Proceeding Paper

White Wine Pomace Mitigates Hyperglycemia-Induced Cell Damage and Oxidative Stress in Caco-2 Cells †

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† Presented at the 4th International Electronic Conference on Foods, 15–30 October 2023; Available online: https://foods2023.sciforum.net/.

Abstract: Hyperglycemia is a significant risk factor in metabolic syndrome, contributing to the development of cardiovascular diseases and diabetes mellitus. Hyperglycemia increases ROS (reactive oxygen species) production via glucose oxidation and protein glycosylation, leading to cell damage. Our previous studies have highlighted the antioxidant properties of wine pomace products (wWPPs), a co-product of winemaking, and their ability to modulate oxidative stress. The objective of this study was to evaluate the protective effect of wWPPs against oxidative stress in hyperglycemic Caco-2 cells. They were treated with 1.5 µg GAE/mL of wWPP bioaccessible fractions, obtained from gastrointestinal digestion (WPGI) and colonic fermentation (WPF), under normoglycemic or hyperglycemic (35 mM glucose) conditions. After 24 h of treatment, cell viability, oxidative stress biomarkers and the expression of transcription factors and enzymes involved in cellular oxidation balance were evaluated. Hyperglycemia induced a 30% reduction in cell viability, which was restored to normoglycemic levels by WPF treatment. The bioaccessible fractions were able to counteract hyperglycemia-induced oxidative stress in intestinal cells, as evidenced by significant decreases in carbonyl groups and MDA levels (10 and 40%, respectively). Furthermore, hyperglycemia-induced NF-κB overexpression was also significantly reduced by WPGI and WPF pre-treatment (between 15 and 53%), modulating the redox activity. In conclusion, the bioaccessible fractions of wWPP, particularly WPF, demonstrated significant potential in mitigating hyperglycemia-induced oxidative stress and enhancing cell viability in Caco-2 cells.

Keywords: polyphenols; hyperglycemia; ROS

Citation: Gutiérrez-González, V.; Gerardi, G.; Muñiz, P.; Cavia-Saiz, M. White Wine Pomace Mitigates Hyperglycemia-Induced Cell Damage and Oxidative Stress in Caco-2 Cells. *Biol. Life Sci. Forum* **2023**, *26*, 31. https://doi.org/10.3390/Foods2023-15000

Academic Editor: Antonello Santini

Published: 13 October 2023

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1. Introduction

Metabolic syndrome is characterized by different metabolic and vascular disorders, including obesity, hypertension and hyperglycemia, that can lead to high mortality diseases like cardiovascular diseases and diabetes mellitus type II [1].

Persistent hyperglycemia can result in a series of chronic complications, with the gastrointestinal tract being one of the target organs [2,3]. Hyperglycemia plays a role in these diseases through the production of reactive oxygen species (ROS) via glucose oxidation and protein glycosylation, causing low-grade inflammation and interfering with homeostatic epithelial integrity [2,4]. Indeed, mechanisms of redox signaling are involved in the development of oxidative stress associated with hyperglycemia by modulating the expression of the transcription factor NF-κB, thus activating the transcription of pro-inflammatory genes [5]. The regulation of ROS production plays an important role in the prevention of epithelial function dysregulation associated with hyperglycemia.

Different studies suggest that antioxidant and anti-inflammatory bioactive compounds present in food could be used in the maintenance of epithelial functions. Wine pomace is the main co-product of the winemaking industry and has a high content in polyphenols

and dietary fiber [5,6]. Our previous studies highlighted the antioxidant properties of wine pomace products (wWPPs), a by-product of winemaking, and their ability to modulate oxidative stress associated with hyperglycemia in endothelial cells [5].

In view of the above, the aim of this study was to determine the potential protective effect of the bioaccessible fractions of white wine pomace against oxidative stress in hyperglycemic intestinal epithelial cells; similar effects might be caused by a high-glucose diet, which may induce alterations of epithelial functions implicated in a multitude of disorders.

