In vitro studies of an aged black garlic extract enriched in S-allylcysteine and polyphenols with cardioprotective effects

A.L. García-Villalón a, S. Amor a, L. Monge a, N. Fernández a, M. Prodanov b, M. Muñoz c, A.M. Inarejos-García c, M. Granado a, *

a Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma de Madrid, Madrid, Spain
b Departamento de Química Física Aplicada, Facultad de Ciencias, CIAL (CEI, CSIC-UAM), Universidad Autónoma de Madrid, Madrid, Spain
c Pharmactive Biotech Products SL, Parque Científico de Madrid, Madrid, Spain

ABSTRACT

Aged black garlic (ABG) exerts metabolic and cardiovascular beneficial effects. The aim of this work was to analyse the in vitro cardiovascular effects of an ABG extract enriched in S-allyl-cysteine and polyphenols (ABG10+) in Sprague-Dawley rats. Hearts were pretreated either with ABG10+ or vehicle and subjected to 30 min ischaemia followed by 45 min reperfusion (IR) using the Langendorff technique. Segments of aorta and tail artery were used for inflammation/oxidative stress and vascular reactivity experiments respectively in presence/absence of ABG10+. ABG10+ induced a nitric oxide (NO) dependent vasodilating effect in tail artery segments and directly increased the release of NO in aorta segments. In the heart, ABG10+ induced a relaxing effect on coronary arteries before and after IR and prevented the IR induced decrease in myocardial contractility. Functional changes were associated with increased expression of both pro- and antioxidant and pro- and anti-inflammatory markers in the myocardium and in aorta.

© 2016 Elsevier Ltd. All rights reserved.

Keywords:
Aged black garlic
Ischaemia-reperfusion
Nitric oxide
Antioxidant
Anti-inflammatory

1. Introduction

Raw garlic has long been used as traditional medicine for treating a diverse range of human diseases, due to the presence of several bioactive components with proven beneficial effects (Bautista et al., 2005; Rees, Minney, Plummer, Slater, & Skyrme, 1993). Among them, the most studied and relevant is allicin and its derivatives; allicin is the major thiosulfinate in fresh crushed garlic, which is responsible for its characteristic taste and aroma (Rosin, Tuorila, & Uutela, 1992; Salazar et al., 2008).

Allicin is highly unstable at pH close to neutral values, at high temperatures or in the presence of oils and rapidly degrades during processing or during storage, limiting its bioaccessibility (Kim, Nam, Rico, & Kang, 2012).

Concerning toxicity, raw garlic must be consumed in moderation because it might be toxic at high doses (Bae, Cho, Won, Lee, & Park, 2014; Kodera et al., 2002). In addition to toxicity, the consumption of unprocessed raw garlic is limited due to its characteristic odour, taste and tendency to cause an upset stomach (Kodera et al., 2002). Therefore, in recent years, various processing methods such as heat treatment, ageing and
fermentation have been used to eliminate the offensive odour and improve garlic palatability. Heat treatment is the most frequently used processing method to improve the taste and flavour of garlic. During heating, non-enzymatic browning (Maillard reaction) produces some physical-chemical changes such as colour, flavour, texture, macronutrients and minor compounds content (Nencini et al., 2011; Toledano-Medina, Perez-Aparicio, Moreno-Rojas, & Merinas-Amo, 2016). Another important change during the ageing process is the increase in polyphenols content (Kim et al., 2011; Park, Park, & Park, 2009), and consequently also in its antioxidant capacity (Nencini et al., 2011).

On the other hand, the increased antioxidant capacity is also due to the transformation of some of the unstable and odorous components of raw garlic into stable and odourless compounds during ageing process, mostly organosulphur compounds such as S-allylcysteine (SAC) (Lee et al., 2009). SAC is a water-soluble bioactive compound known for its high antioxidant capacity (Bae, Cho, Won, Lee, & Park, 2012) formed during the enzymatic hydrolysis of γ-glutamyl-S-allylcysteine, catalyzed by γ-glutamyl transpeptidase (γ-GTP, EC 2.3.2.2). Its content is about 20–30 μg/g in raw garlic, and rises up to six times after the ageing process (Bae et al., 2014; Hanum, Sinha, Guyer, & Cash, 1995). Nevertheless, the γ-GTP activity is affected by heat (Munday, James, Fray, Kirkwood, & Thompson, 1999), and therefore high temperatures can limit its formation during ageing.

