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Selenium, a dietary-antioxidant with cardioprotective effects, prevents the impairments in heart rate and systolic blood pressure in adolescent rats exposed to binge exposure.

Mª Luisa Ojeda[a], Paula Sobrino[a], Rui Manuel Rua[b], María del Carmen Gallego-Lopez [a], Fátima Nogales[a]*, Olimpia Carreras[a].

[a]Department of Physiology, Faculty of Pharmacy, Seville University, 41012 Seville, Spain.

[b]Faculty of Health Sciences, University Fernando Pessoa, Porto, Portugal.

*Address: Dra. Fátima Nogales Bueno.

Department of Physiology.

Faculty of Pharmacy, Seville University.

C/ Profesor García González, nº 2.

41012. Seville. Spain.

Tel: +34 954556518

Fax: +34 954233765

E-mail: fnogales@us.es

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Abstract

Background: Binge drinking (BD) during adolescence has recently been related to cardiovascular alterations. Selenium (Se) is an essential trace element with antioxidant, anti-inflammatory and antiapoptotic properties, essential for correct heart function.

Objectives: To study the protective cardiovascular effects of selenium in adolescent rats exposed to a BD-like procedure.

Methods: 32 adolescent male rats exposed to an intraperitoneally BD-like model or not, and supplemented with 0.4ppm of selenite or not, were divided into 4 groups: control, alcohol, control-selenium and alcohol-selenium. At the end of the experimental period blood pressure and heart rate (HR) were determined. Se deposits, oxidative balance and the expression of glutathione peroxidases (GPxs), NF-kB and caspase-3 were measured in the heart. Also, DNA instability in rat lymphocytes and serum vascular markers were determined. Statistical analysis was performed with the ANOVA model.

Results: The BD-like model depleted Se heart deposits, decreased GPx activity and GPx1 and GPx4 expression, increased NF-kB, caspase-3 expression, and generated oxidation in myocytes. Outside the heart, BD-like model caused double-strand breaks in lymphocyte DNA and increased the vascular markers Vascular Endothelial Growth Factor (VEGF), Connective Tissue Growth Factor (CTGF). All of these cardiovascular alterations were related to higher systolic and diastolic blood pressure as well as HR. In the heart, Se supplementation of BD-exposed rats significantly increased Se deposits and improved oxidative balance and vascular damage;

including increased GPxs and decreased NF-kB and caspase-3 activation, consequently decreasing systolic blood pressure and HR.

Conclusions: Se-supplementation lowered HR and systolic blood pressure in BD-exposed adolescent rats.

Keywords: Binge Drinking Experience; Systolic Blood Pressure; Heart Rate; Selenium; Vascular Markers.

1. INTRODUCTION.

Repeated binge drinking (BD) is currently the most widespread pattern of alcohol consumption during adolescence [1], yet its effects on the cardiovascular system are poorly understood [2]. Recent clinical studies have shown that this pattern of ethanol consumption in adolescents is associated with a higher risk for hypertension [3] and vascular dysfunction [2]. It has also been related to cardiac arrhythmias in animals [4].

Epidemiological studies report a J-shaped curve relationship between the amount of alcohol consumed and cardiovascular disease [5]. The dose and pattern of alcohol consumption appears to be one of the greatest modulators. During binge drinking episodes blood alcohol levels exceed 0.08% and the risk of adverse alcohol-related consequences increases significantly [6].

It is known that in adults acute ethanol-induced heart alterations are related mainly to its oxidative metabolism; i.e., acetaldehyde and aldehyde dehydrogenases. More than one mechanism is often activated, which leads to a multiple changes in cellular proteins and associated cell functions. For instance, Piano and Phillips [7] revealed important roles for oxidative stress (OS) and the hormone angiotensin II. In this context, it is important to remember that acute ethanol administration during the above indicated oxidative metabolism induces cytochrome P450 2E1 (CYP2E1) activity. Noteworthy is the fact that CYP2E1 is a powerful generator of reactive oxygen species (ROS) [8]. Excessive oxidation can, moreover,

activate the sympathetic nervous system (SNS) as well as renin-angiotensin-aldosterone system (RAAS) [9]. BD may also adversely affect lipid profiles and hemostatic/coagulation mechanisms, leading to severe peripheral endothelial dysfunction, which affects coronary arteries and heart function. It is also known that a BD pattern directly affects heart function, since it provokes OS and apoptosis and increases the vulnerability for arrhythmias by altering myocardial electrophysiological properties [10,11].

Selenium (Se) is an essential micronutrient found in grain, meat, seafood, and nuts, as well as in dairy products. Se performs its biological functions via its incorporation into 25 different selenoproteins, such as the antioxidant family GPx or the main Se carrier in the body Selenoprotein P (SelP), which also has antioxidant properties [12]. Se, therefore, acts as an antioxidant. Oxidation is related to mitochondrial dysfunction, apoptosis and NF-kB activation, all of which are intimately linked to cardiovascular biology and function. An Se deficiency is, therefore, associated with cardiac pathology [13–16].

