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# Effects of quercetin on heart nitric oxide metabolism in L-NAME treated rats

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ABSTRACT

This study investigated the effects of a quercetin-supplemented diet on the biochemical changes installed in the heart of NO-deficient rats in terms of oxidants production and NO bioavailability determinants. Sprague-Dawley rats were subjected to $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME) treatment (360 mg/L L-NAME in the drinking water, 4 d) with or without supplementation with quercetin (4 g/kg diet). L-NAME administration led to increased blood pressure (BP) (30%), decreased nitric oxide synthase (NOS) activity (50%), and increases in NADPH oxidase (NOX)-dependent superoxide anion production (60%) and $p47^{phox}$ protein level (65%). The co-administration of quercetin prevented the increase in BP and the activation of NOX but did not modify the decrease in NOS activity caused by L-NAME. In addition, quercetin affected oxidative stress parameters as glutathione oxidation, and the activities of oxidant detoxifying enzymes superoxide dismutase, glutathione peroxidase, and catalase. Thus, quercetin administration counteracts L-NAME effects on NO bioavailability determinants in vivo, essentially through controlling NOX-mediated superoxide anion production.

## 1. Introduction

Quercetin is a flavonoid highly represented in human diet due to its presence in frequently consumed fruits, vegetables and plant-derived products, e.g. onion, dark chocolate, berries and apples [1,2]. Clinical studies showed a decrease in blood pressure (BP) in subjects who received quercetin-enriched onion extracts or pure quercetin [3], as well as in different experimental animal models of hypertension using quercetin-supplemented diets [4–8]. Moreover, quercetin prevented the increase in BP in NO-deficient rats developed by a long-term $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME) treatment [9], in salt sensitive hypertensive Dahl rats [6,10], and also in spontaneously hypertensive rats (SHR) [11]. Regarding quercetin effects on heart, Liu et al. showed a decrease in oxidative stress and inflammation markers in myocardium after ischemia-reperfusion in rabbits [12]. Also, quercetin protected from myocardial dysfunction after LPS-induced inflammation in mice [13].

NO produced by nitric oxide synthase (NOS) enzymes, is a key modulator of vascular and cardiac function, including vessel tone and heart contractility and rate [14,15]. A decrease in NO levels is associated with cardiovascular disease and hypertension [16–18]. NO bioavailability depends on its synthesis by different NOS isoforms, i.e. endothelial (eNOS), neuronal (nNOS), and inducible (iNOS), and on its degradation specially by reacting with superoxide anion [19–21]. NADPH oxidases (NOX) are considered important sources of superoxide anion in the heart, being NOX2 and NOX4 the main isoforms expressed in this tissue [22–24]. Evidence related to quercetin effects on NO bioavailability determinants showed that quercetin administration prevented alterations in NOS and NOX gene and protein expression in the heart of rabbits subjected to ischemia-reperfusion injury [25]. Moreover, Sanchez et al. found a restored activity of NOS and modulation of eNOS expression in rat aorta of SHR after quercetin consumption [26].

The aim of this work was to evaluate the effects of a quercetin-supplemented diet in the heart of NO-deficient rats after a short-term L-NAME treatment in terms of determinants of NO bioavailability. In addition, the effects of quercetin will be discussed considering those obtained for (–)-epicatechin using a similar animal model and experimental conditions [27].

## 2. Materials and methods

### 2.1. Materials and chemicals

Bovine serum albumin (BSA), N,N′-dimethyl-9,9′–biacridinium dinitrate (lucigenin), epinephrine, ethylenediaminetetraacetic acid

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(EDTA), glutathione reductase, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), L-NAME, oxidized glutathione (GSSG), quercetin, reduced glutathione (GSH), and superoxide dismutase (SOD) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies for $p47^{phox}$, eNOS, iNOS, nNOS, and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibody for β-tubulin was from Abcam (Cambridge, MA, USA). [$^{14}$C] L-arginine was from Perkin Elmer Life and Analytical Sciences (Boston, MA, USA). All other reagents were of the highest commercially available purity.

