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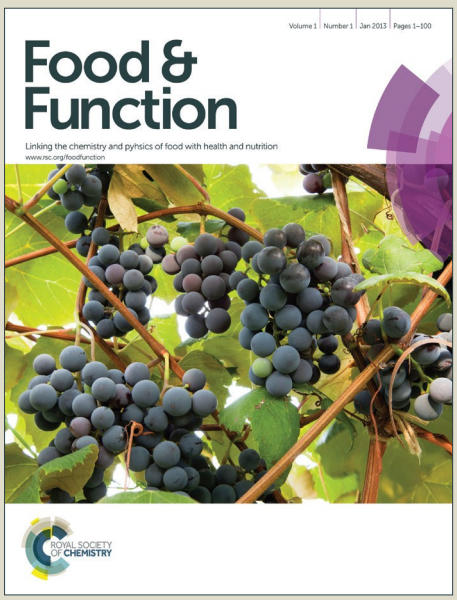

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Reformulated meat products protect against ischemia-induced cardiac damage

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Short title: Reformulated meat and cardioprotection.

Keywords: Antioxidants, Cardiomyocyte, Ischemia, Processed meat

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Abstract

The protective effects of the antioxidants present in food are of great relevance for cardiovascular health. This study evaluates whether the extracts from reformulated meat products with a reduced in fat and/or sodium content exert a cardioprotective effect against ischemia-induced oxidative stress in cardiomyocytes, compared with non-meat foods. Ischemia damage caused loss of cell viability, increased reactive oxygen species and lipid peroxidation and decreased the antioxidant activity. Pretreatment for 24 h with digested or non-digested extracts from reformulated meat products led to protection against ischemia-induced oxidative damage: increased cell viability, reduced oxidative stress and restored the antioxidant activity. Similar results were obtained using extracts from tuna fish, but not with the extracts of green peas, salad or white beans. These results suggest that reformulated meat products have a beneficial impact in protecting cardiac cells against ischemia, and they may represent a source of natural antioxidants with benefits for cardiovascular health.

Keywords: Antioxidants, Cardiomyocyte, Ischemia, Processed meat

Abbreviations

CAT: catalase

CFDA-SE: 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester

CVD: cardiovascular disease

H_2DCFDA: 2', 7'-dichlorodihydrofluorescein diacetate

EDTA: ethylenediaminetetraacetic acid

EGCG: epigallocatechin gallate

GPx-3: glutathione peroxidase-3

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IHD: ischemic heart disease

MDA: malondialdehyde

MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

PBS: phosphate buffered saline

PMSF: phenylmethylsulfonyl fluoride

ROS: reactive oxygen species

TBARS: thiobarbituric acid reactive substances

1. Introduction

Growing social interest between food consumption and health is leading to new insights into the effect of food components on physiological function and health. These insights have moved consumers to become more health-conscious, driving a trend towards healthy and nutritious foods with additional health promoting functions, such as functional foods. So, although the meat is the major source of animal protein for many consumers, and is also a source of some valuable nutrients such as minerals and vitamins[1], regrettably consumers in many countries recognize meat products to be bad for health and even dietary food guides suggest reducing the daily consumption of meat[2]. The main drawback of meat consumption is the high content of both salt and fat added to most meat products during processing. However, consumers are unaware that meat is also a source of substances with antihypertensive, antioxidant or antithrombotic activity, among others [3–5]. Most of these bioactives are small peptides or free amino acids functionally inactive within the native proteins, which must be released during processing (hydrolysis, cooking or fermentation) to achieve their biological activity[6,4]. Bioactive peptide generation can also occur naturally in mammals within the gastrointestinal tract during *in vivo* digestion[7]. Emphasizing these activities is one possible approach for improving the perception of meat as a healthy product and developing functional meat products.

Traditionally, human nutrition research has largely focused on evaluating natural components in food that may retard many chronic diseases, including cardiovascular diseases (CVDs)[3,8,9]. *In vivo* and *ex vivo* studies have provided evidence supporting the key role of oxidative stress in a number of CVDs such as atherosclerosis, ischemia, hypertension, cardiomyopathy, cardiac hypertrophy and congestive heart failure[9–11].

Indeed, CVD is the most commonly identified specific cause of ischemic cardiomyopathy in developed countries and remains the leading cause of death worldwide and a major cause of disability[12]. During ischemia, the inadequate supply of oxygen and glucose to the heart leads to an increase in oxidative stress, cell damage and myocyte death. Endogenous antioxidants represent one of the most potent mechanisms for combating cell damage and death induced by ischemia. These antioxidants act by neutralizing the excess of free radicals, thereby protecting the cells against their toxic effects and contributing to disease prevention[9]. In addition, the dietary intake of food-derived antioxidants plays an important role in helping endogenous antioxidants to maintain a healthy oxidant/antioxidant balance in the body. Indeed, nutrient antioxidant deficiency is one of the causes of numerous chronic and degenerative pathologies[9], and food-derived bioactive peptides - as an alternative to synthetic pharmaceuticals - have gained increased attention in recent years for both the prevention and treatment of CVDs.

In the present study, we evaluate whether extracts from reformulated meat products, reduced in fat and/or sodium before and after simulating an *in vitro* gastrointestinal digestion process, may exert a cardioprotective effect against oxidative stress induced in a cell model of cardiac ischemia.