In the heart, the most studied selenoprotein is the GPx family [16], by rank the selenoprotein mRNAs expressions detected in the heart are: GPx4 > GPx3 >> GPx1. GPx4 reduces hydroxyperoxides in lipoproteins, complex lipids and phospholipids of biomembranes, and plays an essential antioxidant role in mitochondria, modulating their intrinsic apoptotic pathway and the transcriptional factor NF-KB protein [17]. Due to its ability to serve as an ROS scavenger in extracellular spaces [18], GPx3 protects the extracellular matrix from oxidative damage, while cytosolic GPx1 is particularly important for detoxifying intracellular ROS [14]. SelP is also expressed in the heart; it reduces tissue ROS both directly and also by delivering Se to protect selenoproteins in different tissues[19]. Optimal serum Se levels are, moreover, related to atherosclerosis prevention [20,21].

It has recently been found that BD exposure during adolescence increases ROS and disrupts Se homeostasis and Se tissue distribution [22]. In this context, administering an Se-supplemented

diet to BD-exposed rats is a good strategy against liver and kidney BD damage, as it decreases oxidative damage and prevents NF-kB activation and apoptosis [23,24]. The objective of this study was to evaluate heart Selenoprotein balance in BD-exposed adolescent rats, and its relationship with oxidative, apoptotic and inflammatory balance, as well as blood pressure, heart rate (HR) and endothelial function. In order to support Se as a therapy against cardiovascular damage in adolescent BD consumers, the above parameters were analyzed after Se supplementation in this animal model.

2. MATERIAL AND METHODS.

Animals.

Thirty-two adolescent male Wistar rats (Centre of Production and Animal experimentation, Vice-rector's Office for Scientific Research, University of Seville) were used in these experiments. Rats were received at 21 days old and housed in groups of two rats per cage for one week in order to acclimatize them to the housing conditions and handling. The experimental treatment was conducted over a 3–week period, beginning when the rats reached postnatal day (PND) 28 and ending at 47 days of age. This period corresponds to adolescence in Wistar rats [25]. The animals were kept in an automatically controlled temperature (22-23 ºC) vivarium with a 12-hour light-dark cycle (09:00 to 21:00). Animal care procedures and experimental protocols were performed in accordance with EU regulations (Council Directive 86/609/EEC, November 24th 1986) and approved by the Ethics Committee of the University of Seville.

On PND 28, when the adolescent period begins, rats were randomly assigned into four groups (n = 8/group) according to their treatments: control group (C): rats were given control diet and drinking water ad libitum, and on the corresponding days, an physiological saline solution (PSS) intraperitoneally (i.p.); binge drinking group (BD): rats were given control diet and drinking water ad libitum, and on the corresponding days, an ethanol solution 20% (v/v) in isotonic saline (3 g/kg/d) i.p.; control selenium supplemented group (CSe): rats were given control diet

and Se supplemented in drinking water ad libitum, and on the corresponding days an injection of PSS; and binge drinking selenium supplemented group (BDSe): rats were given control diet and drinking water supplemented with Se ad libitum, and on the corresponding days, an alcohol solution 20% (v/v) in isotonic saline (3 g/kg/d) delivered i.p.

Standard pellet diet (2014 Teklad Global 14% Protein Rodent Maintenance Diet, Harlan Laboratories, Barcelona, Spain) that contained 0.23 ppm of Se were available *ad libitum* in all the experimental groups. The Se supplemented groups (CSe and BDSe) received 0.14 ppm of Se of extra as anhydrous sodium selenite (Panreac, Barcelona, Spain) in drinking water during all experimental period. The amount of 0.14 ppm of Se was chosen according to the amount of Se consumed by adolescent rats just from the standard pellet diet, and based on the study of Yang et al. [26], which, states that GPx activities in rats' plasma and liver were maximized at 0.5 ppm of dietary Se. Since C adolescent rats used in this study presented an intake of approximately 12 g of food per day, with a supplemented Se diet of 0.5 ppm they will receive 6 µg/day of Se. With the objective of studying the effects of excessive but not extreme Se doses, the amount of Se supplemented in water was estimated in order not to reach 6 µg/day of Se intake.

Nutritional control.

Body weight and the amount of food consumed by rats were monitored daily until the end of the experimental period. The amount of food and drinking water ingested was calculated by measuring these parameters every day in the morning. Knowing the Se concentration (ppm) in the diet and the drinking water, Se intake was calculated by multiplying by food and water ingested every day. All measurements were taken at 9:00 a.m. to avoid changes due to circadian rhythms.

Ethanol treatment.