2. Materials and Methods

2.1. White Wine Pomace Product (wWPP) and In Vitro Digested Fractions

The white wine pomace product (wWPP) was prepared at the University of Burgos from seedless white wine pomace from the winemaking of *Vitis vinífera* L. cv. *Verdejo* following a previously patented method [7]. The obtained wWPP was submitted to in vitro gastrointestinal digestion [8] with oral, gastric and intestinal phases with solutions containing α-amylase, pepsin and pancreatin and bile salts, respectively. At the end, a soluble digested bioaccessible fraction (WPGI) was obtained. In vitro colonic fermentation was performed in non-bioaccessible fractions with caecal content of healthy rats in a sterile anaerobic environment to mimic the human microbiota [8]. After centrifugation, the supernatant obtained represents the soluble fermented bioaccessible fraction (WPF). WPGI and WPF were freeze-dried and stored.

2.2. Characterization of the Bioaccessible Pomace Fractions WPGI and WPF

The antioxidant capacity of the fractions was assessed with Q-ABTS and Q-FRAP assays following previously described QUENCHER methods [8]. A total of 1 mg of the samples was analyzed at 734 and 593 nm, respectively, after incubation in the dark for 30 min with $ABTS^{\bullet+}$ radical solution or FRAP reagent. Results were expressed as μmol Trolox equivalents (TE)/g wWPP or μmol Fe (II) E/g wWPP, respectively. Total polyphenol content was assessed by incubating 1 mg of the samples with 0.1 mL of Folin–Ciocalteu reagent, and after 2 min, 2 mL of Na_2CO_3 75 g/L solution and Milli-Q water up to 5 mL. The supernatant absorbance was determined at 750 nm and gallic acid was used for calibration. The results were expressed as mg gallic acid equivalents (GAE)/g wWPP.

2.3. Cell Culture and Treatment

Human colon adenocarcinoma cell line Caco-2 (ATCC® HTB–37™) was purchased from the American Type Culture Collection (ATCC, Barcelona, Spain). Cells were cultured as a monolayer using Minimum Essential Medium Eagle (MEM) supplemented with 20% (*v*/*v*) heat-inactivated Fetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1% non-essential amino acids and 0.5 μg/mL amphotericin B. Cells were incubated at 90% humidity, 37 °C and 5% CO_2 atmosphere. Normoglycemic cells were incubated with MEM 5 mM and hyperglycemic cells with a medium high in glucose (35 mM D-glucose) for 48 h in normo- or hyperglycemic conditions. Afterwards, the WPGI and WPF fractions were added at 1.5 μg GAE/mL.

2.4. Cell Viability Assessment

Cell viability was measured using the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium) method according to previous studies [9]. Caco-2 cells were cultured at a density of 10^4 cells in 150 μL per well on a 96-well plate, then the cells were incubated with the bioaccessible fractions of wWPP at concentration of 1.5 μg GAE/mL for 24 h. Subsequently, 40 μL of MTT solution (5 mg/mL) was added and cells were incubated for 2 h at 37 °C. After incubation, MTT formazan crystals were dissolved in 100 μL of DMSO and the optical density was measured at 570 nm. The results were expressed as % cell viability with respect to normoglycemic control cells.

2.5. Assessment of Oxidative Stress Biomarkers

Malondialdehyde (MDA) levels were analyzed to determine lipid oxidation in the normoglycemic and hyperglycemic cell sonicated solution. The samples were incubated with 15 µL of 3M NaOH, for 30 min at 60 °C. After, 75 µL of 6% (*v*/*v*) H_3PO_4 and 75 µL of 0.8% (*w*/*v*) thiobarbituric acid (TBA) were added and incubated at 90 °C for 45 min. The MDA levels were measured by injecting 50 µL into an Agilent 1100 Series HPLC systems (Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with a diode array detector for its detection. Absorbance was measured at 534 nm and a calibration curve of 1,1,3,3-tetramethoxypropane (TMP) was performed to determine results, which were expressed as µM of MDA equivalents.