2. Materials and methods

2.1. Reagents

Claycomb medium, foetal bovine serum, protease inhibitor cocktail, L-glutamine, penicillin, streptomycin, (±)-norepinephrine (+)-bitartrate salt, phenylmethylsulfonyl fluoride (PMSF) and other biochemicals were obtained from (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Food extracts

Reformulated meat products with a low fat and/or sodium content of were assessed; these included: turkey breast, cooked ham, dry-cured ham, mortadella and cured sausage. Canned tuna fish, white beans, green peas and salad (iceberg lettuce, carrot and lombard) were also included as non-meat foods differing in their protein and antioxidant contents. One hundred grams of any food were processed to obtain their respective aqueous total food extract[13] and gastrointestinal soluble fraction[14] using the experimental protocols cited above but adapted for our purposes. Briefly, for total extracts (non-digested), foods were minced and homogenized with 100 ml of 0.01 M HCl in an ultraturrax for 10 min at 4 ºC. The homogenates were centrifuged (1800 xg for 15 min at 4 ºC) and filtered through glass wool. The pH was adjusted to 6.0 with 0.1 M NaOH. For the gastrointestinal soluble fraction (digested), foods were minced and homogenated with 80 ml of 0.1 M HCl in a stomacher for 15 min and the pH was adjusted to 2.0 with 6 M HCl. Pepsin (10 mg/g protein) was added and the mixture was incubated for 2 h at 37 ºC with shaking at 200 rpm in an orbital incubator shaker. The pH was raised to 5.3 by adding 6 M NaOH before adding the pancreatin solution (25 mg/ml pancreatin + 5 mg/ml lipase in 0.1 M $NaHCO_3$). Finally, the pH was increased to 7.5 by the addition of 1 M NaOH, and the samples were incubated for 3 h at 37 ºC with shaking at 100 rpm. To complete the *in vitro* digestion process, the mixture was heated at 100 ºC for 15 min and then cooled in ice water. The homogenate was centrifuged (1800 xg for 15 min at 4 ºC) and filtered through glass wool. The protein content of different extracts was measured by the bicinchoninic acid assay using bovine serum albumin as the standard protein[15]. The fractions were stored at -80 ºC until use.

Epigallocatechin gallate (EGCG, 30 μM), a polyphenol with high antioxidant activity was used as a positive control of cardioprotection. EGCG is the major antioxidant catechin found in green tea and has been associated with cardiovascular benefits[16,17].

2.3. Cell culture, treatments and preparation of cell extracts

HL-1 cells are a line of adult mouse cardiomyocytes that maintain a differentiated cardiac phenotype, which has been widely used in similar experimental studies. Cells in complete culture medium consisting of Claycomb medium, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.1 mM norepinephrine were maintained at 37 ºC in exponential growth phase. The monolayer culture was split in a 1:6 ratio or harvested to perform experiments when confluence was 70-80%[18,19]. Before ischemia induction, plated cells were incubated with food extracts in the CO_2 incubator preset to 37 ºC, 95 % humidity, 5 % CO_2, and 21 % CO_2 for 24 h, unless otherwise stated. To obtain cell extracts, subconfluent cultures harvested with trypsin/EDTA and pooled in Eppendorf tube, were washed twice with phosphate-buffered saline at 4 ºC. Afterwards, cells resuspended in 100 μl of ice-cold medium containing 10 mM Tris-HCl, pH 7.4, 1% (v/v) Triton X-100 and 0.1 mM PMSF, were lysed on ice-water bath with 80 strokes in glass-teflon Dounce homogenizer. Samples were centrifuged at 10,000 xg for 20 min at 4 °C. The supernatant was aliquoted, and stored at −80 °C for further study. The protein content of the cellular extracts was determined by the bicinchoninic acid method[15].

156 *2.4. Simulated ischemia*

157 To closely simulate the conditions in the ischemic myocardium, ischemia
158 exposure was carried out by replacing the Claycomb medium by a modified saline
159 medium [ischemia Ringer solution (IR)], which contained: 1.25 mM $CaCl_2$, 1 mM
160 MgCl2, 120 mM NaCl, 8 mM KCl, 6 mM HEPES, and 0.9 mM NaH_2PO_4 at pH 6.3;
161 and cells were transferred to the hypoxia chamber (INCO 153, O_2/CO_2 incubator;
162 Memmert GmbH, Schwabach, Germany) in which the oxygen level was lowered to 2%
163 by replacing with nitrogen gas at 37 ºC, 5% CO_2 concentration and 95% relative
164 humidity. The ischemic protocol used consisted in a reduction of the oxygen level to 2%
165 for 15 h in IR buffer, unless otherwise stated. This time was selected according to the
166 results of the trials depicted in figure 2A. For control conditions, HL-1 cells were
167 maintained under normoxic conditions (21% O_2+5% CO_2) for equivalent periods of
168 time in normal Ringer solution (NR): 1.25 mM $CaCl_2$, 1 mM $MgCl_2$, 137 mM NaCl, 6
169 mM KCl, 10 mM glucose, 6 mM HEPES and 0.9 mM NaH_2PO_4 at pH 7.4.
170
171 *2.5. MTT assay*
172 Cells at ~15×10^3/well were seeded in quadruplicate in 24-well plates and
173 grown at 37 °C for 4 days in complete culture medium. Subconfluent cultures were
174 subjected to the indicated treatments. Culture medium from treated or untreated cells
175 was aspirated and cells were washed twice with pre-warmed PBS. The redox potential
176 of live cells was evaluated after incubation with 300 µl/well 1 mg/ml 3-(4,5-
177 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 30 min in the CO_2
178 incubator. The formazan precipitate was dissolved by adding 250 µl/well dimethyl
179 sulfoxide and shaking the plate for 5 min at room temperature. Then, 25 µl of 2 M Tris
180 at pH 10.5 was added to each well. Absorbance of the samples was read at 570 nm and

the background value at 690 nm was subtracted. Cell viability at each time point is expressed as a percentage with respect to control.