Alcohol-exposed groups (BD and BDSe) received an i.p. injection of alcohol (20 % v/v) in PSS (3 g/kg/d). Alcohol injections were given starting at 7:00 p.m., when the dark cycle began, for 3 consecutive days each week for 3 weeks. No i.p. injections were given during the remaining 4 days of each week [27]. Control groups (C and CSe) received an i.p. injection of an equal volume of PSS at the same time as the alcohol-exposed group's injections.

Blood pressure (mm Hg) and heart rate (beats/min).

Systolic and diastolic blood pressure (SBP and DBP), and heart rate (HR) were monitored with a pressure meter (NIPREM 645, CIBERTEC, Spain) using the indirect tail occlusion method. Measurements were taken in adolescent rats 24 hours after last alcohol exposure or last injection with saline solution. Each animal was measured 4-5 times successively in order to calculate the arithmetic mean, this being the value analyzed. Mean blood pressure (MBP) was calculated from the SBP and DBP data.

Samples.

At the end of the experimental period the rats were fasted for 12 h and feces and urine samples were collected using individual metabolic cages. Then, 24 hours after their last alcohol exposure or treatment with saline solution, adolescent rats were anesthetized with an i.p. injection of 28% w/v urethane (0.5 ml/100 g of body weight). The blood was obtained by heart puncture and collected in tubes. The serum was prepared using low-speed centrifugation for 15 min. at 1300 x g. The abdomen was opened by a midline incision and the whole heart was removed, weighed, and frozen in liquid nitrogen and stored at −80 ºC for future biochemical determinations.

Selenium Analysis.

Serum and heart Se levels were determined by graphite-furnace atomic absorption spectrometry following the procedure described in Ojeda et al. [28]. We used a PerkinElmer AAnalyst™ 800 high-performance atomic absorption spectrometer with WinLab32 for AA

software, equipped with a Transversely Heated Graphite Furnace (THGA) with longitudinal Zeeman-effect background corrector and an AS-furnace autosampler (PerkinElmer, Ueberlingen, Germany) and electrodeless Se discharge lamps (EDLs). Further details are provided in the supplementary material.

Antioxidant enzymes and oxidative stress markers.

The activity of antioxidant enzymes (Superoxide dismutase (SOD), catalase (CAT), GPx and glutathione reductase (GR)), as well as lipid and protein oxidation, were determined in the heart, employing the biochemical assays described in Ojeda et al. [29]. Further details are provided in the supplementary material.

Immunoblotting assays.

The expression of the selenoproteins GPx1, GPx3, GPx4 and SelP as well as NF-κB p65 and cleaved caspase-3 were determined in whole-heart samples of the adolescent rats by Western blotting. The samples contained 150 μg of protein. The specific primary antibodies (rabbit polyclonal IgG, Santa Cruz Biotechnology) were diluted: GPx1 and GPx3 (1:2000), GPx4 (1:5000), and SelP (1:2500), NF κB p-65 and cleaved caspase-3 (1:1000). Secondary antibody (anti-rabbit IgG HRP conjugate, Santa Cruz Biotechnology) was utilized in dilutions of 1:5000 for GPx1, GPx3 and SelP, 1:10000 for GPx4, 1:2500 for NF κB p-65 and caspase-3. Further details are provided in the supplementary material.

Lymphocytes isolation and determination of DNA stability by comet analysis.

DNA instability (strand breaks) was measured in rat lymphocytes at the age of 47 days by comet analysis [30]. The percentage of fluorescence in the comet tail, head and Olive Tail Moment (OTM, defined as the product of the tail length and the fraction of total DNA in the tail, OTM = [Tail mean − Head mean] × Tail % DNA/100) were measured. Further details are provided in the supplementary material.

Vascular markers.

Serum Caveolin 1 (Cav-1), Vascular Endothelial Growth Factor (VEGF), Connective Tissue Growth Factor (CTGF), and Plasminogen Activator Inhibitor-1 (tPAI-1) were measured by The Luminex xMAP (Millipore, Darmstadt, Germany). The Luminex system is a combination of three basic xMAP technologies.

Statistical Analysis

The results are expressed as means ± standard error of the mean (SEM). The data were analyzed using a mixed ANOVA model. The subject variability factor within each group was analyzed using standardized data to the dependent variables of each animal and using a repeated measures two-way ANOVA (Statistica Version 12.0 (StatSoft, Tulsa, OK, USA)). There were no significant differences between the subjects within each group. The dependent variables determined for each treatment were analyzed separately by one-way ANOVA (GraphPad InStat 3, CA, USA) and Fisher's test. When ANOVA detected significant differences, multiple comparisons between the means were studied by the Tukey-Kramer test. The statistical significance was established at $p<0.05$.

RESULTS.

