, washed twice with cold PBS and suspended at a final concentration of 0.5×10^6 cell/mL in pre-warmed PBS containing 10 μM probe. After incubation for 30 minutes in the dark at 37°C, cells were washed twice in PBS and stained with 10 μg/mL propidium iodide (PI). Fluorescence of labelled cells was measured on a "Coulter EPICS XL" flow cytometer (Beckman Coulter, USA) using an excitation wavelength of 488 nm. Emissions were recorded using the green channel for carboxy-DCF and the red channel for PI. The cells permeable to PI were excluded from the cell population considered for the ROS production to avoid false negatives. The data acquired were analyzed by the FCS Express Program (De Novo Software, CA, USA).

2.6. *Lipid peroxidation products*

Lipid peroxidation products were quantified by measuring thiobarbituric acid reactive substances (TBARS). One mL of 20% (w/v) trichloroacetic acid containing 0.8% (w/v) thiobarbituric acid (TBA) was added to each culture dish. The cells were scratched off and the suspensions were transferred to glass centrifuge tubes and boiled for 45 min. After centrifugation the absorbance of the supernatant at 535 nm was determined. Using the molar extinction coefficient of the (Malondialdehyde) MDA–TBA complex of 1.49×10^5 M−1 cm−1 the amount of TBARS was expressed as nmol MDA equivalents formed per mg protein [28].

2.7. *Cell total antioxidant activity*

The antioxidant activity of CaCo-2 cells treated in different experimental conditions was performed by oxygen radical absorbance capacity (ORAC) assay. Briefly, Trolox standard and samples was added to triplicate wells in a black 96-well microplate. Fluorescein (0.08 mM) was added to each well and incubated at 37 °C for 20 min before the addition of 17.5mM freshly prepared AAPH in working buffer. The microplate was immediately inserted into a microplate reader (BioTek Synergy HP, VT, USA). The decay of fluorescence at 530 nm was measured with excitation at 485 nm every 4 min for 3 hours. The area under the fluorescence versus time curve was used for quantification.[29]. Antioxidant activity was expressed as mM trolox equivalents (TE)/10^6 cells.

2.8. *Quantitative Determination of Glutathione*

Total levels of glutathione were measured spectrophotometrically at 412 nm using the glutathione reductase (GR) recycling assay in the presence of 5,5-dithiobis (2-nitrobenzoic acid) (DTNB), with a calibration curve obtain with known concentrations of GSH [19,30]. Cells were trypsinized, washed twice in cold PBS, and quickly centrifuged. For determination of total glutathione levels, the pellet was resuspended with 1% sulfosalicylic acid, vortexed, and incubated 30 min at 4 °C. Samples were then centrifuged at 2300 x g for 2 min, and the supernatant was recovered and analyzed for glutathione quantification. Finally, the pellet was resuspended with 1 M NaOH for recovery and quantification of proteins by Bradford methods using BSA as standard. Intracellular total glutathione is expressed as nmol/mg protein

2.9. *Glyoxalase system enzymatic assay*

Glyoxalase I activity was determined spectrophotometrically at 25°C by monitoring the intermediate S-D-lactoylglutathione (SLG) formation at 240 nm for 1 min (ε = 2.86 mM−1 cm−1). The hemithioacetal is pre-formed in situ by incubation of 2mM GSH (freshly prepared) and 2 mM MG in 100 mM phosphate-buffer pH 6.8 at room temperature in the dark for 15min. Then, 50µl of each sample were used per test. GlxI activity is given in units per mg of protein, where one enzyme unit (U) is defined as the amount of enzyme catalyzing the formation of 1 µmol of SLG per minute under assay conditions [31].

Glyoxalase II activity was measured spectrophotometrically at 25°C by monitoring the increase of GSH for 1 min at formation at 412 nm (ε = 13.6 mM−1 cm−1) [32]. Briefly, samples were incubated in a reaction mixture containing 100 mM MOPS buffer pH 7.2, 0.8 mM SLG, and 0.2 mM 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB). One enzyme unit (U) is defined as the amount of enzyme catalyzing the hydrolysis of 1 µmol of SLG per minute at saturating substrate concentration. For specific activity, U was referred to protein-concentration.

2.10. *Statistical analysis*

Data are expressed as the mean of measurements conducted separately ± Standard deviation. The Tukey-Kramer multiple comparison test or Kruskal-Wallis 1-way ANOVA (metabolomic) were used to determine whether the differences in results between treated and non-statistically significant cell groups ($p < 0.05$).

3. Results

3.1 *Effect of High glucose on AGEs formation and oxidative stress*

As shown in table 2, we observed a significant increase in intracellular ROS and a significant decrease of total intracellular antioxidant activity in cells incubated with HG compared with control cells. There was also an increase in lipid peroxidation ($p < 0.001$). All these results support that our experimental conditions trigger glyco-oxidative stress.