Several studies have reported that SAC has cardioprotective effects (Banerjee, Mukherjee, & Maulik, 2003; Ried, Frank, & Stocks, 2010; Steiner, Khan, Holbert, & Lin, 1996) although the mechanisms involved are not completely understood. As reactive oxygen species (ROS) play a central role in these alterations, research has focused on the antioxidant properties of garlic products (Yamasaki, Li, & Lau, 1994).

The wide range of beneficial effects of aged black garlic include the protection of vascular endothelial cells against hydrogen peroxide induced injury (Reeve, Bosnic, Rozinova, & Boehm-Wilcox, 1993), protection against damage caused by ionizing radiation (Kojima, Toyama, & Ohnishi, 1994) or toxic substances (Aikreathy et al., 2010; Lee et al., 2011), inhibition of oxidative and inflammatory markers (Weiss, Papatheodorou, Morihara, Hilge, & Ide, 2013) and an increase in nitric oxide availability in endothelial cells (Munday et al., 1999) are reported. In addition, aged garlic has enhanced metabolic beneficial effects compared to row garlic, for example, decreasing LDL oxidation and thus preventing atherosclerosis (Arora, Arora, & Gupta, 1981; Ide & Lau, 1999).

Oxidative damage is involved in many cardiovascular diseases, including heart disease (Kiesewetter et al., 1993), peripheral arterial occlusive disease (Foushee, Ruffin, & Banerjee, 1982) and hypertension (Zhang et al., 2001); therefore these diseases may be improved and/or prevented by garlic products. Raw garlic extract reduces infarct size in isolated rabbit hearts (Sharma et al., 2012) and rat hearts (Banerjee, Dinda, Manchanda, & Maulik, 2002), although high doses were cardiotoxic and improved oxidative myocardial damage (Ku et al., 2002). Moreover, it produces arterial dilatation mediated by nitric oxide (Benavides et al., 2007; Grman et al., 2011) or hydrogen sulfide (Chuah, Moore, & Zhu, 2007). These beneficial effects might be enhanced in aged garlic, as SAC present in ABG10+ is reported to induce myocardial protection during ischaemia (Numagami, Sato, & Ohnishi, 1996). Indeed, aged garlic has been shown to protect against ischaemia–reperfusion injury in rat brain (Augusti, 1996), but the effects of this particular preparation have not been studied during cardiac ischaemia.

The preventive and/or therapeutic use of garlic products on the cardiovascular system needs to be improved by a better characterization of their effects as there is evidence that there may be undesirable effects at high doses (Banerjee et al., 2001; Egen-Schwind, Eckard, & Kemper, 1992; Joseph, Rao, & Sundaresh, 1989; Nakagawa, Masamoto, Sumiyoshi, Kunihiro, & Fuwa, 1980; Robards, Frenzler, Tucker, Swatsitang, & Glover, 1999).

The chemical characterization of SAC and its derivatives is still a challenge to be overcome in aged black garlic, mainly due to its complexity and the very limited absorption of ultraviolet (UV) light of these compounds, 205–210 nm. This is the range, where the most common solvents used in reversed phase HPLC, acetonitrile and/or methanol, also absorb. In addition, fine separation of these compounds requires the use of salt-containing mobile phases, which is a serious problem in HPLC maintenance and mass detection (Beato, Sanchez, de Castro, & Montano, 2012). This is why most of the analytical techniques developed for determination of amino acids are based on their derivatization to chromophores (o-phthaldialdehyde, OPA) or fluorophores (9-fluorenylmethyl chloroformate, FMOC or 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, AQCl) (Bae et al., 2012; Cronin, Pizzarello, & Gandy, 1979; Gartenmann & Kochhar, 1999). However, while they considerably improve the detection selectivity and sensitivity, they are also time-consuming, laborious and/or require specific instrumentation for carrying out a pre- or post-column derivatization, which often leads to low reproducibility. Consequently, it is necessary to develop a more simple and efficient methodology for the assessment of these molecules.

Therefore, in this study we have used a newly developed fast methodology for the determination of SAC and its main derivatives in an aged black garlic extract ABG10+ and analysed its in vitro effects on isolated rat arteries and isolated perfused rat hearts during coronary ischaemia–reperfusion.

### 2. Materials and methods

Aged Black Garlic Extract ABG10+ was obtained from Pharmactive Biotech Products SL (Madrid, Spain); eight samples from different batches during the year 2015 were used in this study.