Intermittent i.p. BD exposure did not alter total kcal intake, weight gain, relative heart weight or heart protein concentration (Table 1). However, Se deposits in the heart of BD animals decreased significantly ($F(3, 28) = 7.07$, $p<0.01$). The Se-supplemented therapy was effective, since supplemented groups consumed a greater amount of total Se than their respective counterparts ($F(3, 28) = 28.615$, $p<0.001$) and had significantly higher Se deposits in the heart ($F(3, 28) = 33.80$, $p<0.001$). BD animals had significantly higher levels of SBP ($F(3, 28) = 16.89$, $p<0.001$), DBP ($F(3, 28) = 3.83$, $p<0.05$), MBP ($F(3, 28) = 5.64$, $p<0.01$) and HR ($F(3, 28) = 3.16$, $p<0.05$) than control rats (Figure 1). Supplementing Se in BD animals significantly reduced SBP ($F(3, 28) = 3.69$, $p<0.05$) and HR ($F(3, 28) = 5.76$, $p<0.01$); whereas the BDSe group had higher SBP values but lower HR values than CSe rats.

In the heart, BD exposure significantly increased CAT and GR antioxidant activity ($F(3, 28) = 11.88$ and 6.87, $p<0.001$ to CAT and GR, respectively) and decreased GPx activity ($F(3, 28) = 6.19$, $p<0.01$), while protein and lipid oxidation took place (Table 2). Supplementing these animals with Se decreased CAT activity ($F(3, 28) = 3.67$, $p<0.05$), but Se mainly acted by increasing the selenoprotein GPx activity and decreasing GR activity ($F(3, 28) = 48.08$ and 15.10, $p<0.001$ to GPx and GR, respectively); thus, Se modulated the activity of this pair of enzymes, which in turn contributed to a decrease in lipid ($F(3, 28) = 92.70$, $p<0.001$) and heart protein oxidation ($F(3, 28) = 4.26$, $p<0.05$). In Se-supplemented control rats, an increase in GPx activity, which did not affect biomolecular oxidation was observed.

Binge ethanol consumption significantly decreased the expression of the selenoproteins GPx1 ($F(3, 28) = 26.58$, $p<0.001$) and GPx4 ($F(3, 28) = 4.449$, $p<0.05$) (Figure 2A and 2C). According to the reduction of the GPxs expression found, NF-kBp65 and cleaved-caspase-3 expression increased significantly in the heart of BD rats ($F(3, 28) = 8.66$, $p<0.01$; $F(3, 28) = 19.96$, $p<0.001$) (Figure 3A and 3B). Se supplementation prevented this by increasing GPxs expression and reducing NF-kB and caspase-3 expression. Control Se-supplemented rats displayed significantly increased GPx1 and GPx4 in the heart ($F(3, 28) = 6.624$, $p<0.01$), but there was no modification of NF-kB or caspase-3 expression compared to control animals.

Figure 4 displays the DNA oxidative profile in blood/serum since DNA stability was studied in peripheral blood lymphocytes. BD group had significantly lower head % DNA and higher tail % DNA and OTM ($F(3, 28) = 53.56$, 94.73 and 11.60, $p<0.001$ to head % DNA, Tail % DNA and OTM, respectively), which suggests DNA damage. Se supplementation to BD-exposed rats prevented this ($F(3, 28) = 53.56$, 94.73 and 11.60, $p<0.001$ to head % DNA, Tail % DNA and OTM, respectively) completely.

BD exposure increased all of the vascular markers measured: tPAI-1 ($F(3, 28) = 3.95$, $p<0.05$), Cav-1 ($F(3, 28) = 7.06$, $p<0.01$), VEGF ($F(3, 28) = 9.06$, $p<0.01$) and CTGF ($F(3, 28) = 16.85$,

p<0.001) (Figure 5). Se supplementation to BD rats significantly decreased VEGF and CTGF serum levels ($F(3, 28) = 9.06$ and 7.18, $p<0.01$ to VEGF and CTGF, respectively); however tPAI-1 and Cav-1 were increased (($F(3, 28) = 15.93$, $p<0.01$; $F(3, 28) = 71.36$, $p<0.001$). Control Se-supplemented animals had higher serum tPAI-1 and Cav-1 values than the control rats ($F(3, 28) = 3.82$, $p<0.05$; $F(3, 28) = 71.36$, $p<0.001$), with tPAI-1 serum levels significantly lower in CSe rats than in their BDSe counterparts ($p<0.01$).

3. DISCUSSION.

Previous studies have described that repeated BD consumption produces ROS and decreases antioxidant response. Excessive oxidation could, moreover, activate the sympathetic nervous system (SNS) and renin-angiotensin-aldosterone system (RAAS) [9]. In this context, an integrative review of the cardiovascular consequences of BD concluded that BD in young adults may produce vascular OS, inflammation, activation of the SNS and RAAS, impairment of the baroreceptors, changes in endothelial and smooth cell function and vascular reactivity [31]. It is also known that BD directly affects heart function by generating OS in myocardium by different cellular mechanisms [10,11].