3.2 Effect of polyphenols on glyco-oxidative stress

Significant differences were observed in cells incubated in the presence of high glucose and polyphenols. Lower levels of ROS, and lipid peroxidation were observed. Moreover, total antioxidant capacity was increased. These results demonstrate that polyphenol treatment protects Caco 2 cells against glyco-oxidative stress (table 2).

Table 2. The oxidative stress in HG-treated CaCo-2 cells whit or without polyphenols extract. The oxidative stress in HG-treated CaCo-2 cells whit or without polyphenols extract. Intracellular ROS production; Total antioxidant capacity and Lipid peroxidation; in intestinal CaCo-2 cells treated for one week with physiological (25mM) glucose (CTRL), or with high glucose (50mM) concentrations in the absence (HG) or in the presence of Calville White Winter polyphenols extract (150 µg polyphenols/mL). Results are represented as mean ± SD of 5 determinations carried out in triplicate. Different letters indicate significant differences between samples ($p < 0.05$).

	Intracellular ROS production (Intensity of fluorescence A.U.)	Antioxidant capacity (µmol/TE/mg protein)	Lipid peroxidation (nmol MDA equivalents/mg protein)
CTRL	14±2	806±161	0.55±0.21
HG	29±2*	286±152*	3.01±0.62*
HG + extract	10±1*#	715±157#	0.54±0.26#

3.1. Effect of High glucose and polyphenols on AGEs formation.

To evaluate the effect of high glucose exposure and antiglycation effect of apple polyphenolic extract we compared the AGEs level in physiological (25mM) or HG (50mM) condition, and in cells incubated with HG plus polyphenol extract from Calville W.W. apple. As shown in figure 2, we observed a significant increase in AGEs formation in cells incubated with HG compared with control cells. Both fluorescent AGEs and GA-modified proteins were significantly increased. HG plus polyphenolic extract treatment decreased significantly AGEs formation with respect HG treatment (Figure 2).

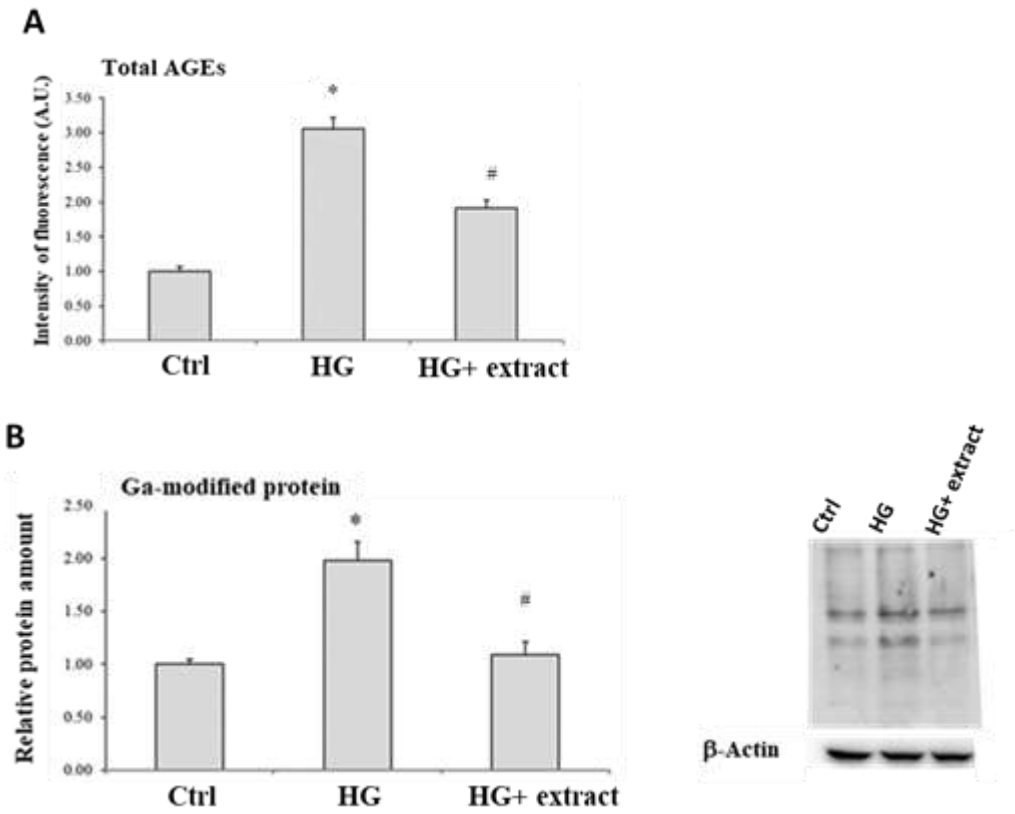

Figure 2. AGEs formation and Ga-modified protein in HG-treated CaCo-2 cells whit or without polyphenols extract. CaCo-2 cells was treated for one week with physiological (25mM) glucose (CTRL), or with high glucose (HG) (50mM) concentrations in the absence or in the presence of apple polyphenols extract (HG+Calville W.W.). A) Levels of total fluorescent AGEs and B) Representative western blot of GA-modified proteins and relative densitometric analysis. Densitometric data are normalized on β-actin. Results are presented as mean ± SD of 5 determinations carried out in triplicate. Different letters indicate significant statistic differences between samples ($p < 0.05$).