Folin–Ciocalteu reagent, gallic acid, sodium carbonate, S-allylcysteine, U46619 (9,11-dideoxy-1a,9a-epoxy methanopros-
taglandin F20) and l-NAME (N-omega-nitro-l-arginine methyl ester) were purchased from Sigma-Aldrich (Madrid, Spain); heptanesulfonic acid sodium salt and orthophosphoric acid were from Fisher Scientific (Santa Clara, CA, USA). HPLC solvents were from Merck (VWR International, Spain). Ultrapure water for chromatographic use was obtained from a MilliQ system (Millipore Corp., Bedford, MA, USA). U46619 (9,11-dideoxy-9a,9a-epoxymethanoprostaglandin F20) and l-NAME (N-omega-nitro-l-arginine methyl ester) were obtained from Sigma-Aldrich (Madrid, Spain).

2.1. Analytical characterization of ABG10+ extract

2.1.1. Determination of total phenol content

Total phenol content of ABG10+ was ascertained by colorimetry employing the Folin–Ciocalteu reagent (Singleton & Rossi, 1965). Briefly, ABG10+ was dissolved in water and mixed with Folin–Ciocalteu reagent. After 3 min a sodium carbonate solution was added. Total phenols were determined after a 2 h incubation at room temperature. The absorbance of the resulting blue colour solution was measured at 765 nm with a Beckman Coulter DU Series 730 Life Science UV/Vis Scanning Spectrophotometer (Fullerton, CA, USA). The quantification of total phenol content was expressed as grams of gallic acid per 100 grams of ABG10+ (dry basis).

2.1.2. HPLC analysis (SAC quantification)

The exclusive quantification of SAC in ABG10+ by HPLC was initially performed according to Bae et al. (2012) employing an Agilent Technologies 1220 Infinity series system (Palo Alto, CA, USA) equipped with autosampler and photo-diode array detector; the separation of SAC was carried out on a C18-PFP Ultra-Inert HPLC Column (250 × 4.6 mm × 5 μm; ACE, Scotland).

2.1.3. HPLC-PAD-MS assessment

For the identification and quantitative assessment of SAC together with its derivatives, a simple HPLC-MS method with direct injection of the sample (without any purification and/or derivatization step) was developed. The chromatographic system was an Agilent 1200 series, (binary pump, autosampler, photodiode array detector), coupled to an electrospray ionization (ESI) quadrupole mass analyser. The stationary phase was a Liquid Purple ODS column from Análisis vinicos, S.L. (Tomelloso, Spain) (250 × 4.6 mm and 5 μm particle size), at ambient temperature. The mobile phase was pumped at a flow rate of 0.5 mL/min and was a linear gradient of component A (0.1% (v/v) formic acid in water) and component B (acetonitrile), as follows: from 0 to 20 min, 10 to 90% of B; for 10 min, 90% of B; from 90 to 10% of B at 1 min and 10 min at 90% B for conditioning the column for the next analysis. The PAD was set at 208 nm and the injection volume was 20 μL. The total run time was 41 min. ESI(MS) was tuned as follows: mass range (SCAN): from 50 to 1500 umas, ionization mode: ESI+, drying gas flow: 9 L/min, nebulizer pressure: 60 psig, drying gas temperature: 250 °C, vaporizer temperature: 150 °C, capillary tension: 2000 V, charging tension: 2000 V, fragmentator tension: 40 V.

2.2. Methods to test the in vitro cardiovascular effects of ABG10+ in experimental animals

In the present study male Sprague-Dawley rats with a body weight of 300–350 g were used. All the experiments were conducted in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and in compliance with all relevant laws and regulations. The use of these animals was also approved by the Animal Care and Use Committee of the Universidad Autónoma de Madrid. After anaesthesia with sodium pentobarbital (100 mg/kg i.p.) and following i.v. injection of heparin (1000 UI), the heart, the aorta and the tail artery were collected.

2.2.1. Perfused hearts

Immediately after removal of the heart, the ascending aorta was cannulated and the heart was subjected to retrograde perfusion with Krebs–Henseleit buffer (115 mM NaCl, 4.6 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2.5 mM CaCl2, 25 mM NaHCO3 and 11 mM glucose) equilibrated with 95% oxygen and 5% carbon dioxide to a pH of 7.3–7.4. Perfusion was initiated in a non-recirculating Langendorff heart perfusion apparatus at a constant flow rate of 11–15 mL/min to provide a basal perfusion pressure of approximately 70 mmHg. Both the perfusion solution and the heart were maintained at 37 °C. Coronary perfusion pressure was measured through a lateral connection in the perfusion cannula and left ventricular pressure was measured using a latex balloon inflated to a diastolic pressure of 5–10 mmHg, both connected to Statham transducers. Left ventricular developed pressure (systolic left ventricular pressure minus diastolic left ventricular pressure), left ventricular end-diastolic pressure, the first derivative of the left ventricular pressure curve (dP/dt) and heart rate were calculated from the left ventricular pressure curve. These parameters were recorded on a computer using the PowerLab/8e data acquisition system (AD instruments).