It is known that BD drastically reduces the bioavailability of the antioxidant Se in adolescent rats even though they have eaten a proper amount of Se in diet [23]. This fact could be due to the body consuming this element in order to synthesize active antioxidant selenoproteins to fight against the acute ethanol-generated OS. This theory is in consonance with the depletion of Se found in the heart of BD-exposed animals, the lower selenoprotein GPx activity and the higher protein, and especially lipid, oxidation found in their hearts. In terms of the antioxidant enzyme balance and contrary to GPx activity, CAT and GR activity increased after BD exposure. The increased CAT activity could be due to the fact that this enzyme, specifically in heart, acts as a principal ethanol metabolizing enzyme, as well as acting upon myocardial morphology and hemodynamics [32]. Therefore, the acute levels of ethanol found in blood after BD exposure could also have been metabolized by secondary tissues such as heart, by stimulating CAT

activity in order to metabolize it. GR activity also increased significantly in BD rats. This enzyme leads to glutathione (GSH) generation which is necessary for a correct GPx function. Despite this generation of GSH in the heart, GPx was decreased significantly, suggesting Se depletion as a key factor in the dysregulation of GPx activity and heart oxidative balance. When extra Se was supplied to BD rats, cardiac Se deposits increased and therefore so did GPx activity, preventing lipid and partial protein oxidation in the heart.

Accordingly, cardiac Se depletion found in BD rats resulted in decreased GPx1 and GPx4 expression, with GPx3 remaining unaffected. This is unexpected since GPx4 is a selenoprotein with very low dependence on Se deposits and in this study it decreased despite the conservation of GPx3 [33]. Iwata et al. [34] found that GPx3 expression increased significantly in hyperglycemic mice, suggesting that this enzyme was dependent on insulin and serum glucose balance, playing a significant role in protecting cardiomyocytes from OS caused by hyperglycemia [32]. It is important to note that BD-exposed adolescent rats also presented sings of hyperglycemia, as we have previously found [23]. For this reason Gpx3 is either conserved or derived from blood to extracellular heart matrix. However, due to its low dependence on Se deposits, the decrease in GPx4 expression should be further examined. In this study, it appears that the lower GPx4 expression depends on mitochondrial oxidation more than on Se deposits. GPx4 is the only GPx enzyme which reduces hydroxyperoxides in lipoproteins, complex lipids and phospholipids of biomembranes, playing an essential antioxidant role in mitochondria [17]. Mitochondria are the main organelles damaged in the heart by OS after BD consumption [35]. It is known that the mitochondrial oxidation caused by acute ethanol leads to apoptosis in cardiomyocytes via the intrinsic apoptosis pathway [11,36]. Probably, GPx4 is consumed in order to prevent this situation and, thus, its expression decreases. More recently, Park et al. [37] found that the downregulation of GPx4 contributes to ferroptotic cell death in cardiomyocytes upon metabolic stress. Ferroptosis is a lipid ROS-induced cell death programme that is dependent on intracellular iron. These facts suggest that

BD exposure induces mitochondrial oxidation, GPx4, cardiomyocyte cell death and compromised heart function.

According to its extreme dependence on Se deposits, GPx1 is the selenoprotein that presents the greatest decrease in the heart of BD animals. This is in consonance with the lower GPx activity measured, implying higher intracellular OS in heart. BD exposure does not affect SelP heart expression. This is consistent with the literature, since this selenoprotein has a low dependence on Se deposits, including in heart [18].

Se supplementation increased GPx1 and GPx4 heart expressions. The increase in GPx1 expression is related to the higher antioxidant GPx activity found in the heart, decreasing intracellular myocyte lipid and protein oxidation. Interestingly, GPx4 is highly up-regulated after supplementation in BD-exposed animals. Since it is associated with protection from mitochondrial oxidation [38], decreasing the release of cytochrome c owing to its inner membrane location in mitochondria and its ability to repair cardiolipin peroxidation [39]. This increase in GPx4 is important for a correct cardiac mitochondrial function. In fact, BDSe rats had lower HR levels than those of the BD rats.

An increase in activation of caspase-3 and NF-kB was found in the heart of BD animals, showing that BD exposure is related to cardiac oxidation, apoptosis and inflammation. Previous studies found, *in vitro*, that rat cardiac tissue and cardiocytes acutely exposed to high ethanol levels led to an increase in NF-kB, OS and caspase 3/7 activities [40]. The same study found *in vivo* that acute high ethanol intake increased stroke volume and systolic pressure. The authors defend that these changes are related to OS where PI3K/Akt plays a pivotal role. In the current study the changes observed in oxidative balance and GPxs, NF-kBp65 and cleaved caspase-3 expression after BD exposure could be related to alterations in heart function. In fact, BD exposure led to higher SBP, DBP, MBP and HR. The high HR found is probably due to the OS and apoptosis found in the heart, since it is known that these are two factors which increase the vulnerability of the myocardium to developing arrhythmias by

altering myocardial electrophysiological properties [10,11]. The higher HR also leads to a higher SBP, which displayed a large increase. However, these cellular changes caused by BD in the heart, are not the only mechanisms involved in the increases of HR and BP detected. Repeated BD ethanol intake during adolescence is known to specifically increase sympathetic activation related to impaired baroreceptors and higher HR [10,41]. Acute exposure to ethanol by interaction with receptors in the brainstem also decreases baroreceptors sensitivity and its modulation on HR control, decreasing vagal control [42]. Using the same BD rat model of the present study, BD exposition has been found to stimulate the Hypothalamic-pituitary-adrenal-axes (HPA) [27] and RAAS [24] contributing to increase HR and BP.