2.6. Carboxyfluorescein diacetate N-succinimidyl ester (CFDA-SE) assay

Cell viability was also evaluated by 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) assay adapted for our purposes[18]. Briefly, HL-1 cells were grown in complete culture medium (2×10^6 cells per flask) in 25-cm^2 flasks at 37 ºC for 4 days. After the indicated treatment, HL-1 cells were loaded with the CFDA-SE fluorescent probe (2 mM) and the decrease in fluorescence was used to calculate the loss of cell viability. The fluorescence of the cells was monitored with an FACSort flow cytofluorimeter (BectonDickinson, San Jose, CA, USA) equipped with a 15 mW argon laser, tuned to 488 nm, and standard sets of filters for green (FL1) and red (FL3) fluorescence. Propidium iodide (PI) was added to all the samples before data collection to identify dead cells. The flow-cytometry results presented as dot plots are representative of four experiments performed under selected assay conditions. Cell viability was calculated as the percentage of fluorescence compared with the control.

2.7. Determination of reactive oxygen species (ROS)

Oxidative damage was measured with the aid of the cell-permeable indicator H_2-DCFDA[20]. After intracellular deacetylation, the non-fluorescent dye 2',7'-dichlorodihydrofluorescein is oxidized by ROS to the fluorescent 2',7'-dichlorofluorescein (DCF). Cells in a 96-well microtiter plate were grown at 37 ºC for 2 days in complete culture medium to get ~8×10^4 cells/well. After the indicated treatment, cells were washed with prewarmed phosphate-buffered saline and loaded in the dark at 37 ºC for 20 min with 10 μM H_2-DCFDA in Claycomb medium. Excess probe was

washed out using ice-cold PBS. Immediately after that, of green fluorescence was monitored at excitation and emission wavelengths of 485 and 530 nm, respectively using a Fluostar Omega microplate reader (BMG Labtech, GmbH Hanns-Martin-Schleyer-Str., 10. Offenburg, Germany). ROS production was calculated as the percentage of fluorescence compared with a control. In parallel, cells (2×10^4) were plated on each 35-mm glass-bottomed dish and grown at 37 ºC in complete medium for 2 days. Treated and untreated cells were loaded at 37 ºC with DCFDA and the fluorescence images were captured with Leica equipment consisting of TCS SP2 confocal scanning head coupled to a DM IRE II inverted fluorescence microscope. DCFDA-loaded cells were visualized at room temperature through an HCX PL APO 63x oil immersion objective. The numerical aperture was 1.32 and the confocal pinhole for detection was 140 mm, equivalent to 1.1 mm thickness in the z direction. Samples were illuminated with the 488-nm argon laser line and the emitted fluorescence was selected through the wavelength range of 500-530 nm.

2.8. Spectrophotometric determination of TBARS. Lipid peroxidation.

Lipid peroxidation was estimated spectrophotometrically according to the thiobarbituric acid-malondialdehyde (TBA-MDA) assay, using the method of Ohkawa, Ohishi and Yagi (1979), and adapted for our purposes[21]. Lipid peroxidation occurs when ROS oxidize phospholipids, and the lipid peroxides form carbonyl compounds, such as malondialdehyde (MDA). At high temperatures, MDA forms adducts with thiobarbituric acid (TBA) to produce a red colored product (TBA-MDA) quantified by colorimetry. Subconfluent cultures in 6-well plates at 6×10^4 cells/well, after the indicated treatment, were washed with cold PBS, scraped and lysed by homogenization in ice-cold 1.15% (v/v) KCl. Samples containing 100 µl of cell lysates were combined

with 0.2 ml of 8.1% (p/v) SDS, 1.5 ml of 20% (v/v) acetic acid adjusted to pH 3.5 and 1.5 ml of 0.8% (v/v) TBA. The mixture was brought to a final volume of 4 ml with distilled water and heated at 95°C for 120 min. After cooling to room temperature, 5 ml of mixture of n-butanol and pyridine (15:1, (v:v, n-butanol/pyridine)) was added to each sample and the mixture was shaken vigorously. After centrifugation at 14,500 xg for 10 min, the supernatant fraction was isolated and the TBA-MDA adducts (TBARS) were measured colorimetrically at 532 nm with an Infinite® 200 PRO spectrophotometer (Tecan Group AG, Seestrasse, Männedorf, Schweiz). The concentration of malondialdehyde (MDA) equivalents was calculated by using a determination curve elaborated with 1,1,3,3-tetraethoxipropane (TEP) as standard reagent in the range from 0.25 to 4 μM.

2.9. Antioxidant activities

Antioxidant activities were assessed using the procedure adopted by Asensio-Lopez et al., with some modifications adapted to this study[19]. Cells at 2×10^6/well were seeded in 25-cm^2 plates and grown at 37 °C for 1 day before being subjected to the specific treatments. After incubation, the cells were harvested and disrupted by three cycles of freezing/thawing in 40 μl of Tris–HCl (pH 7.4) containing 0.1% (v/v) Triton X-100, 100 mM PMSF, and 10 ml/1×10^6 cells protease inhibitor. After centrifugation at 300 xg for 20 min at 4 °C, the supernatants were used to determine catalase (CAT) and glutathione peroxidase (GPx-3) activities. The Amplex red catalase assay kit from Molecular Probes (Eugene, OR, USA) and the glutathione peroxidase activity kit from Enzo Life Sciences (Farmingdale, NY, USA) were used for CAT and GPx-3 activities, respectively. Data were showed as the percentage of activity compared with the control.

256 *2.10. Statistical analysis*

257 Experimental data in the histograms are expressed as mean values of at least five
258 independent assays and standard deviations are indicated by error bars. Differences
259 among groups were tested by Student's t test; p values of less than 0.05 were regarded as
260 significant. The Pearson correlation coefficient was used to measure the strength of the
261 linear relationship between normally distributed variables. Analyses were carried out
262 using the IBM SPSS Statistics 19.0 software.