After a 30 min equilibration period with constant flow perfusion, ABG10+ at concentrations of 50–500 mg/L was added to the perfusion solution, and the hearts were perfused for a further 30 min. After 30 min perfusion with ABG10+, the hearts were exposed to global zero-flow ischaemia for 30 min, and reperfused for 45 min at the same flow rate used before ischaemia. Coronary perfusion pressure, left developed ventricular pressure, dP/dt and heart rate were measured before and after ischaemia–reperfusion, in the absence and in the presence of ABG10+. After the experiment, the hearts were removed from the perfusion system and stored at −80 °C for further analysis.

2.2.2. Tail arteries

After collection, tail arteries were cut into 2 mm long segments and each segment was prepared for isometric tension recording in a 4-mL organ bath containing modified Krebs–Henseleit solution at 37 °C (mM): NaCl, 115; KCl, 4.6; KH2PO4, 1.2; MgSO4, 1.2; CaCl2, 2.5; NaHCO3, 25; glucose, 11. The solution was equilibrated with 95% oxygen and 5% carbon dioxide to a pH of 7.3–7.4. Briefly, two fine 100 μm diameter steel wires were passed through the lumen of the vascular segment; one
wire was fixed to the organ bath wall and the other was connected to a strain gauge for isometric tension recording (Universal Transducing Cell UC3 and Statham Microscale Accessory ULS; Statham Instruments, Inc, Oxnard, CA, USA). This arrangement enables the application of passive tension in a plane perpendicular to the long axis of the vascular cylinder. The changes in isometric force were recorded using a PowerLab data acquisition system (ADInstruments). An optimal passive tension of 1 g was applied to the vascular segments and then they were allowed to equilibrate for 60–90 min. Before beginning the experiment, the vascular segments were stimulated with potassium chloride (100 mM) to determine the contractility of smooth muscle, and any segments that failed to contract at least 0.5 g were discarded.

After equilibration, the relaxation to ABG10+ (10–100 mg/L) was recorded in the segments precontracted with the thromboxane A2 analogue U46619. First, 10-8 M U46619 was added to the bath, and when the contraction reached a stable level, ABG10+ (10–100 mg/L) was added cumulatively, and the relaxation recorded. The relaxation to ABG10+ was also recorded in the presence of an inhibitor of nitric oxide synthase L-NAME, which was added to the bath 20 min before the concentration–response curve.

2.2.3. Incubation of aorta segments
Aorta was carefully dissected from each animal and cut in 2 mm segments. Segments were placed in 24 well plates (1 segment per well) and incubated for 24 hours (37 °C, 5% CO₂) either with culture medium DMEM-F12 or with ABG10+ at two different concentrations: 50 mg/L and 500 mg/L. After 24-hour incubation both culture medium and segments were collected and immediately frozen and stored at −80 °C to further analyze nitrates and nitrates concentrations and the gene expression of different genes in arterial tissue.

2.2.4. Nitrite and nitrate determination in the culture medium
Nitrite and nitrate concentrations were measured in the culture medium by a modified Griess assay, described by Miranda, Espey, and Wink (2001). Briefly, 100 μL of vanadium chloride was added to 100 μL of culture medium in 96-well plate. Immediately after Griess reagent (1:1 mixture of 1% sulfanilamide, and 0.1% naphthylethylenediamine dihydrochloride) was added to each well and incubated at 37 °C for 30 min. The absorbance was measured at 540 nm. Nitrite and nitrate concentration was calculated using a NaNO₂ standard curve and expressed as μM.

2.2.5. RNA preparation and purification
Total RNA was extracted from myocardial and arterial tissue according to the Tri-Reagent protocol (Chomczynski, 1993). cDNA was then synthesized from 1 μg of total RNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA).