Carbonyl groups (CGs) were determined in 50 µL of the cell suspension that was mixed with 250 µL of 2,4-dinitrophenylhydrazine (DNPH) 0.2% and incubated 1 h at room temperature. Then, 250 µL of 20% trichloroacetic acid (TCA) was added and incubated for 15 min at 4 °C. It was washed with ethyl acetate:ethanol (1:1, *v*/*v*) and resuspended in 200 µL of 6M acidized guanidine. Samples were measured at 373 nm and results were expressed as nmol of GCs/mg of protein.

2.6. Quantitative Real-Time PCR Analysis (qPCR)

Total RNA was extracted from the frozen Caco-2 suspensions using TRI Reagent (Applied Biosystems, Foster City, CA, USA). After quantification with NanoDrop (BioTek, Winooski, VT, USA), 3 µg was treated with DNase I (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the RNA was reverse-transcribed using a First Strand cDNA Synthesis kit (Thermo Fisher Scientific). qPCRs were performed using specific primers: NF-Kb (F:5′- GGCGAGAGGAGCACAGATAC-3′ and R:5′- CTGATAGCCTGCTCCAGGTC-3′) and GADPH (F:5′- GCTCTCCAGAACATCATCCC-3′ and R:5′-GTCCACCACTGACACGTTG-3′) and SYBR Green q PCR Master Mix (EURx Sp. z. o. o., Gdansk, Poland) with ROX.

qPCR was carried out with a Quant Studio 5 Real-time PCR instrument (Applied Biosystems, Thermo Fisher Scientific Inc.). Results were calculated using the efficiency ΔΔCt method, with GADPH as the housekeeping gene. Results were expressed as folds of change compared with the normoglycemic non-treated cells.

2.7. Statistical Analysis

Statistical analysis was performed using StatGraphics® Centurion 18.1.13 (Statpoint Technologies Inc., Warrenton, VA, USA). Data were expressed as means ± standard deviation of independent experiments performed in triplicate and One-way analysis of variance (ANOVA) to determine significant differences ($p < 0.05$) between data.

3. Results and Discussion

3.1. Antioxidant Activity and Total Polyphenol Content of the wWPP Fractions

The wWPP was subjected to in vitro gastrointestinal digestion and colonic fermentation to obtain the fractions that would be bioaccessible in the small and large intestine, respectively. The antioxidant capacity and the polyphenol content are shown in Table 1.

The analysis of the bioaccessible fractions showed a high phenolic content and antioxidant activity. The antioxidant capacity of the WPF was significantly higher than that of the WPGI when they were analysed using the Q-ABTS method. The higher antioxidant activity in the WPF may be associated with a higher content of phenolic acids as a consequence of intestinal microorganism actions that release and modify more complex polyphenols [10]. These results show that the high antioxidant activity and phenolic composition of the wWPP is not altered by the digestion and fermentation processes, and in fact is enhanced during digestion, especially the antioxidant activity [5].

Table 1. Total antioxidant capacity and polyphenol content of the bioaccessible gastrointestinal (WPGI) and the bioaccessible fermented (WPF) fractions.

	WPGI	WPF
Q-ABTS (μmol TE/g wWPP)	261 ± 10.5	760 ± 92.3 *
Q-FRAP (μmol Fe(II)E/g wWPP)	31.6 ± 1.4	30.0 ± 0.7
Total polyphenols (mg GAE/g wWPP)	11.7 ± 0.6	12.8 ± 2.3

Antioxidant capacity of the WPGI and WPF was determined with Q-ABTS and Q-FRAP. Values represent mean (n = 3) ± SD. Statistical analysis was performed with Student's *t*-test and significant differences ($p < 0.05$) are indicated by asterisk (*). WPGI: bioaccessible digested fraction of the white wine pomace product; WPF bioaccessible fermented fraction of the white wine pomace product. ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); FRAP: Ferric reducing antioxidant power; TE: Trolox equivalent; GAE: gallic acid equivalent.