From a cellular point of view, Se supplementation to BD-exposed rats decreased caspase-3 and NF-kB activation in myocytes, mainly by decreasing OS and preventing apoptosis and inflammation. These actions are intimately related to the higher heart GPx4 expression which protects mitochondria from oxidation, preventing apoptosis. It is also inversely related to NF-kB activation in different tissues such as endothelial cells [43,44]. GPx4 specifically interferes with NF-kB activation by interleukin-1, decreasing the synthesis of leukotrienes and prostanoids, which modulate inflammatory processes [45]. Looking for the existence of other possible mechanisms of action of Se supplementation to BD-exposed adolescent rats on cardiovascular regulation, it is important to remember that this therapy decreased the activation of HPA and SRAA in the same experimental BD-exposed rats [24]. Furthermore, although it is not measured in this study, the antioxidant Se could be decreasing vascular ROS. Vascular ROS decrease the activity of baroreceptors [46], increasing these receptors activity and function on HR and vagal control. All of these actions affect heart function by sharply decreasing cardiac HR, preventing tachycardia. Despite Se supplementation's important protective roles in heart functioning, this therapy only partially decreased SBP, and for this reason vascular studies were undertaken.

As it dramatically increases DNA damage in lymphocytes, BD leads to higher systemic oxidation. It is established that acute ethanol induces DNA damage in peripheral lymphocytes via an oxidative pathway [47]. Therefore, in this study BD increased the OTM of lymphocytes, indicating the breaking of the double helix, one of the most severe types of DNA damage, due to the oxidation generated. Se supplementation completely prevents this. This prevention probably occurs by increasing GPx activity and decreasing OS in these cells. In fact, in other studies, Se supplementation administered to adolescent rats exposed to the same BD protocol as the one used in this study increased serum GPx3 activity and decreased serum lipid peroxidation, both of which were altered in ethanol-exposed animals [24]. These results are interesting, since systemic oxidation and serum GPx3 activity are intimately related to vascular function and blood pressure [48]. These are both conditions which are highly compromised in our BD model, such that a single acute ethanol exposure enhances vascular OS and induces vascular dysfunction through RAAS activation as well as vascular and systemic lipid peroxidation [49]. We have previously found that adolescent BD rats have high serum aldosterone levels and lipid peroxidation [24] and that Se supplementation decreases both parameters, but only modestly. Perhaps this is the reason why the effects of Se upon blood pressure are lower than in heart function.

The intermittent BD pattern used in adolescent rats increased all of the vascular markers studied here in. VEGF is considered the most potent proangiogenic growth factor involved in vascular permeability, vascular dilation, endothelial proliferation and angiogenesis. Previous data established that acute ethanol exposure significantly increases serum VEGF values, but also perturbs endothelial VEGF signalling and action [50,51]. The growth factor CTGF plays important roles in cell adhesion, migration, proliferation, and angiogenesis and is critically involved in fibrotic process. Different studies relate CTGF to VEGF production and angiogenesis [52]. CTGF is upregulated by stimuli involved in cardiovascular damage, including OS [53]. Since ethanol exposure increases OS, both proteins (CTGF and VEGF) increased in this study and

endothelial function is compromised. Se supplementation improves vascular function, lowering SBP values by decreasing both parameters to normal values, probably due to its antioxidant properties. The observation of beneficial actions of Se on vasculature are not new, since anti-atherosclerotic activity of Se has been described previously [21].

tPAI-1 is an acute phase protein expressed in adipocytes and endothelial cells, and is highly expressed by most cells in response to stress. tPAI-1´s role has been identified as the inhibition of plasminogen activator which blocks fibrinolysis. Liver can produce large amounts of tPAI-1 in response to stress, such as ethanol exposure; furthermore, tPAI-1 plays a critical role in alcohol-induced steatosis [49,50]. Therefore, BD exposure can increase this parameter greatly, resulting in vascular and hepatic damage. However, despite the fact that Se supplementation in adolescent BD animals improved hepatic inflammatory, oxidative and apoptotic profile and function [23], although it does not decrease tPAI-1 serum levels. Moreover, tPAI-1 serum levels also increased in control Se-supplemented animals. Although this result was new to our laboratory, Viezeliene et al. [55] found similar results when examining aluminum-induced OS and the effects of supplementing the animals with Se. Se is a mineral related to lipid homeostasis [56]. It is also known that t-PAI-1 secretion in HepG2 cells is modulated by triacylglycerols and by linoleic acid and/or its metabolic products [57]. Therefore, perhaps Se and tPAI-1 synthesis could be related via lipid homeostasis.