2.2.6. Quantitative real-time PCR
The gene expression of inducible nitric oxide synthase (iNOS; Rn01525859_g1), interleukin 10 (IL-10; Rn01483988_g1), NADPH oxidase 1 (NOX1; Rn00586652_m1), NADPH oxidase 4 (NOX4; Rn00585380_m1), glutathione peroxidase 3 (GPX3; Rn00574703_m1), glutathione reductase (GR; Rn01482159_m1) and superoxide dismutase-1 (SOD1; Rn00566938_m1) was assessed in myocardial and arterial tissue by quantitative real-time PCR. Quantitative real-time PCR was performed using assay-on-demand kits (Applied Biosystems) for each gene. TaqMan Universal PCR Master Mix (Applied Biosystems) was used for amplification according to the manufacturer’s protocol in a Step One machine (Applied Biosystems). Values were normalized to the housekeeping gene 18S (Rn01428915_g1). The ΔΔCT method was used to determine relative expression levels. Statistics were performed using ΔΔCT values (Livak & Schmittgen, 2001).

3. Results

3.1. Bioactive components analysis
The total phenol content (TPC) of the ABG10+ samples analysed by Folin–Ciocalteu (as gallic acid, dry basis) was homogeneous (Table 1). The analysis was performed on eight different batches to establish the corresponding percentiles distribution (P25, P50, P75), the minimum (P0) and the maximum (P100) with 3.41 and 3.65 mg/100 mg ABG10+ respectively (Table 1). During ageing the TPC increases due to the higher levels of complex polyphenols from the later phase of the browning reaction as suggested by Robards et al. (1999), which together with the SAC content are responsible for the antioxidant capacity (Lee et al., 2009).

Likewise SAC concentration throughout the different batches analysed by HPLC was also homogeneous (Table 1); the percentile distribution shows that the SAC content was within 0.15 and 0.17 mg/100 mg ABG10+. The increase in SAC and TPC in aged black garlic when compared to raw garlic agrees with the findings of other authors (Park et al., 2009; Toledano-Medina et al., 2016).

In order to confirm the presence of SA and its derivatives, and to perform the corresponding quantitative assessment, a
new simple HPLC-MS method was developed. This included a common octadecylsilane stationary phase, a binary water/acetonitrile mobile phase and 1% formic acid, as an ionization agent. The SAC reference solution was injected directly into the ESI source and the ionization conditions were tuned as described above. PAD was used only for the qualitative control of the separation. The identification and quantification of SAC and its derivatives was carried out on the basis of their MS spectral characteristics. The ability of the MS detector to extract specific ions enabled their selective identification. Fig. 1 shows the UV chromatogram of ABG10+ analysed by HPLC-PAD-MS, registered at 208 nm, the total ion current and extractions of the ion current, corresponding to the ions of m/z 162, 291 and 265.

Furthermore, the SAC reference solution was diluted to obtain different concentrations and a calibration curve was plotted according to the previously described HPLC-MS/ESI method. The results showed a concentration between 0.26 and 0.34 mg SAC per 100 mg ABG10+. The other compounds detected such as iso-SAC, GSAC and GSMC had concentrations below the quantification limit of the detector (1.1 ppm).

### 3.2. Effects of ABG10+ in perfused hearts

Treatment of the perfused rat heart with ABG10+ at 50 mg/L or 500 mg/L induced coronary vasodilatation with a reduction in perfusion pressure, and an increase in the left ventricle developed pressure and dP/dt (Table 2), without modifying the heart rate. The changes in the left ventricle developed pressure and dP/dt were transitory and returned to basal values after 30 min of treatment, whereas the reduction in the coronary perfusion pressure persisted. After ischaemia–reperfusion, coronary perfusion pressure increased in untreated hearts, but not in the hearts treated with ABG10+ at a dose of 50 mg/L (P < 0.05). The left intraventricular developed pressure and dP/dt
were reduced after ischaemia–reperfusion in both treated and untreated hearts, but dP/dt was significantly higher ($P < 0.05$) in the hearts treated with ABG10 50 mg/L after 45 min of reperfusion, compared with untreated hearts. In the hearts treated with ABG10+ at a dose of 500 mg/L, coronary perfusion pressure, the left intraventricular developed pressure and dP/dt after ischaemia–reperfusion were not improved compared with the untreated hearts.