3.2. Effects of Hyperglycemia in Cell Viability and Oxidative Stress Biomarkers

The hyperglycemic effect on epithelial cells is well known and is characterized by a decrease in cell proliferation and an increase in apoptosis due to an increase in oxidative stress [4,11]. Caco-2 cell viability significantly decreased in hyperglycemic conditions compared with that with normoglycemia (Figure 1). However, this effect was regulated by the WPF fractions, which increased the cell viability of the hyperglycemic cells to the level of the normoglycemic control.

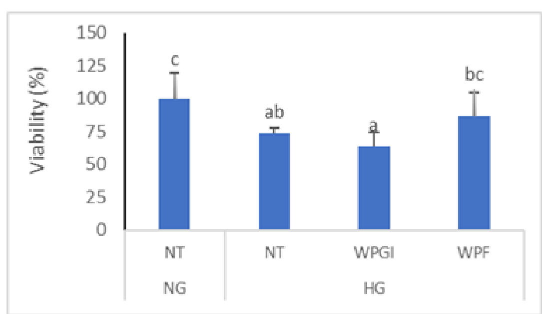

Figure 1. Cell viability in normoglycemic and hyperglycemic Caco-2 cells treated with WPGI and WPF fractions. Values represent mean (n = 3) ± SD. Significant differences ($p < 0.05$) between the non-treated and the treated fractions for both conditions are indicated by Latin letters (a, b, c). NG: normoglycemic; HG: hyperglycemic; NT: non-treated with pomace fractions; WPGI: bioaccessible digested fraction of the white wine pomace product; WPF: bioaccessible fermented fraction of the white wine pomace product.

Chronic hyperglycemia increases the expression of NOX4, ROS, apoptosis-related proteins and inflammatory factors in intestinal epithelial cells [11]. To determine the protective effect of bioaccessible fractions on intestinal epithelial cells in hyperglycemic conditions, we evaluated the malondialdehyde levels and carbonyl group content as biomarkers of oxidative stress. Both biomarkers significantly increased in hyperglycemia (Figure 2A,B).

MDA levels, an indicator of lipid damage and peroxidation, were increased by 20% with hyperglycemia; this increase was reduced by both bioaccessible fractions, and the WPGI fraction could significantly reduce it to normoglycemic levels. The lipid peroxidation, as well as the carbohydrate oxidation, can cause the production of reactive carbonyl species, which can be introduced as carbonyl groups into proteins, damaging their structure [12]. This agrees with our results showing a 50% increase in carbonyl group content in hyperglycemia compared with that in normoglycemic conditions. Both WPGI and WPF fractions were able to reduce it significantly to normoglycemic levels. These results are in line with other studies showing that polyphenols were able to reduce the formation of Schiff bases and dicarbonyl groups via oxidative stress [13].

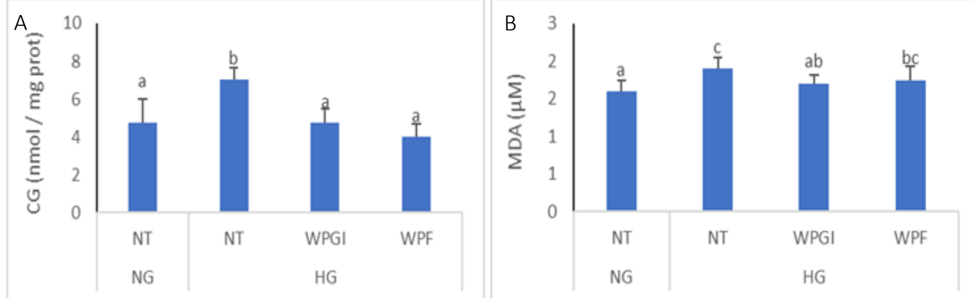

Figure 2. Cell biomarkers of oxidative stress in normoglycemic and hyperglycemic Caco-2 cells treated with WPGI and WPF fractions. (**A**) Carbonyl group (GC) levels and (**B**) malondialdehyde (MDA) levels. Values represent mean (n = 3) ± SD. Significant differences ($p < 0.05$) between the non-treated and the treated fractions for both conditions are indicated by Latin letters (a, b, c). NG: normoglycemic; HG: hyperglycemic; NT: non-treated with pomace fractions; WPGI: bioaccessible digested fraction of the white wine pomace product; WPF: bioaccessible fermented fraction of the white wine pomace product.