263

264 **3. Results and discussion**

265 *3.1. Selection of work conditions*

266 First of all, in order to select the optimal working dilution for each food extract,
267 HL-1 cells were incubated for 24 h with different dilutions of each extract and cell
268 viability was assayed by measuring the mitochondrial function with the MTT reagent.
269 (Figure 1). The effect of any of the digested extracts on cell viability is dependent on the
270 dilution used. As shown in panel A, the 1:1 (v/v, food extract/culture medium) dilution
271 of any of the digested extracts resulted in a significant reduction of cell viability. This
272 decrease in cell viability was related with changes in the osmolarity of the medium[22,23].
273 As shown in panel B, the assays conducted with a 1:1 (v/v, food extract/culture
274 medium) dilution showed osmolarities higher than 450 mOsm/kg or close to 200
275 mOsm/kg. Cell culture mediums are designed to have osmolarities in the range of 260-
276 320 mOsm/kg, basically to mimic the osmolarity of serum at 290 mOsm/kg[24]. Our
277 results suggest that the hyper- or hypo-osmotic stress was the cause of cardiomyocyte
278 death when they were exposed to 1:1 (v/v, food extract/culture medium) dilution of any
279 of digested food extracts. Based on these results, we selected a 1:5 (v/v, food
280 extract/culture medium) dilution as the working dilution for either extracts, because at

this dilution level osmolarity was 295±12 mOsm/kg and there was no effect on cell viability (Figure 1A).

3.2. Meat extracts protect cardiomyocytes against ischemia-induced oxidative stress

Once the working dilution of the digested extracts had been selected, the next step was to evaluate whether these extracts protect cardiomyocytes against oxidative stress induced by ischemia. The use of an *in vitro* assays using culture cardiomyocytes as experimental model of ischemia to mimic in vivo system, represents a powerful and useful tool to investigate cardioprotective strategies that we have used against the myocardial infarction[25,26]. Despite the obvious differences between these experimental systems, there are surprisingly pleasing similarities under conditions of stress, and the way the same cells behave under the catastrophic conditions of ischemia, in animal models. Thus, we have used a simple, highly controlled experimental system that providing detailed basic information as to how that cell type responds to an oxygen and glucose deprivation (ischemia) or as different strategies may protect against oxidative damage associated with the ischemia.

Oxidative stress is a condition in which oxidant metabolites exert their toxic effect because of an increased production of ROS, impaired antioxidant capacities, or both. A high ROS level is a critical factor in modulating cardiac stress response signaling during ischemia[9,10,27]. As mentioned, we wished to assess whether the food extracts in question may exert a functional preventive effect on the high ROS level induced by ischemia. As expected, ischemia increased the ROS levels in a time-dependent manner, which negatively correlated with the cell viability ($r=-0.977$, $p<0.001$) (Figure 2A). The increase in ROS levels was clearly visible after 6 h of simulated ischemia. The increase of ROS, as a result of ischemia, was also evaluated by

confocal microscopy, using the cell-permeable indicator H_2-DCFDA (Figure 2C): ischemia induced an increase in fluorescence green, as consequence of oxidation of the probe by the generated ROS, as compared to the condition of normoxia. In the following tests, the exposure time to ischemia was 15 hours, after which the cell viability had decreased to 25±6% and the ROS levels had increased to 2.3±0.1 (Figure 2A). Interestingly, pretreatment with any of the meat extracts prevented the increase in ROS level induced by ischemia (1.3±0.2, turkey breast; 1.1±0.3, cooked ham; 0.9±0.2, cured ham; 1.2±0.3, mortadella and 1.2±0.1, sausage) (Figure 3A). This protective effect was similar to that obtained with the extract from tuna fish (0.8±0.2) or by the catechin EGCG (0.6±0.4, EGCG). Contrary to expected, pretreatment with extracts of green peas (2.1±0.1), salad (2.3±0.1) or beans (2.3±0.3) did not prevent the increase in ROS levels induced by ischemia. The lack of effect of these extracts may be explained by the fact that the assays were adjusted by food quantity and not by protein concentration. Indeed, the protein concentration in salad extract was lower (2-5 times) than the other food extracts. Therefore, the low concentration of functional proteins, or peptides, with antioxidant capacity in salad extract may justify the fact that one hundred grams of this product were not able to obtain a significant antioxidant effect. However, green peas and beans extracts had similar protein concentration that tuna or meat extracts, which suggest that the antioxidant effect is also dependent of protein source. On the other hand, the sole administration of digested extracts in the absence of ischemic damage had no effect on ROS levels compared with the control (Supplementary figure 1A). The protective effect of these digested extracts against ROS generation induced by ischemia was also evaluated by confocal microscopy (Supplementary figure 2). The results obtained corroborate previous data.

In pathological conditions, ROS attack unsaturated fatty acids of lipid membranes and induce lipid peroxidation, which can lead to changes in the permeability and fluidity of the membrane lipid bilayer and can dramatically alter cell integrity[28]. The effect of digested food extracts on ischemia-induced lipid peroxidation in cardiomyocytes was evaluated by measuring TBARS levels[21]. Figure 3B shows the protective effect of digested meat extracts against lipid peroxidation. After 15 h of simulated ischemia, a significant increase in lipid peroxidation was observed (11.8±0.4). However, pretreatment with digested meat extracts prevented the increase in lipid peroxidation induced by ischemia to a similar extent as the other digested food extracts (5.7±1, turkey breast; 6.4±0.1, cooked ham; 7.9±0.3, cured ham; 6.3±0.1, mortadella; 5.4±0.2, sausage, 6.9±0.4, tuna fish; 9.7±0.7, green peas; 8.4±0.7, salad; 7.7±0.1, beans and 4.5±0.2, EGCG). Again, the sole administration of extracts in the absence of ischemic damage had no effect in itself (Supplementary figure 1B).