### 2.2. Animal groups and animal care

Male Sprague-Dawley rats were housed under controlled temperature and humidity conditions (21-25 °C) with a 12 h light/dark cycle. The animals were randomly divided into four experimental groups (8 rats/group) that were fed: i) water ad libitum and standard rat chow (control group, C); ii) water ad libitum and standard rat chow added with 4 g quercetin/kg diet (quercetin group, Q); iii) 360 mg/L L-NAME in the drinking water and standard rat chow (L-NAME group, L); and iv) 360 mg/L L-NAME in the drinking water and standard rat chow added with 4 g quercetin/kg diet (L-NAME-quercetin group, LQ). The 4 g of quercetin/kg of diet used in this study are high when compared with human consumption but it is significantly lower than the NOAEL (no observed adverse effect level) established for rats fed other flavonoids, e.g. rutin [28], green tea catechins [29], or α-glycosyl isoquercitrin [30]. Food and beverage intake were recorded every day. Systolic BP (SBP) was measured daily by tail plethysmography in conscious, pre-warmed, slightly restrained rats and recorded on a polygraph (SC1000, Hatteras Instruments, Cary, North Carolina, USA). After the experimental period, rats were weighted, and euthanized with $CO_2$. The heart was excised, and left ventricle was separated and fractionated to be used for further determinations. All the procedures were carried out according to protocols for animal use and care approved by the Animal Ethics Committee (School of Pharmacy and Biochemistry, University of Buenos Aires) (Protocol #3999/15).

### 2.3. Creatine kinase-MB

Creatine kinase-MB activity was measured in plasma using a commercial kit following the providers instructions (Wiener Lab, Rosario, Argentina).

### 2.4. Heart homogenates preparation

Heart left ventricle was minced and homogenized in phosphate buffer, pH 7.4 at 4 °C. After homogenates centrifugation at 600 × g for 10 min, the pellet was discarded. A fraction of the supernatant was separated (total homogenate) and the remaining portion was further centrifuged at 10000 × g, for 10 min at 4 °C, to obtain a fraction free of mitochondria and peroxisomes to measure superoxide anion production. Protein content was assayed using BSA as standard [31].

### 2.5. Nitric oxide synthase activity

NOS activity was evaluated in left ventricle slices by measuring [$^{14}$C]-L-citrulline formation using [$^{14}$C]-L-arginine as substrate [32]. Specific NOS activity was assessed by subtracting citrulline formation measured in the presence of 100 μM L-NAME. [$^{14}$C]-L-citrulline values are expressed as pmol/g tissue.min.

### 2.6. Superoxide anion production

Superoxide anion production was determined in homogenates free of mitochondria and peroxisomes following the chemiluminescence of samples added with 25 μM lucigenin and 40 μM NADPH [22]. Chemiluminescence was monitored for 7 min. To confirm superoxide anion detection, SOD was added to samples. Superoxide anion production was calculated as percentage of SOD-inhibitable chemiluminescence and referred to control group values.

### 2.7. Western blot analysis

A portion of left ventricle was homogenized in RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 1% (v/v) sodium deoxycholate, 5 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate, and protease inhibitors). Homogenates were centrifuged at 10000 × g for 10 min at 4 °C, and the resulting supernatant was used for western blot analysis. Aliquots containing 40–50 μg of protein were denatured with sample buffer, separated on SDS polyacrylamide gels and then transferred to poly-vinylidene fluoride membranes by liquid electroblotting (MiniProtean 3, Bio-Rad) in a transfer solution containing 25 mM Tris, pH 8.3, 190 mM glycine, and 20% (v/v) methanol, at 110 V for 90 min. Membranes were blocked with phosphate buffered saline containing 5% (w/v) dry low-fat milk, for 2 h at room temperature, and subsequently incubated in the presence of the corresponding primary antibody (1:1000 dilution) overnight at 4 °C. After incubation for 90 min with the corresponding peroxidase-conjugated secondary antibody (1:10000 dilution), complexes were detected using luminol-based enhanced chemiluminiscence system. Films were scanned and densitometric analysis was performed using Image J (National Institute of Health, Bethesda, MD, USA). Samples protein content was normalized to β-actin or β-tubulin.