Cav-1 is the main component of the caveolae plasma membranes found in most cell types and it interacts with different cell components, regulating multiple cellular events such as cell growth, apoptosis and cholesterol trafficking. It allows blood vessels to sense, organise and mediate signal transduction in the face of altered shear stress conditions [58]. The underlying mechanism by which Cav-1 regulates ethanol induced damage is, however, not well-understood. In a BD model [59], it was found that an increase in serum and hepatic Cav-1, which could be a cellular defense through inhibiting reactive nitrogen species and iNOS-

signalling cascades. In our BD model, serum Cav-1 values increased and Se supplementation increased this value even further. Control Se animals also had high Cav-1 serum levels. Thus, Se appears to be related to serum Cav-1 levels, yet there are no bibliographical data which reflect or could explain this effect. We think that since this mineral is related to lipid homeostasis and Cav-1 is also important in cholesterol trafficking and insulin resistance, they could be related at different points in these pathways [60].

In summary, BD exposure during adolescence severely damages cardiac tissue by generating oxidation, inflammation and apoptosis, leading to high HR. These disturbances could be related in part to the low Se and GPx4 levels found in the heart, since they contribute to decrease mitochondrial oxidation, increasing their survival and function. Therefore, Se supplementation appears to be a good strategy for preventing heart oxidation, inflammation and apoptosis, improving heart function by reducing the tachycardia generated by BD exposure. BD-exposed rats also developed high SBP and DBP, due to higher systemic oxidation generated, which leads to endothelial dysfunction. Se supplementation in BD-exposed rats reverses DNA oxidation and the serum levels of the proangiogenic markers. However, these actions result in a partial decrease in SBP only. Public health messages regarding BD should, therefore, include information on the cardiovascular effects of this consumption pattern, since it provokes heart damage and vascular dysfunction, which also are important risk factors during the third and fourth decades of life. In this context, Se therapy should be investigated further as an antioxidant strategy for preventing such cardiovascular damage.

Conflict of interest: All authors of this manuscript declare that there are no conflicts of interest.

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Table 1. Nutritional and heart parameters.

	C	BD	CSe	BDSe
Kcal intake (kcal/day)	54.6 ± 3.4	50.1 ± 3.3	56.9 ± 3.5	50.5 ± 2.9
Increased body weight (g/day)	5.7 ± 0.2	5.1 ± 0.3	5.9 ± 0.2	5.2 ± 0.2
Se intake (µg/day)	3.1 ± 0.2	2.8 ± 0.2 ^{aaa}	5.6 ± 0.3 ^{ccc}	4.9 ± 0.3
HRW (%)	0.52 ± 0.024	0.52 ± 0.011	0.47 ± 0.009	0.50 ± 0.023
Heart Protein (mg prot/ml)	9.82 ± 0.64	9.03 ± 0.67	8.34 ± 0.50	9.04 ± 0.73
Se in heart (µg Se/g dry weight)	0.12 ± 0.005	0.09 ± 0.01 $^{**, aaa}$	0.18 ± 0.01 ^{cc}	0.17 ± 0.01

The results are expressed as mean ± SEM and analysed by a multifactorial analysis of variance (one-way ANOVA) followed by the Tukey´s test. The number of animals in each group is 8. HRW: heart relative weight; Se: Selenium. Groups: C: control group, BD: binge drinking group, CSe: control selenium supplemented group, and BDSe: binge drinking selenium supplemented group. Statistic difference between groups was expressed as: A vs C: **p<0.01; BD vs BDSe: ^{aaa}p<0.001; C vs CSe: ^{cc}p<0.01, ^{ccc}p<0.001.

Table 2. Oxidative balance in heart: antioxidant enzymes activities (SOD, CAT, GPx and GR) and lipid (MDA) and protein (PC) oxidation.