These results demonstrate for the first time that processed meat products, with low sodium and fat, and after simulating an *in vitro* gastrointestinal digestion process, are able to protect cardiomyocytes against ischemia-induced oxidative stress. Our results are consistent with the fact that several meat compounds have been identified as having antioxidant activity[29,30]. Indeed, recent studies have shown that natural postmortem processing or the industrial processing of meat results in an intense proteolysis, leading to the generation of bioactive peptides of different sizes and composition, which have been shown to exert protective effects (antihypertensive, antioxidant and prebiotic peptides)[4,7] . The endogenous histidine-containing dipeptides, carnosine and anserine, are the most abundant antioxidants in meat[31]. These dipeptides have been reported to prevent diseases related to oxidative stress such as diabetes, cancer and neurodegenerative diseases and to reduce ischemia/reperfusion damage in

different animal models and organs including brain, liver and testis[32]. Other peptides generated by enzymatic treatment of porcine muscle proteins (actin, tropomyosin, myosin heavy chain, integrin α3 or collagen α1) also showed antioxidant activity[33,34,35]. Recently, a processed meat product, dry-cured ham, has been also reported as a natural source of antihypertensive and antioxidant peptides[36,37]. To the best of our knowledge, no studies have evaluated the efficacy of these meat-derived antioxidants on cardiovascular diseases or in cardiac models of oxidative stress. The results obtained in this study suggest that processed meat products represent an important source of antioxidants, which are able to protect cardiomyocytes against the increase in ROS levels and lipid peroxidation induced by ischemia.

3.3. *Meat extracts protect against the decrease of endogenous antioxidant activity induced by ischemia*

As reported in other studies, ischemia causes alterations in the defense mechanisms used to combat oxygen free radicals, mainly through a reduction in the activity of Gpx-3 and CAT[38]. The next step was to assess the impact of digested meat extracts on the decrease in antioxidant activity induced by ischemia (Figure 4). Compared to normoxia, ischemia caused a significant decrease in GPx-3 activity of HL-1 cells (38±7.3%) (Figure 4A). However, pretreatment with the digested meat extracts prevented this inhibition of GPx-3 induced by ischemia (81±5.4%, turkey breast; 82±6.1%, cooked ham; 86±6.8%, cured ham; 75±3.2%, mortadella and 80±7.3%, sausage). The pretreatment with tuna fish extract or EGCG also prevented the inhibition of Gpx-3 induced by ischemia (86±5.6%, tuna-fish and 89±6.5%, EGCG). However, green peas (34±1.6%), salad (37±11.1%) or beans (38±8.4%) were not able to do so. Similar behavior was observed when assessing CAT activity (Figure 4 B). The

treatment with digested meat extracts in the absence of ischemia had no effect on the endogenous GPx-3 and CAT activities of HL-1 cells (Suplementary figure 1C and D). Our findings agree with a previous study. In which, treatment with a peptide purified from venison muscle protein hydrolysates increased Gpx-3 and CAT activities and reduced ROS levels and lipid peroxidation in cultured neuronal cells subjected to H_2O_2-induced damage[38]. The results of the present study demonstrate that the water-soluble components extracted after *in vitro* gastrointestinal digestion of processed meat products are able to restore endogenous antioxidant activity in a cardiac model of ischemic damage. This increase in antioxidant activity may explain the decrease in ROS levels and lipid peroxidation shown above[40,41].

Similar assays were performed with total extracts not subjected to gastrointestinal digestion (Supplementary figure 4). The non-digested extracts showed a similar antioxidant effect to that observed with digested extracts, which suggests that the protective bioactives (peptides or non-protein compounds), are not a result of *in vitro* digestion and can resist digestive processes and so reach the bloodstream. However, this hypothesis should be demonstrated in further *in vivo* assays.

3.4. *Meat extracts reduced ischemia-induced cardiomyocyte death*

After we have evaluated the functional effect of these derivatives on the ischemia-induced oxidative damage, the next step was to evaluate whether the antioxidant properties of meat extracts resulted in a protective effect against ischemia-induced cell death. As previously shown in figure 2A, after15 h exposure to ischemia, the percentage of cell viability was reduced to 25±6% in MTT assays. As shown figure 5A, pre-treatment for 24 h with any of meat extracts significantly reduced the loss of cell viability induced by ischemia (59±5%, turkey breast; 82±9.6%, cooked ham;

89±2.4%, cured ham; 77±3.2%, mortadella and 96±3.3%, sausage). As expected, pretreatment with tuna fish or EGCG prevented ischemia-induced cell death (75±4.1%, tuna-fish and 110±3.9%, EGCG), while pretreatment with green pea (14±2%), salad (21±1%) or bean (18±7%) extracts did not prevent the loss of cell viability. These findings confirm previous results obtained. The antioxidant effect of any of the digested extracts against ischemia damage leads to reduce the loss of cell viability[42–44]. Again, the treatment of HL-1 cells with meat extracts in the absence of ischemia had no effect on cell viability (Supplementary figure 1E). Similar results were obtained when cell viability was measured using the CFDA-SE assay (Figure 5B). A representative dot plot for any extract is also shown in Supplementary figure 3.