### Table 2 – Haemodynamic parameters of perfused rat hearts before and after ischaemia–reperfusion treated with vehicle (n = 6) or with ABG10 50 mg/L (n = 6) or 500 mg/L (n = 4).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Treated</th>
<th>Ischaemia-reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15 min</td>
<td>30 min</td>
</tr>
<tr>
<td><strong>Coronary perfusion pressure (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>70 ± 3</td>
<td>73 ± 5</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>ABG10 50 mg/L</td>
<td>75 ± 2</td>
<td>47 ± 3**</td>
<td>63 ± 5</td>
</tr>
<tr>
<td>ABG10 500 mg/L</td>
<td>75 ± 3</td>
<td>53 ± 4††</td>
<td>82 ± 6</td>
</tr>
<tr>
<td><strong>Left ventricle developed pressure (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>114 ± 7</td>
<td>106 ± 7</td>
<td>61 ± 14*</td>
</tr>
<tr>
<td>ABG10 50 mg/L</td>
<td>108 ± 9</td>
<td>103 ± 6</td>
<td>73 ± 11</td>
</tr>
<tr>
<td>ABG10 500 mg/L</td>
<td>107 ± 11</td>
<td>163 ± 3**</td>
<td>48 ± 16</td>
</tr>
<tr>
<td><strong>dP/dt (mmHg/s)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>2670 ± 167</td>
<td>2625 ± 173</td>
<td>1196 ± 293**</td>
</tr>
<tr>
<td>ABG10 50 mg/L</td>
<td>2811 ± 233</td>
<td>2789 ± 123</td>
<td>1369 ± 247*</td>
</tr>
<tr>
<td>ABG10 500 mg/L</td>
<td>2812 ± 197</td>
<td>3687 ± 400**</td>
<td>702 ± 287††</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. *$P < 0.05$ and **$P < 0.01$ compared with control, †$P < 0.05$, ††$P < 0.01$ compared with vehicle.

Fig. 2 – Myocardial gene expression of inducible nitric oxide synthase (iNOS) (A), endothelial nitric oxide synthase (eNOS) (B), cyclooxygenase-2 (C), interleukin 1β (IL-1β) (D), interleukin 6 (IL-6) (E) and tumoral necrosis factor alpha (TNF-α) (F) in non-treated hearts (Control; n = 6) and ABG10+ (50 mg/L) pre-treated hearts (n = 6) after ischaemia–reperfusion (IR). Values are represented as mean ± SEM. *$P < 0.05$ vs control; **$P < 0.01$ vs control.
3.3. Cell death in myocardial tissue

ABG10+ (50 mg/L) administered before ischaemia and during 45 min of reperfusion significantly decreased ischaemia–reperfusion induced cell death in the myocardium (IR = 100 ± 18; IR – ABG10+ = 50 ± 9; P < 0.05).

3.4. Gene expression of cytokines and enzymes involved in the production of vasoactive substances and inflammatory factors in myocardial tissue

The gene expression of inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) as producers of nitric oxide, and cyclooxygenase-2 (COX-2) as a producer of prostanoids was measured in myocardial tissue after IR both in non-treated hearts and in hearts treated with ABG10+ before ischaemia and during reperfusion (Fig. 2A, 2B and 2C respectively). In addition the gene expression of different pro-inflammatory cytokines such as interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) was also measured (Fig. 2D, 2E and 2F respectively). There was an up-regulation of both iNOS and eNOS gene expression in ABG10+ treated hearts compared to untreated hearts (P < 0.05 and P < 0.01 respectively). Likewise the myocardial mRNA levels of IL-1β and IL-6 were also increased in ABG10+ treated hearts compared to non-treated ones (P < 0.05 and P < 0.01 respectively), whereas the gene expression of COX-2 and TNF-α was unchanged between groups.

3.5. Gene expression of pro-oxidative and anti-oxidative enzymes and cytokines in myocardial tissue

The mRNA levels of pro-oxidative enzymes such as NADPH oxidase 1 (NOX1) and NADPH oxidase 4 (NOX4) and anti-oxidative enzymes such as glutathione peroxidase 3 (GPX3), glutathione reductase (GSR) and superoxide dismutase-1 (SOD1) were analysed in hearts treated and untreated with ABG10+ before ischaemia and during reperfusion (Fig. 3A, 3B, 3C, 3D and 3F respectively). In addition the gene expression of the anti-inflammatory cytokine interleukin 10 (IL-10) was also measured (Fig. 3E). Treatment with ABG10+ induced an overexpression in the mRNA levels of NOX-1, NOX-4, GSR and SOD1 (P < 0.05 for all), whereas the gene expression of GPX3 and IL-10 was not modified.