3.2. *Effect of High glucose and polyphenols on glyoxalase system*

We studied the effects of incubation of cells with HG on glyoxalase system. Therefore, we compared the activities of GlxI and GlxII and levels of total glutathione in control cells and cells incubated with HG. Glx I activity increased significantly after HG treatment compared with control cells (Figure 3A), while GlxII activity was lower in HG-treated cells (Figure 3B). GSH levels were not significantly modified (Figure 4).

Glx I activity was lower in HG-treated cells incubated with polyphenols and the activity was getting closer to control cells (Figure 3A), Glx II was not significantly modified after incubation with polyphenols (Figure 3B). Intracellular levels of GSH were increased with respect to HG treated cells in absence of polyphenols (Figure 4).

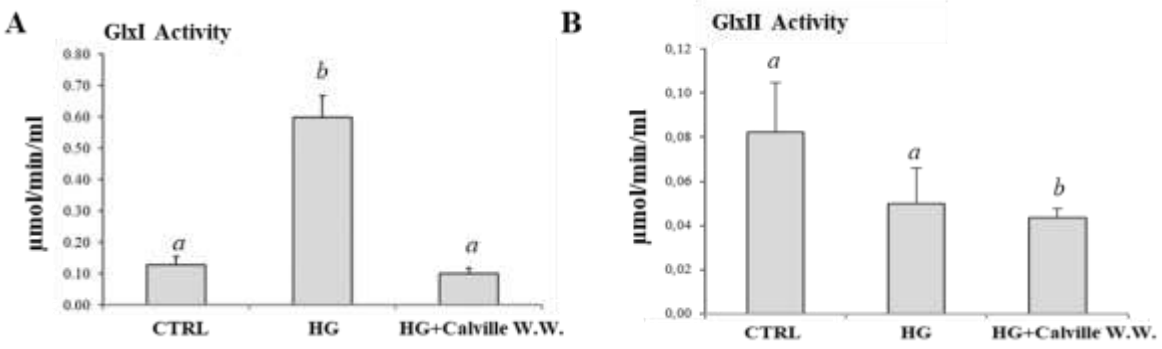

Figure 3. The glyoxalase system in HG-treated CaCo-2 cells whit or without polyphenols extract A) Glyoxalase I and B) Glyoxalase II activity in intestinal CaCo-2 cells treated for one week with physiological (25mM) glucose(CTRL), or with high glucose (50mM) concentrations in the absence (HG) or in the presence of Calville White Winter polyphenols extract (150 µg polyphenols/mL), Results are presented as mean ± SD of 5 determinations carried out in triplicate. Different letters indicate significant statistic differences between samples ($p < 0.05$).

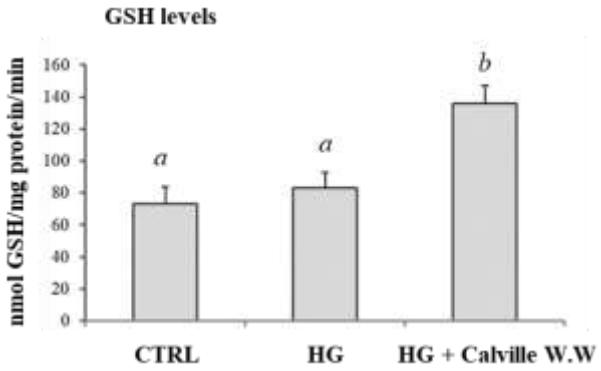

Figure 4. Glutathione level in HG-treated CaCo-2 cells whit or without polyphenols extract. Glutathione level in intestinal CaCo-2 cells treated intestinal CaCo-2 cells treated for one week with physiological (25mM) glucose (CTRL), or with high glucose (50mM) concentrations in the absence (HG) or in the presence of Calville White Winter polyphenols extract (150 µg polyphenols/mL). Results are represented as mean ± SD of 5 determinations carried out in triplicate. Different letters indicate significant differences between samples ($p < 0.05$).