### 2.8. Superoxide dismutase, glutathione peroxidase, and catalase activities

SOD activity was determined in total homogenates through the inhibition of epinephrine oxidation by superoxide anion at 480 nm. Results are expressed as units of SOD/mg protein [33]. One unit of SOD is defined as the amount of enzyme necessary to cause a 50% inhibition of epinephrine oxidation. Glutathione peroxidase (GPx) activity was determined in total homogenates in the presence of glutathione reductase following NADPH oxidation at 340 nm. Values are expressed as units per mg protein (U/mg protein). One unit of GPx is defined as the amount of enzyme necessary to oxidize 1 μmol of NADPH/min [34]. Catalase (CAT) activity was evaluated following the consumption of hydrogen peroxide at 240 nm, and calculating the pseudo-first order constant of the reaction. CAT activity is expressed as pmoles of CAT/mg of protein [35].

### 2.9. Reduced and oxidized glutathione

Approximately 100 mg of heart left ventricle were homogenized in 1 M $HClO_4$ containing 2 mM EDTA. Samples were centrifuged for 20 min at 12000 × g. Supernatants were filtered through 0.22 μm cellulose acetate membranes and subjected to HPLC separation using a LC-18-DB column (25 cm × 4.6 mm, 5-μm particle size). GSH and GSSG were eluted with 20 mM sodium phosphate (pH 2.7) and detected electrochemically at 0.800 V [36]. Glutathione oxidation ratio was calculated as $GSSG/GSH^2$.

### 2.10. Statistical analyses

Values in the text, table, and figures are expressed as the mean ± SEM. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by the Multiple Comparison Bonferroni's test with Graph Pad Prism 4 (GraphPad Software Inc., San Diego, CA, USA). To establish the significance of between-group differences, $p$ values $< 0.05$ were considered significant.

**Table 1**
Body and heart weight, food and water intake, plasma creatine kinase-MB and systolic blood pressure.

| Parameter | Groups | | | |
|---|---|---|---|---|
| | C | Q | L | LQ |
| Initial body weight (g) | 122 ± 5 | 133 ± 4 | 126 ± 3 | 136 ± 4 |
| Final body weight (g) | 140 ± 5 | 148 ± 5 | 144 ± 3 | 153 ± 5 |
| Heart weight (g) | 0.65 ± 0.03 | 0.68 ± 0.04 | 0.60 ± 0.08 | 0.65 ± 0.08 |
| Heart/total weight ratio ($10^{-3}$) | 4.6 ± 0.2 | 4.6 ± 0.3 | 4.2 ± 0.2 | 4.3 ± 0.1 |
| Creatine kinase-MB (U $L^{-1}$) | 477 ± 52 | 472 ± 67 | 680 ± 113 | 458 ± 32 |
| Water intake (ml $d^{-1}$) | 49 ± 6 | 40 ± 5 | 51 ± 1 | 53 ± 1 |
| Systolic blood pressure (mm Hg) | | | | |
| Initial | 112 ± 2 | 118 ± 3 | 111 ± 2 | 114 ± 3 |
| Final | 103 ± 2 | 110 ± 2 | 144 ± 2* | 104 ± 3 |

C, control group; Q, quercetin group; L, L-NAME group; and LQ, L-NAME + quercetin group. The final values were taken after 4 days of treatment. *statistically different from C, Q and LQ ($p < 0.001$). Values are expressed as mean ± SEM (n = 8 per group).

## 3. Results and discussion

NO has been shown to regulate cardiac function, modulating, for example, cardiomyocytes contractility, angiogenesis, thrombogenicity and mitochondria respiration [14,15,37]. Moreover, NO is associated to oxidative stress not only because of its reaction with superoxide anion, but also through the actions of both NO and superoxide anion, on NOX and NOS [38–41]. Thus, NO and superoxide anion production and modulation of NOS and NOX, are crucial for NO bioavailability in most tissues. This study shows that dietary quercetin prevented undesirable changes in the heart induced by L-NAME in terms of oxidant production that ultimately affect NO bioavailability. Essentially, downregulation of NOX-mediated superoxide anion production appears to be a major event triggered by quercetin to counteract L-NAME effects.