Although additional studies are necessary, our results suggesting an important functional role of these derivatives against ischemia-induced oxidative damage. To date, to our knowledge, all studies that have evaluated the antioxidant capacity of meat extracts have been performed in the laboratory using test tubes experiments[33,45,46]. However, these experimental models do not evaluate the antioxidant capacity of the meat in a physiological context. Indeed, very little is known about the antioxidant effect of meat extracts on cellular systems. Kim et al. reported the antiapoptotic role of a peptide (APVPH I) purified from venison protein by enzymatic proteolisis[39]. Pretreatment with this peptide, protected neuronal cells of oxidative stress and cell death induced by H_2O_2[39]. To date, there is no research that has evaluated the antioxidant role of meat extracts on an experimental model of ischemia. The data shown in our study demonstrate for the first time in cultured cardiomyocytes the antioxidant and protective effect of meat extracts from processed products against ischemia-induced damage.

Dietary-guidelines recommend eating less red and processed meat in order to avoid CVDs[2]. Recently, a meta-analysis of thirteen cohort studies found an association

between the consumption of meat (total, red, white and processed) and all-cause, CVD and ischemic heart disease (IHD) mortality[47]. This analysis concluded that processed meat consumption could increase the risk of mortality from any cause, included CVD, while red meat consumption was positively but weakly associated with CVD mortality. No significant associations were observed between the consumption of any type of meat and IHD mortality. Another meta-analysis concluded that the consumption of processed meat, but not red meat, is associated with a higher incidence of CHD and diabetes[48]. These differences may be explained by the fact that, while unprocessed and processed meats contain similar saturated fat and dietary cholesterol levels, processed meat contains much higher concentrations of salt and nitrate preservatives. Due to increased concern, much effort has been made by the food industry to develop processed meat products with low content of saturated fats and sodium. Future research should consider separately the effects of these new processed nutritional products, given that previous population studies did not differentiate between new and traditional processed meat products. In addition, as shown in the present and other previous studies, meat-derived extracts exert a wide variety of health-promoting benefits (antihypertensive, antioxidant or antithrombotic). The identification of these meat-derived bioactive compounds and the way in which they can be increased in meat products should be a strategy for developing novel functional and healthier products.

4. Conclusions

Meat is the main source of protein in our diet and peptides generated from the digestion of meat proteins are reported to have antioxidant properties. However, little research has focused on the potential benefits of consuming processed meat products. The main finding of this study was that the hydrolysates obtained from processed meat

455 products with a low fat and sodium content exert a protective effect against oxidative
456 stress and cell damage induced by ischemia in cardiac cells. These results suggest that
457 such meat products, besides their nutritional benefit for their low fat and sodium
458 content, may also represent a source of natural antioxidants with potential benefits for
459 cardiac health. Further research in physiological models (animal or human) is necessary
460 to confirm the antioxidant role of processed meat products *in vivo*.
461

462 **5. Limitations**

463 We have evaluated the preventive effect of each digested food extract in a
464 simulated ischemia model. It would be of additional interest to assess the protective
465 effects of these food extracts on cardiac damage induced by ischemia-reperfusion. The
466 choice of the extract dilution (1:5) was based on the lowest one that did not cause loss
467 of cell viability. We assumed that higher dilutions have a similar effect. Before
468 assessing the antioxidant effect of processed meat in an animal or human experimental
469 model of cardiac ischemic, further experimental studies are necessary to identify which
470 biopeptides, or non-protein compounds, are involved in the cardioprotective effect. In
471 this initial study we have used a HL-1 cell line. Further studies, by using primary
472 cardiomyocytes or in vivo animal models, will be of interest in order to confirm these
473 results. These studies will allow us evaluate the safety, dose response, bioavailability
474 and efficacy after ingestion for each product, before encouraging the incorporation of
475 these bioactive compounds as an approach to obtaining functional meat products.
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Conflict of Interest

Study in collaboration with ElPozo Alimentación S.A., which supplied meat products and the funding to reagents. The work was carried out as a blind study in the laboratory belonging to Cardiology Service and Department of Internal Medicine, School of Medicine, University of Murcia.

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589 **Figure legends**

590 **Figure 1.** Selection of optimal work dilution. HL-1 cells were treated for 24 h with
591 different dilutions of each digested food extract (v:v, food extract:culture medium) in
592 normoxia conditions. The percentage of cell viability (A) was measured as described in
593 Methods. Medium osmolarity (B) was measured using a vapor pressure osmometer
594 (Vapro 5600, Wescor Inc, Utah, USA). Turkey breast (●), cooked ham (□), cured ham
595 (■), mortadella (◇), sausage (○), tuna fish (△), green peas (▽), salad (◇), beans (◆).
596

597 **Figure 2.** Time-dependent effect of ischemia on cell viability and ROS production. A)
598 HL-1 cells were exposed to different times of ischemic conditions (0-48 h), and cell
599 viability (●) and ROS levels (○) were measured. Cell viability is shown as percentage
600 with respect to control (HL-1 cells under normoxia). ROS levels are shown as fold of
601 control (a.u., arbitrary units). B) Schematic diagram showing the experimental protocol.
602 Upper: The cells were pre-incubated with the different extracts for 24 hours under
603 normoxia conditions. Next, the cells were washed and maintained under ischemic
604 conditions in the absence of the extracts for 15 h, prior to the measurements. Down:
605 control assay, the experimental procedure is similar to described before, but in this case
606 the last period of 15 hours was under normoxic conditions. C) The increase in ROS was
607 also visualized by confocal microscopy in HL-1 cells exposed to 15 h of ischemia (scale
608 bar, 70 µm). The largest picture shows the ROS of a single cardiomyocyte subjected to
609 ischemia (scale bar, 10 µm).
610

611 **Figure 3**. Meat extracts prevented oxidative stress induced by ischemia. ROS and MDA
612 levels were measured in untreated HL-1 cells (normoxia), HL-1 cells subjected to
613 ischemia for 15 h and HL-1 cells pretreated with a 1:5 (v:v, food extract:culture