Fig. 3 – Myocardial gene expression of NADPH oxidase 1 (NOX1) (A), NADPH oxidase 4 (NOX4) (B), glutathione peroxidase 3 (GPX3) (C), glutathione reductase (GSR) (D), interleukin 10 (IL-10) (E) and superoxide dismutase (SOD) (F) in non-treated hearts (Control; n = 6) and ABG10+ (50 mg/L) pre-treated hearts (n = 6) after ischaemia–reperfusion (IR). Values are represented as mean ± SEM. * P < 0.05 vs control.
3.6. Vasoactive response to ABG10 in tail arteries and release of nitrites and nitrates

ABG10+ (30–1000 mg/L) induced a concentration-dependent relaxation in isolated rat tail arteries, which was reduced by the inhibition of nitric oxide synthesis with L-NAME (Fig. 4A; P < 0.05). In addition two different doses of ABG10+ (50 and 500 mg/L) significantly increased the release of nitrites and nitrates when incubated with aorta segments for 24 hours (Fig. 4B; P < 0.001 for both).

3.7. Gene expression of cytokines and enzymes involved in the production of vasoactive substances and inflammatory factors in arterial tissue

Fig. 5 shows the gene expression of iNOS, eNOS, COX-2, IL-1β, IL-6 and TNF-α in rat aorta segments incubated either with vehicle (control) or either with two different doses of ABG10+ (50 and 500 mg/L) for 24 h. The gene expression of eNOS and TNF-α was not modified in segments incubated with ABG10+ compared to controls. On the contrary iNOS (Fig. 5A) and COX-2 (Fig. 5C) gene expression was significantly increased in arterial tissue after incubation with ABG10+ at both doses (P < 0.05 and P < 0.001, and P < 0.05 for both). In addition IL-1β and IL-6 were also upregulated when incubated with ABG10+ 500 and ABG10+ 50 mg/L respectively (Fig. 5D and 5E; P < 0.001 for both).

3.8. Gene expression of pro-oxidative and anti-oxidative enzymes and cytokines in arterial tissue

The mRNA levels of NOX-1, NOX-4, GPX3 and GSR were unchanged between groups (Fig. 6A, 6B, 6C and 6D respectively). However IL-10 and SOD1 gene expression was significantly increased in aorta segments incubated with ABG10+ 500 (Fig. 6E; P < 0.01) and ABG10+ 50 mg/L respectively (Fig. 6F; P < 0.05).

4. Discussion

The results of the present study suggest that the aged garlic extract ABG10+ exerts beneficial effects on the cardiovascular system. In the systemic circulation, ABG10+ increased the production of nitric oxide in the aorta and produced relaxation of tail arteries mediated, at least in part, by nitric oxide. This agrees with previous studies showing that garlic induces nitric oxide release (Kim et al., 2001; Weiss et al., 2013) and endothelium-dependent relaxation in rat pulmonary arteries (Ku et al., 2002) and aorta (Grman et al., 2011). Nitric oxide exerts different beneficial effects on the vascular system protecting against atherosclerosis, hypertension and other pathologies (Desjardins & Balligand, 2006).

Since myocardial ischaemia is the most important cause of cardiovascular disease in developed countries, we have
studied the effect of two different doses of ABG10+ on the isolated heart during ischaemia–reperfusion. After ischaemia–reperfusion a concentration of 50 mg/L ABG10+ improved myocardial contractility as assessed by dP/dt. This concentration of ABG10+ also reduced cellular death in the myocardium after ischaemia–reperfusion. The cardioprotective effect of garlic has previously been reported. Garlic extract reduced infarct size and enhanced the protective effect of preconditioning in perfused rat or rabbit (Zhang et al., 2001) hearts, although higher doses were toxic (Sharma et al., 2012). Similarly our results show that the effects of ABG10+ in the perfused heart were concentration dependent, as no improvement in coronary perfusion and myocardial contractility after ischaemia–reperfusion was observed with a higher concentration (500 mg/L). This suggests that the beneficial effect of ABG10+ ameliorating ischaemia–reperfusion induced alterations in the myocardium may be present at an intermediate range of concentrations. A similar biphasic dose–response curve has been described for several chemicals and agents (Mattson, 2008), including garlic that is reported to exert beneficial effects at low doses but it is not beneficial or even toxic at higher doses (Banerjee et al., 2001; Egen-Schwind et al., 1992).