We aimed to identify early biochemical alterations in NO bioavailability and oxidative stress induced by L-NAME administration that are not related to major and/or long-term modifications in heart structure and function. Confirming the absence of major changes in animal and heart physiology, at the end of the four days treatment, body weight, food consumption, water intake, heart weight, and plasma creatine kinase-MB were not significantly different among the studied groups (Table 1). A recent study revealed that L-NAME administered for longer periods of time (6–18 w) increased plasma levels of creatine kinase-MB and lactate dehydrogenase [42]. The non-significant increase in creatine kinase-MB observed in the L group, is possibly an indication of the onset of cardiac damage that would occur upon the extension of the treatment.

As we previously demonstrated, administration of L-NAME for 4 d leads to increased BP [43]. Initial SBP was similar in all groups; after L-NAME treatment, SBP in the L group increased 30% over the initial value, being significantly different from all other groups at day 4 (Table 1). Moreover, L-NAME effect on SBP was observed as early as day 2 ($p < 0.05$, L vs C and Q) (data not shown). The BP lowering effect of quercetin in the LQ group was not observed in control rats, suggesting that quercetin is either preventing the actions of L-NAME, and/or is affecting conditions that regulate BP, e.g. NO bioavailability.

L-NAME primary effect is to inhibit NOS in a competitive manner; but as it was reported in a previous study a decrease in NO production can lead to a subsequent regulation of NOS protein expression [44]. Then, we determined NOS activity as well as nNOS, eNOS, and iNOS protein expression in the heart. NOS activity in the LQ group was similar to that in the L group and significantly lower than in C and Q groups (50% and 62% respect to C and Q, respectively, $p < 0.01$)

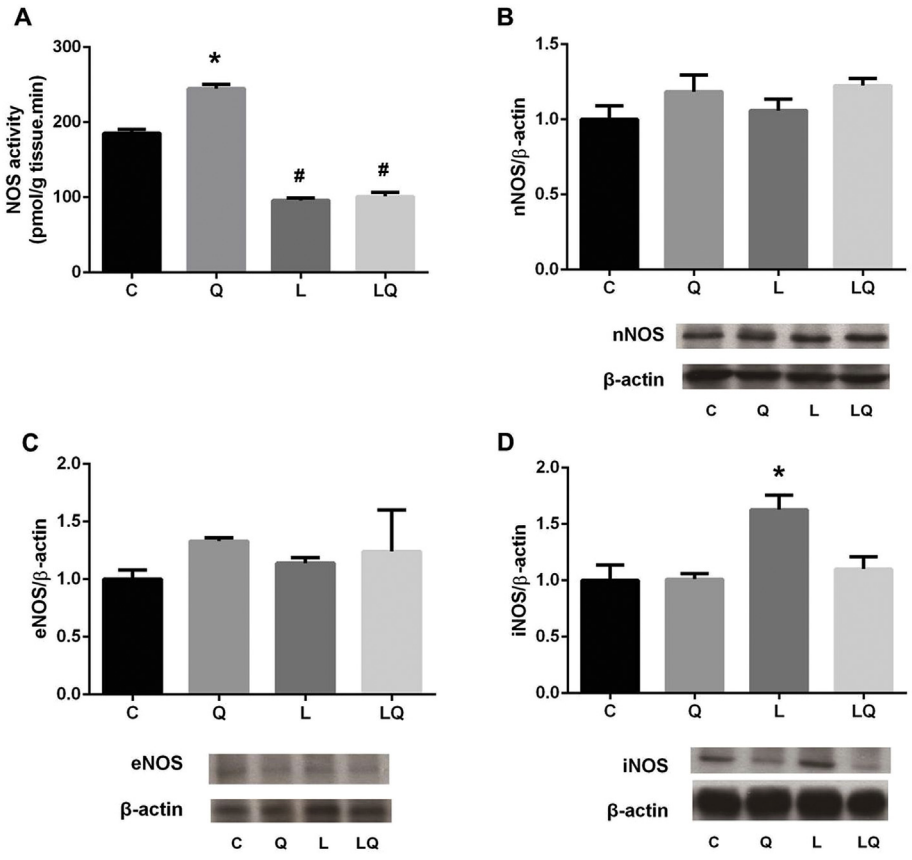


**Fig. 1. Quercetin effects on NOS**. NOS activity, measured as the formation of [$^{14}$C]-L-citrulline (A), and NOS isoforms expression measured by Western blot (B-D) in heart homogenates from the other groups after 4 d of treatment. *Statistically different from C, Q and LQ ($p < 0.05$); #statistically different from C ($p < 0.05$). Values are expressed as mean ± SEM (n = 3, and n = 8 per group, for NOS activity and Western blots, respectively).