614 medium) dilution of any given digested food extract for 24 h before ischemia induction.
615 30 µM EGCG was used as positive control of antioxidative effect. Arbitrary units: a.u.
616 ***p<0.001 vs. normoxia. #p<0.05, ##p<0.01, ###p<0.001 vs. ischemia.
617
618 **Figure 4**. Meat extracts prevented the attenuation of antioxidant activities induced by
619 ischemia. The activities of antioxidant enzymes GpX-3 and catalase were measured in
620 untreated HL-1 cells (normoxia), HL-1 cells subjected to ischemia for 15 h and HL-1
621 cells pretreated with 1:5 (v:v, food extract:culture medium) dilution of any given
622 digested food extract for 24 h before ischemia induction. 30 µM EGCG was used as
623 positive control of antioxidative effect. Data are shown as a percentage of activity
624 compared with untreated cells (normoxia). Glutathione peroxidase 3: Gpx3. ***p<0.001
625 vs. normoxia. ##p<0.01, ###p<0.001 vs. ischemia.
626
627 **Figure 5**. Meat extracts prevented cell death induced by ischemia. Cell viability was
628 measured by MTT assay (A) and CFDA-SE assay (B) in HL-1 cardiomyocytes
629 pretreated with 1:5 (v:v, food extract:culture medium) dilution of any given digested
630 food extract for 24 h before ischemia induction. Cell viability data are shown as a
631 percentage with respect to untreated cells (normoxia). 30 µM EGCG was used as
632 positive control of cardioprotection. ***p<0.001 vs. normoxia. ##p<0.01, ###p<0.001 vs.
633 ischemia.

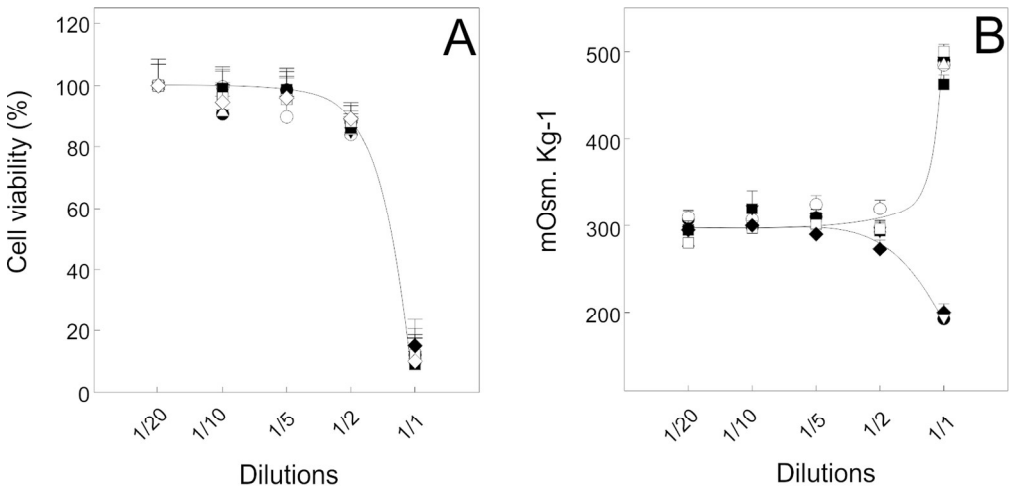

Figure 1

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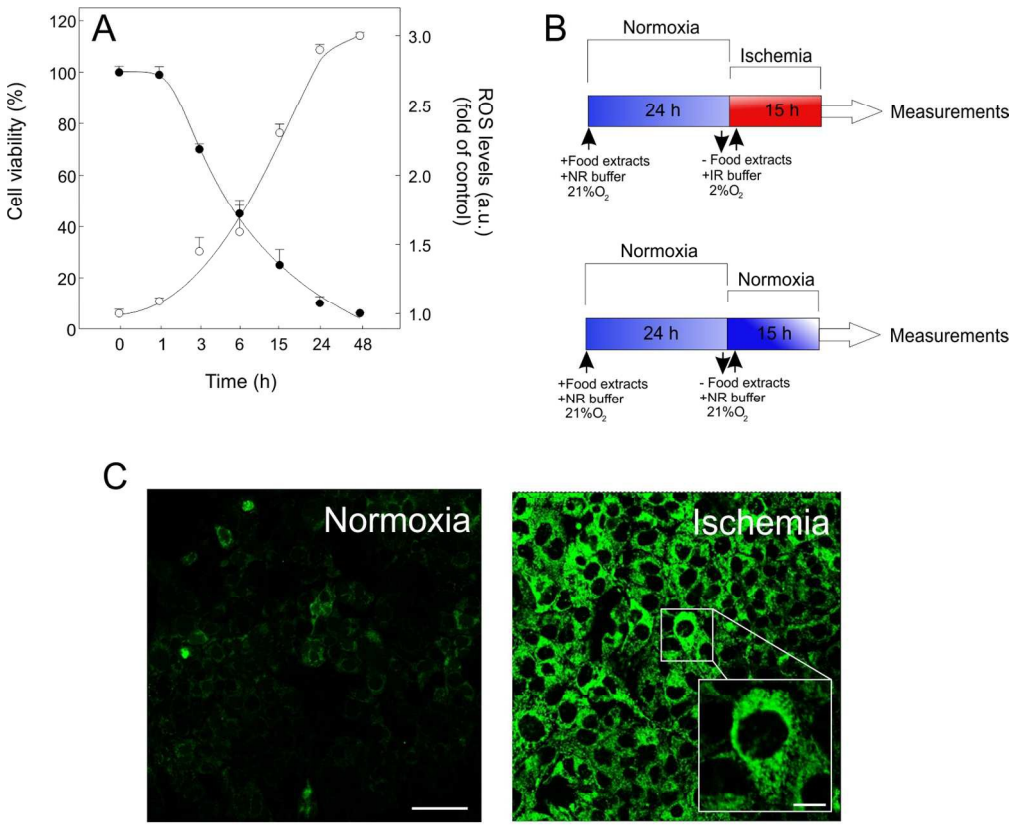