The cardioprotective effect of ABG10+ may be related, at least in part, to an improvement in coronary blood flow before and after ischaemia–reperfusion, as coronary vascular resistance in perfused hearts was lower in hearts treated with ABG10+ compared with untreated hearts. One of the pathophysiological mechanisms of heart damage after ischaemia is the phenomenon of “no-reflow”, by which coronary blood flow remains reduced during reperfusion (Bouleti, Mewton, & Germain, 2015). This was observed in the present study as an increase in coronary vascular resistance after ischaemia–reperfusion, which was abrogated by ABG10+ treatment. The vasodilator effect of ABG10+ in coronary arteries is most likely due to an increase in nitric oxide production as ABG10+ (50 mg/L) significantly increased the gene expression of eNOS and iNOS in myocardial tissue after ischaemia–reperfusion, possibly improving coronary perfusion and myocardial protection in this condition. In addition it is important to point out that the vasodilator effect of ABG10+ may be different depending

---

**Fig. 5** – Gene expression of inducible nitric oxide synthase (iNOS) (A), endothelial nitric oxide synthase (eNOS) (B), cyclooxygenase-2 (C), interleukin 1β (IL-1β) (D), interleukin 6 (IL-6) (E) and tumoral necrosis factor alpha (TNF-α) (F) in aorta segments in presence/absence of ABG10+ (50 and 500 mg/L). (n = 6 segments from 6 different rats.) Values are represented as mean ± SEM. * P < 0.05 vs control; ***P < 0.001 vs control.
on the vascular bed. Our results show that ABG10+ improved coronary blood flow when administered in a low concentration (50 mg/L) but it only induced vasodilation in tail arteries when administered at high concentrations (300 and 1000 mg/L). This fact possibly suggests that coronary arteries are more sensitive than systemic arteries to low doses of ABG10+. This may be beneficial as therapeutic doses for the heart in terms of vasodilation do not produce vasodilation in systemic arteries which could induce hypotension.

As ABG10+ contains multiple compounds it is difficult to assess which one is responsible and in what extent for its beneficial effects in the myocardium. However the protective effects of ABG10+ increasing heart contractility after ischaemia–reperfusion are most likely mediated by SAC as this molecule has been previously described as protecting against acute myocardial infarction (Chuah et al., 2007) by reducing the level of ROS and increasing the activity of antioxidant enzymes such as SOD (Xue et al., 2011). In addition, the quantity of SAC is much higher compared to the quantity of other compounds, as shown by the HPLC analysis. However we cannot exclude the possibility that compounds other than SAC could contribute to the protective effect of ABG10+ against ischaemia–reperfusion injury, because raw garlic has also been reported to exert protection against this type of injury (Banerjee et al., 2002; Rietz, Isensee, Strobach, Makdessi, & Jacob, 1993).

Our results are in agreement with these previous studies as we found that hearts treated with ABG10+ (50 mg/L) before ischaemia and during 45 min of reperfusion showed increased gene expression of the antioxidant enzymes GSR and SOD-1. However, contrary to what was expected, we also found increased in the myocardium of ABG10+ treated hearts the mRNA levels of different genes related to inflammation such as IL-1β, IL-6 and oxidative stress such as NOX-1 and NOX-4. Similarly aorta segments incubated for 24 hours with ABG10+ also presented not only increased gene expression in both anti-inflammatory and anti-oxidative markers such as IL-10 and SOD-1 but also an upregulation of pro-inflammatory cytokines such as IL-1β and IL-6 and enzymes such as iNOS and COX-2.

These observations may be understood by the concept of hormesis. This term describes the phenomenon by which low levels of stressor agents may induce adaptive responses which have overall beneficial effects (Gems & Partridge, 2008; Mattson, 2008). This theory might explain the favourable effects of caloric restriction, physical exercise or ischaemic preconditioning. Also,

![Fig. 6 – Gene expression of NADPH oxidase 1 (NOX1) (A), NADPH oxidase 4 (NOX4) (B), glutathione peroxidase 3 (GPX3) (C), glutathione reductase (GSR) (D), interleukin 10 (IL-10) (E) and superoxide dismutase (SOD) (F) in aorta segments in presence/absence of ABG10+ (50 and 500 mg/L). (n = 6 segments from 6 different rats.) Values are represented as mean ± SEM. * P < 0.05 vs control; ** P < 0.01 vs control.](image-url)
5. Conclusion

ABG10+ exerts a protective effect on the heart during ischaemia–reperfusion, which could be due to the induction of adaptive antioxidant mechanisms in the tissue. In our study ABG10+ was applied before ischaemia, suggesting that it may be useful for prevention of ischaemia–reperfusion in subjects with coronary risk factors. In addition, our results also emphasize the importance of adjusting the concentration range to optimize the beneficial effects.

Acknowledgments

This work has been funded by Pharmactive Biotech Products SL (Madrid, Spain).

REFERENCES