(Fig. 1A). Even though quercetin administration was associated to an increased NOS activity in the absence of L-NAME (25% Q vs C; $p < 0.05$), this effect was not observed in the presence of L-NAME. Regarding NOS isoforms expression, nNOS and eNOS protein levels were similar among groups (Fig. 1 B, C). However, iNOS protein level was higher in the L group compared to all other groups (65%, $p < 0.05$) (Fig. 1D). The effect of L-NAME increasing iNOS protein level agrees with a previous report [44]. Our results showing that quercetin prevented such increase, suggest that this change may be related to an anti-inflammatory action. Accordingly, this quercetin action was also found in animal models of inflammation [12,45] and in cardiomyoblasts challenged with lipopolysaccharides (LPS) [46]. Besides, other flavonoids, e.g., (+)-catechin and (−)-epigallocatechin gallate that decreased the expression of iNOS, also downregulate other inflammatory pathways (NF-κB) [47] and molecules (TNF-α) [48] in rat heart.

The fact that NOS activity measured in heart homogenates of L-NAME treated groups was not affected by quercetin treatment, suggests that the L-NAME active metabolite, i.e. $N^{\omega}$-nitro-L-arginine, remains bound and blocking NOS active site during the determination of the enzyme activity. This suggests that quercetin effect on BP is not related to the direct prevention of L-NAME-mediated NOS inhibition, but to changes occurring because of such inhibition.

Analyzing the other main determinant of NO bioavailability, we studied SOD-inhibitable NADPH-dependent lucigenin-chemiluminscence in heart homogenates as a parameter of changes in the sources of superoxide anion production induced by L-NAME treatment. Superoxide anion production was higher in L compared to all other experimental groups (60%, 88% and 33% respect to C, Q and LQ, respectively; $p < 0.05$) (Fig. 2A). These results indicate that L-NAME treatment increased the heart tissue capacity to generate NADPH-induced superoxide anion and that this effect was not observed when quercetin was present in the diet. As the determination was carried out in free-mitochondria homogenates NOX emerges as the source of superoxide anion.

Thus, the expression of the major heart isoforms of NOX, i.e. NOX2 and NOX4, was evaluated. In line with the results obtained for superoxide anion production, $p47^{phox}$ protein level was significantly higher in L compared to C, Q and LQ ($p < 0.05$) (Fig. 2B). NOX4 protein levels were similar among the groups (Fig. 2C). Both effects of L-NAME, the increase in superoxide anion production and the higher $p47^{phox}$ levels, were prevented by quercetin. These results suggest that quercetin can modulate NOX2 activity, since $p47^{phox}$ regulatory subunit is critical for the assembling and activation of NOX2. Consistent with these results, other authors found decreased $p47^{phox}$ protein levels in aorta of SHR or Ang II-induced hypertensive rats, after quercetin administration [25,26,49]. Moreover, quercetin treatment was associated with increased NO bioavailability by modulating NO and superoxide anion levels in aorta of SHR rats and myocardium of rabbits after ischemia-reperfusion [9,25,26,49].

To investigate possible changes in heart enzymes responsible for detoxifying superoxide anion and its dismutation product, hydrogen peroxide, SOD, GPx and CAT activities were evaluated. SOD activity in L was higher compared to the other groups (57%, 37% and 44% compared to C, Q and LQ, respectively; $p < 0.05$). Similarly, GPx activity in L was higher compared to the other groups studied (22%, 14% and 30% respect to C, Q and LQ, respectively; $p < 0.05$) (Fig. 3 A, B). CAT activity did not change significantly among the different groups (Fig. 3C). The observed increase in SOD and GPx activities are in accordance with previous reports in the heart from L-NAME-treated rats [27,50]. This could be considered as an adaptative response due to a higher superoxide anion production, and consequently, hydrogen peroxide levels, in NO-deficient rats. In this context, the effect of quercetin could be ascribed to the maintenance of controlled superoxide anion production rather than to a direct action on SOD, GPx and/or CAT expression or activity.