The effect of wWPPs in reducing biomarkers of oxidative damage is consistent with previous studies on endothelial cells exposed to hyperglycemia and in vivo models of oxidative stress-related diseases [5,13], and may result from the modulation of several cellular pathways by the phenolic compounds present in wine pomace products, such as hydroxycinnamic acids and resveratrol. In this regard, we evaluated the NF-κB transcription factor, a mechanism involved in hyperglycemic-induced oxidative stress.

Under hyperglycemic conditions, a significant increase was observed in the mRNA levels of NF-κB by almost two-fold (Figure 3). The increase in the NF-kB could explain the increase in the biomarkers of oxidative stress observed and associated with inflammation processes in hyperglycemia [3]. The treatment with WPGI fractions decreased the expression of NF-κB to non-treated values, in line with that observed for oxidative stress biomarkers.

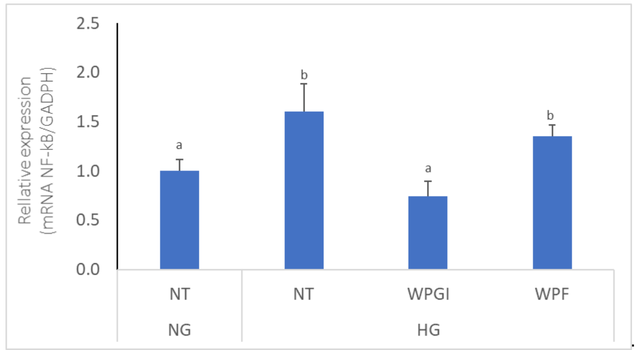

Figure 3. Nf-κB gene expression. Values represent mean (n = 3) ± SD. Significant differences ($p < 0.05$) between the non-treated and the treated fractions for normoglycemic and hyperglycemic conditions are indicated by Latin letters (a, b). NT: non-treated with pomace fractions; WPGI: bioaccessible digested fraction of the white wine pomace product; WPF: bioaccessible fermented fraction of the white wine pomace product. Nf-κB: nuclear factor kappa-light-chain-enhancer of activated B cells.

4. Conclusions

In conclusion, our study has demonstrated that wine pomace products offer promising benefits in attenuating hyperglycemic-related disorders through their capacity to ameliorate intestinal inflammation and oxidative stress. Both the gastrointestinal and fermented bioaccessible fractions of wine pomace have shown the ability to reduce lipid and protein oxidation and inhibit the proinflammatory transcriptional factor NF-κB.

From our perspective, these findings suggest the potential of wine pomace as a functional ingredient, obtained from a winery co-product, with substantial health-promoting properties for preventing hyperglycemia-associated complications. Further studies are needed to develop this product as a nutraceutical or to explore its applications as a functional food.

Author Contributions: V.G.-G.: Investigation, Formal analysis, Methodology, Writing; G.G.: Investigation, Writing—review; P.M.: Funding acquisition, Project administration, Writing—review; M.C.-S.: Investigation, Supervision, Writing—review. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Ministry of Science, Innovation and Universities, Spanish State Research Agency and the European Regional Development Fund (Project PGC2018-097113-B-I00).

Institutional Review Board Statement: Experimentation with animals was approved by the Ethics Committee for Experimental Animal Care at the University of Burgos (PEAUBU012018) and was carried out in accordance with Spanish and European laws (RD53/2013 of Spanish Ministry and European Directive (2010/63/EU).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data from the present study are available upon request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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