Figure 2

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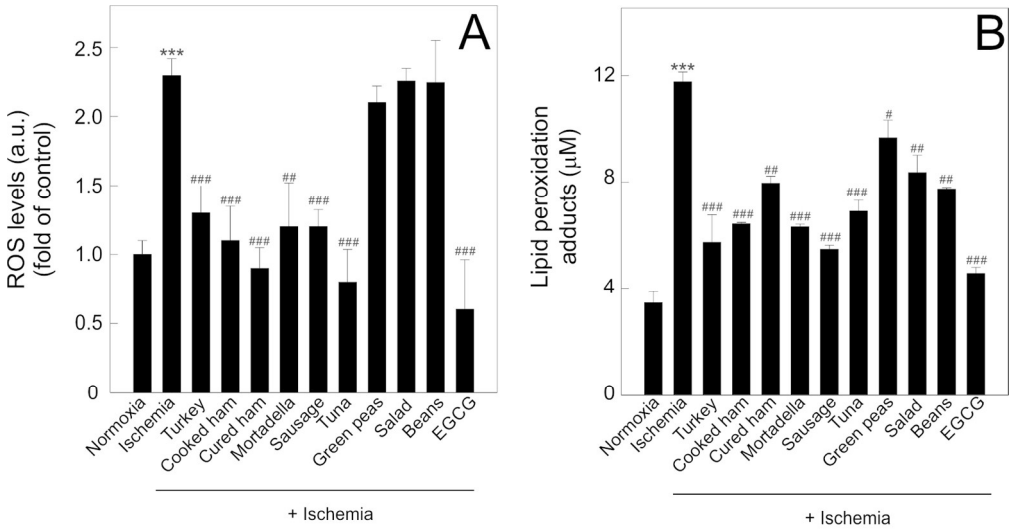

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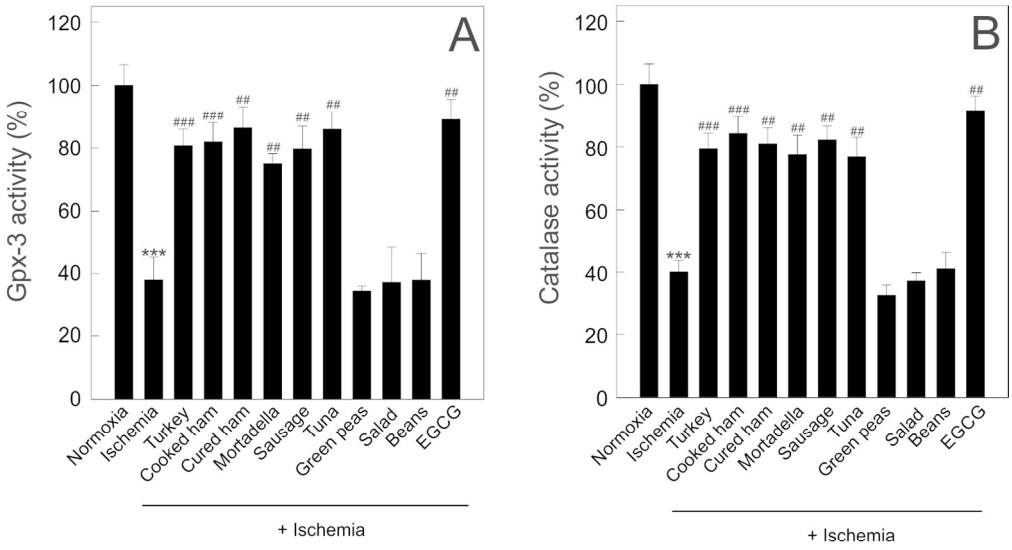

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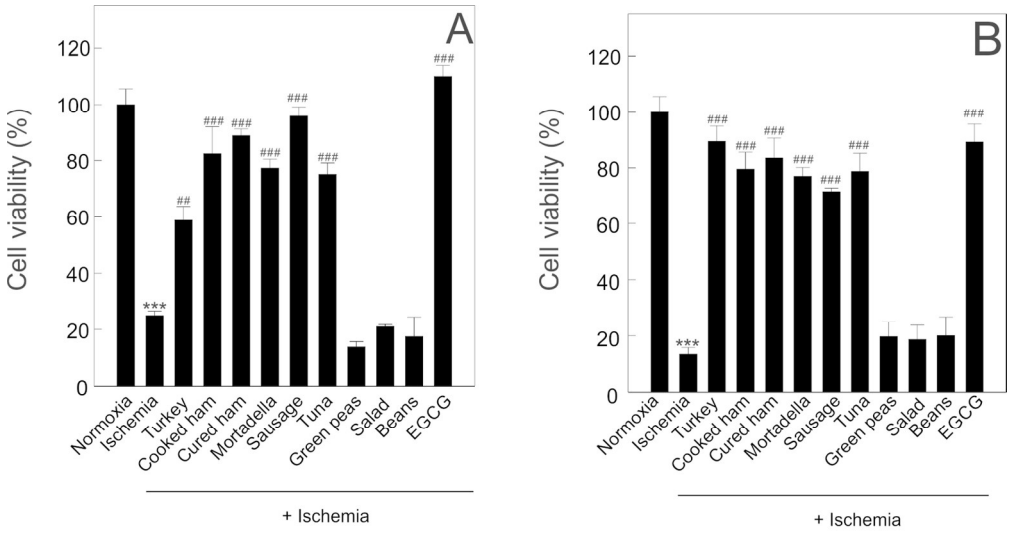

Figure 5

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