Finally, to evaluate oxidative stress in the heart, we determined the level of oxidation of glutathione by calculating $GSSG/GSH^2$ ratio. Levels of GSH (89 ± 8 ng/mg of wet tissue) were similar among the groups. L-NAME treatment did not modify heart glutathione oxidation

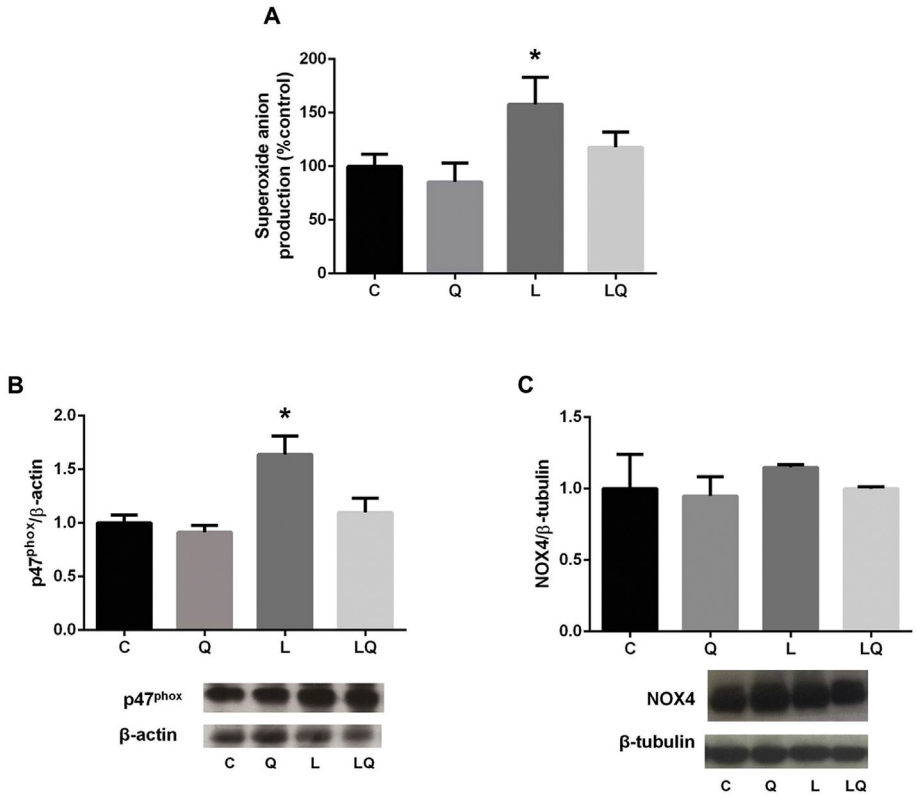


**Fig. 2. Quercetin effects on NADPH oxidase.** NADPH dependent superoxide anion production measured by lucigenin chemiluminiscence (A) and NADPH oxidase subunits protein levels determined by Western blot (B and C) in heart homogenates from C, Q, L and LQ groups after 4 d of treatment. *statistically different from C, Q and LQ ($p < 0.05$). Values are expressed as mean ± SEM (n = 8 per group).