	C	BD	CSe	BDSe
SOD activity (U/mg proteins)	3.9 ± 0.2	4.3 ± 0.3	3.9 ± 0.1	4.2 ± 0.3
CAT activity (U/mg proteins)	28.1 ± 1.9	48.1 ± 2.7 ***,a	32.7 ± 1.9	37.5 ± 3.2
GPx activity (U/mg proteins)	106.8 ± 4.2	86.3 ± 3.3 **,aaa	169.5 ± 6.1 cc	146.5 ± 7.1
GR activity (U/mg proteins)	7.8 ± 0.6	11.2 ± 0.6 ***,aaa	7.7 ± 0.5	6.1 ± 0.5
MDA (mol/mg proteins)	0.056 ± 0.004	0.249 ± 0.019 ***,aaa	0.056 ± 0.004	0.051 ± 0.004
PC (mol/mg proteins)	5.3 ± 0.1	7.2 ± 0.2 ***,a	5.8 ± 0.2	6.4 ± 0.2

The results are expressed as mean ± SEM and analyzed by a multifactorial analysis of variance (one-way ANOVA) followed by the Tukey´s test. The number of animals in each group is 8. SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; GR: glutathione reductase; MDA: malondialdehyde; PC: protein carbonyl. Groups: C: control group, BD: binge drinking group, CSe: control selenium supplemented group, and BDSe: binge drinking selenium supplemented group. Statistic difference between groups was expressed as: BD vs C: **p<0.01, ***p<0.001; BD vs BDSe: ^{a} p<0.05, ^{aaa} p<0.001; C vs CSe: ^{cc}p<0.01.

Figure captions.

Figure 1: Systolic Blood Pressure (SBP: A), Heart Rate activity (HR: B), Diastolic blood Pressure (DBP: C) and Mean Blood Pressure (MBP: D). The results are expressed as mean ± SEM and analysed by a multifactorial analysis of variance (one-way ANOVA) followed by the Tukey´s test. The number of animals in each group is 8. Groups: C: control group, BD: binge drinking group, CSe: control selenium supplemented group, and BDSe: binge drinking selenium supplemented group. Statistic difference between groups was expressed as: BD vs C: *p<0.05, **p<0.01, ***p<0.001; BD vs BDSe: ^{a}p<0.05, ^{aa}p<0.01; BDSe vs CSe: $^{+}$p<0.05.

Figure 2. Expression of heart selenoproteins (GPx1: A; GPx3: B; GPx-4: C; SelP: D). Representative western blots of selenoproteins (normalized to β-actin). The results are expressed as mean ± SEM and analysed by a multifactorial analysis of variance (one-way ANOVA) followed by the Tukey´s test. The number of animals in each group is 8. Groups: C: control group, BD: binge drinking group, CSe: control selenium supplemented group, and BDSe: binge drinking selenium supplemented group. Statistic difference between groups was expressed as: BD vs C: *p<0.05, ***p<0.001; BD vs BDSe: ^{aaa} p<0.001.

Figure 3. Expression of heart NFkBp65 and cleaved-caspase-3. Representative western blots of these proteins (normalized to β-actin). The results are expressed as mean ± SEM and analysed by a multifactorial analysis of variance (one-way ANOVA) followed by the Tukey´s test. The number of animals in each group is 8. Groups: C: control group, BD: binge drinking group, CSe: control selenium supplemented group, and BDSe: binge drinking selenium supplemented group. Statistic difference between groups was expressed as: BD vs C: **p<0.01, ***p<0.001; BD vs BDSe: ^{aa}p<0.01, ^{aaa} p<0.001.

Figure 4. Percentage of DNA in the head (A) and the tail (B), and OTM (C) in lymphocytes. The results are expressed as mean ± SEM and analysed by a multifactorial analysis of variance (one-way ANOVA) followed by the Tukey´s test. The number of animals in each group is 8. OTM: Olive Tail Moment [OTM= (tail mean−head mean) × tail percentage of DNA/100]. Groups: C: control group, BD: binge drinking group, CSe: control selenium supplemented group, and BDSe: binge drinking selenium supplemented group. Statistic difference between groups was expressed as: BD vs C: ***p<0.001; BD vs BDSe: ^{aaa} p<0.001.

Figure 5. Vascular markers: Caveolin 1 (CAv-1) (A), Vascular Endothelial Growth Factor (VEGF) (B), Connective Tissue Growth Factor (CTGF) (C), Plasminogen Activator Inhibitor-1 (tPAI-1) (D). The results are expressed as mean ± SEM and analysed by a multifactorial analysis of variance (one-way ANOVA) followed by the Tukey´s test. The number of animals in each group is 8. Groups: C: control group, BD: binge drinking group, CSe: control selenium supplemented group, and BDSe: binge drinking selenium supplemented group. Statistic difference between groups was expressed as: BD vs C: *p<0.05, **p<0.01, ***p<0.001; BD vs BDSe: ^{aa} p<0.01; C vs CSe: ^{C}p<0.05, ^{CCC}p<0.001; BDSe vs CSe: $^{++}$p<0.01.

Figure 6. Cardiovascular effects of Binge Drinking and selenium supplementation. Glutathione Peroxidase (GPx), Nuclear Factor kappa-light-chain-enhancer of activated B cells

(NFkB), Caveolin 1 (Cav-1), Vascular Endothelial Growth Factor (VEGF), Connective Tissue Growth Factor (CTGF), Plasminogen Activator Inhibitor-1 (tPAI-1), Heart Rate (HR), Mean Blood Pressure (MBP).