4. Discussion

The close relationship between high glucose, oxidative stress and AGEs formation has been previously demonstrated in different cell lines [33,34]. Several studies have shown higher AGEs levels in HG condition [35,36]. As previously demonstrated, intestinal cells are sensitive to glyco-oxidative stress when exposed to high glucose concentrations [21]. Glyco-oxidative stress was confirmed in this study with an increase of AGEs formation, increase of intracellular ROS production, lipid peroxidation and a decrease of total antioxidant capacity.

Glyoxalase system plays a key role against dycarbonyl stress. Indeed, MGO is maintained at not-toxic levels by glyoxalase system. We observed an increase in Glx I activity in HG treated cells with respect to control cells while no changes were detected in Glx II activity and GSH levels. An increase in Glx I activity has been observed by other authors in HG treated cells and it has been suggested a possible protection from high MG formation in HG condition.[37].

In our experimental conditions, despite the significant increase in Glx I activity, levels of AGEs were higher in HG treated cells. Other authors have demonstrated that the GlxI enzyme was ineffective to normalize MO [38] in HG-treated cells. It has also to be stressed that AGEs formation does not only occur due to the increase of intracellular MGO but also as a consequence of glucose auto-oxidation and the non-enzymatic Maillard reaction and/or lipid peroxidation.

Contrasting results have been reported on the effects of hyperglycemic condition on glyoxalase system in different experimental models. In vivo studies in streptozotocin induced diabetic rat, an increase in GlxI and GlxII activity in red blood cells and skeletal muscle was observed while the activity decreased in the liver [39,40]. Other authors have demonstrated that GlxI increased in glomeruli of diabetic mice, while decreased in renal cortex [41]. A decrease of Glx I activity has been shown in SH-SY5Y neuroblastoma cells, and on human brain microvascular endothelial cell line (IHEC) during hyperglycemia. Furthermore, the effects of high glucose on GLX I and GLX II differ. For instance, no effects on Glx II activity have been observed [42]. All these findings suggest that the effects of high glucose concentrations on glyoxalase enzymes are strongly tissue specific.

In our experimental conditions, incubation with polyphenols during HG treatment, was associated with a decrease of AGEs formation, ROS production, lipid peroxidation. Furthermore, an increase of total antioxidant capacity and GSH levels was observed. All these results demonstrate that apple polyphenols were able to counteract the glycol-oxidation induced by HG in good agreement with previous studies on apple polyphenols. Other studies have demonstrated the ability of fruit polyphenols to exert a protective effect against formation of AGEs [43,44]. There are many steps in ROS and AGEs production therefore different mechanisms may occur to delay or decrease of intracellular ROS and AGEs formation [45] Purified phenolic compounds have been confirmed to reduce glycative stress through various pathways, including reduction of ROS production during the glycation process and trapping of dicarbonyl species [46]. A large amount of RO is produced by the early stage of the Maillard reaction and Schiff bases produce ROS and RCS. Therefore, capturing ROS at the early stage of glycation to reduce oxidative stress can inhibit glycation. Moreover, polyphenolic compounds with specific chemical structural arrangement exert a high reactivity with MG [47,48]. An MGO-trapping effect of apple polyphenols has been recently demonstrated [46,49], while the effects of isolated polyphenols on glyoxalase system has not been studied.

Our data showed significant reduction of ROS formation and increase in GSH levels after polyphenols treatment during HG condition compared to no-treated cells. GlxI activity increased in HG cells but polyphenols treatment restores to the control levels GlxI activity. In the glyoxalase pathway, GSH catalyzes the first step in the conversion of MG to D-lactate. In some cell models, an increase of intracellular concentration of GSH, modulates glyoxalase pathway. For instance, flavonoid treatment (morin and quercetin) lead to a significant increase in GSH concentrations and GLX I activity in MGO-treated cerebellar neurons [50]. Glutathione is important in redox regulation of transcription factors and enzymes for signal transduction [51], therefore our results suggest that polyphenol-mediated regulation of glutathione levels could modulate cellular processes in intestinal cells.

Different mechanisms could be advanced to explain the effect of apple polyphenols on GSH. Apple contain several flavonoids. Some authors have suggested that the increase in GSH levels in flavonoid treated cells, could be related to transactivation of the gamma-glutamyl-cysteine synthetase catalytical subunit promoter [52].

It has to be stressed that flavonoid antioxidant treatment and the decrease of intracellular levels of free radicals and ROS, could reflect in modulation of performance of the glyoxalase system

by modulating signaling pathways involved in cellular behavior glutathione synthesis and expression of antioxidant enzymes.

We confirm that apple polyphenols exert a protective effect against oxidative stress and dicarbonyl stress. In this scenario we can suggest that the benefic effect of apples extract was due at combining reduction of free radical production thorough increase in GSH levels and trapping of MG.

Further studies are necessary to understand better the effects of high glucose and polyphenols on dicarbonyl stress and glyoxalase system in intestinal cells and the molecular mechanisms that could be involved.

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