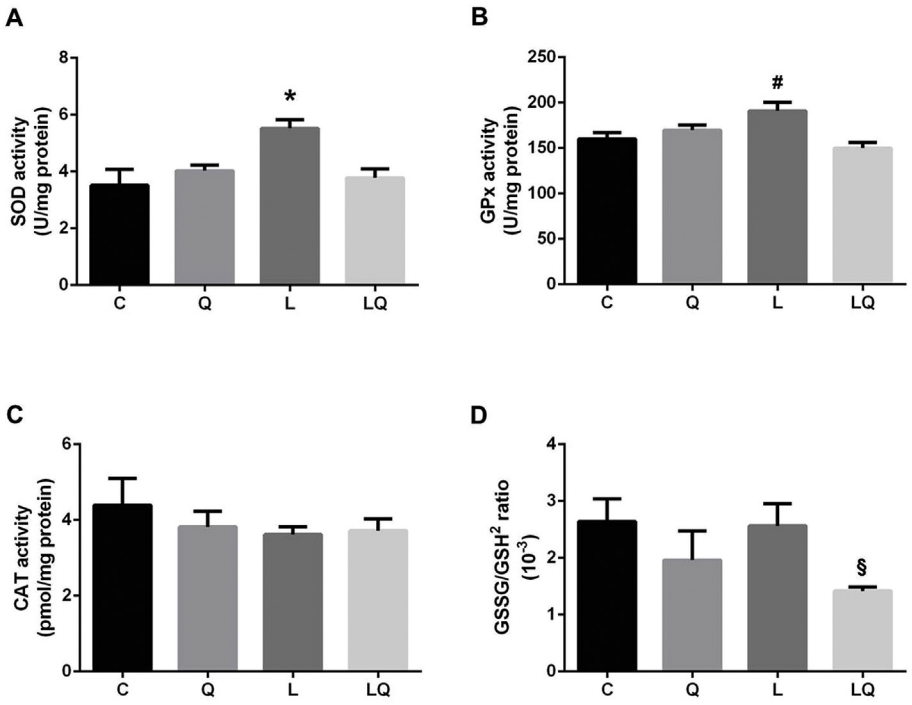


**Fig. 3. Quercetin effects on superoxide anion/hydrogen peroxide metabolizing enzymes, and glutathione in heart**. Superoxide dismutase (SOD) (A), glutathione peroxidase (GPx) (B) and catalase (CAT) (C) activities, and GSSG/GSH$^2$ ratio (D), in heart homogenates from C, Q, L and LQ groups, after 4 d of treatment. *statistically different from C, Q and LQ ($p < 0.05$); $^{\#}$statistically different from C and LQ ($p < 0.05$); $^{\S}$statistically different from C and L ($p < 0.05$). Values are expressed as mean ± SEM (n = 8 per group).

ratio, while quercetin reduced it (Fig. 3D). This reduction was only significant in the LQ group compared with C and L. This antioxidant effect of quercetin has been extensively reported, however, it should be considered as an indirect antioxidant action, and not a free radical scavenging action (direct antioxidant action) [51,52].

Under the same L-NAME challenge and strategy of flavonoid administration, we observed very similar effects for (−)-epicatechin supplementation. Essentially, (−)-epicatechin administration prevented the increases in SBP, superoxide anion production and p47$^{phox}$ expression, observed in the heart from L-NAME treated rats [27]. In line with these results, both (−)-epicatechin and quercetin prevented high-fat diet induced changes in mice [53]. Also in rats fed a high-fructose diet, (+)-catechin, a stereoisomer of (−)-epicatechin, and quercetin attenuate adipose inflammation [54]. In humans, administration of (−)-epicatechin or quercetin led to more complex results depending on the individuals under study. In patients with 125–160 mmHg SBP, both flavonoids decreased soluble endothelial-selectin, a marker of endothelial dysfunction, but only quercetin decreased inflammation markers [55]. In healthy men, only (−)-epicatechin improved parameters of insulin resistance [56]. Interestingly, no effects of (−)-epicatechin or quercetin were observed on the NO related parameters evaluated in these human studies. The similarities between (−)-epicatechin and quercetin actions can be explained considering the high equivalence in the chemical structures of these flavonoids. In addition, based on the current understanding of quercetin and (−)-epicatechin metabolism [57–60], it can be speculated that the ultimate effectors are chemically unique molecules, e.g. hippuric acid, or very structurally similar metabolites generated by host or microbiota metabolism [61].

## 4. Conclusions

In summary, this study shows that L-NAME leads to changes in oxidant production and NO bioavailability that can be prevented by quercetin administration. Quercetin effects would be mediated by the modulation of the expression and/or activity of specific proteins (NOX), resulting in a controlled oxidant production that favors NO bioavailability. Given the relevant role of NO in maintaining heart physiology, and the implication of oxidative stress in several unhealthy conditions, e.g. inflammation and high BP, the observed effects of quercetin appear as highly relevant. The fact that quercetin is commonly present in human diets underlines its health actions within the cardiovascular benefits associated to the high consumption of fruits and vegetables.

## Declarations of interest

None.

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