

General oxidative stress during doxorubicin-induced cardiotoxicity in rats: absence of cardioprotection and low antioxidant efficiency of alpha-lipoic acid.

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After two months, body weight was higher in DOX-AL group than in DOX (+16%), but was due to ascites. Histological liver alterations were observed in DOX and DOX-AL groups. Plasma ROOH concentrations decreased after 10 days of AL treatment, but were greater in DOX and DOX-AL groups. After two months, a decrease (-27%) in cardiac contractility index and a cardiac hypertrophy were observed in DOX and DOX-AL. These dysfunctions were associated with 1) a reduction in plasma ascorbate levels and an increase in ascorbyl/ascorbate ratio and 3) an increase in cardiac tissue MDA and O₂^{•-} content.

In conclusion, a cumulative dose of 10 mg/kg doxorubicin induced liver and heart functional alterations associated with plasma and cardiac oxidative stress. The co-administration of the antioxidant compound AL seemed to accentuate hepatic dysfunction.

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Dijon, October 27th 2010

Dear Editor,

On behalf of my co-authors, I am submitting the enclosed material for possible publication in BIOCHIMIE.

"General oxidative stress during doxorubicin-induced cardiotoxicity in rats: absence of cardioprotection and low antioxidant efficiency of alpha-lipoic acid."

S. Ghibu, S. Delemasure, C. Richard, J.-C. Guillard, L. Martin, L. Rochette and C. Vergely

The manuscript, or part of it, neither has been published (except in the form of abstract or as part of a published lecture or academic thesis) nor is currently under consideration for publication by any other journal.

All the co-authors have read the manuscript and approved its submission to BIOCHIMIE. Tacitly or explicitly, the publication is approved by the responsible authorities where the work was carried out. If this article is accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language without the written consent of the copyright-holder.

Yours sincerely,



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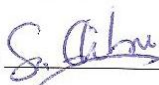
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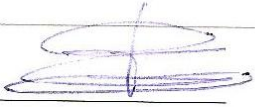
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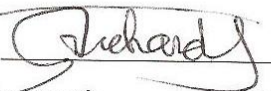
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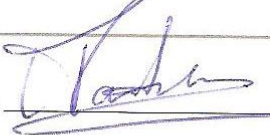
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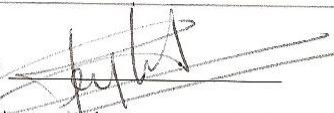
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Author's own work

Abstract:

1 To evaluate the effects of alpha-lipoic acid (AL) in a model of doxorubicin (DOX)-
2 induced cardiotoxicity, male Wistar rats were treated with DOX (1mg/kg/d; 10d) in addition
3 or not with AL (50mg/kg/d; 15d). Plasma oxidative stress was determined by hydroperoxides
4 (ROOH) and ascorbyl radical/ascorbate ratio. Two months after, heart's functional parameters
5 were determined *in vivo* by catheterization and cardiac oxidative stress by malonedialdehyde
6 (MDA) and O₂^{•-} (dihydroethidium fluorescence) tissue content.
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12 After two months, body weight was higher in DOX-AL group than in DOX (+16%),
13 but was due to ascites. Histological liver alterations were observed in DOX and DOX-AL
14 groups. Plasma ROOH concentrations decreased after 10 days of AL treatment, but were
15 greater in DOX and DOX-AL groups. After two months, a decrease (-27%) in cardiac
16 contractility index and a cardiac hypertrophy were observed in DOX and DOX-AL. These
17 dysfunctions were associated with 1) a reduction in plasma ascorbate levels and an increase in
18 ascorbyl/ascorbate ratio and 3) an increase in cardiac tissue MDA and O₂^{•-} content.
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25 In conclusion, a cumulative dose of 10 mg/kg doxorubicin induced liver and heart
26 functional alterations associated with plasma and cardiac oxidative stress. The co-
27 administration of the antioxidant compound AL seemed to accentuate hepatic dysfunction.
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35 **Keywords:** doxorubicin, alpha-lipoic acid, rat hearts, oxidative stress, cardioprotection,
36 anthracyclines
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Highlights:

- Doxorubicin induces cardiotoxicity via oxidative stress-mediated injury
- Lipoic acid and its metabolite dihydrolipoic acid are potent antioxidants both in vitro and in vivo
- Some antioxidants may exert pro-oxidant capacities in certain conditions
- Lipoic acid was unable to decrease oxidative stress in doxorubicin-treated rats
- Moreover lipoic acid seemed to accentuate cardiac and hepatic injury

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4 **General oxidative stress during doxorubicin-induced cardiotoxicity in rats:**
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6 **absence of cardioprotection and low antioxidant efficiency of alpha-lipoic acid**

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31 **Running title:** Lipoic acid and doxorubicin cardiotoxicity

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35 **Conflict of Interest Statement:**

36
37 The authors declare to have no personal, financial or other relationships with other people or
38 organizations within 3 years of beginning the work submitted that could inappropriately
39 influence, or be perceived to influence, the work submitted.
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54 *Abbreviations* : AFR, ascorbyl free radical; AL, alpha-lipoic acid; ANOVA, analysis of variance;
55 AU, arbitrary units; DHE, dihydroethidium; DHLA, dihydrolipoic acid; DOX, Doxorubicin;
56 ESR, electron spin resonance; HR, heart rate; Ht, hematocrit; LVDP, left ventricular developed
57 pressure; LVEDP, left ventricular end-diastolic pressure; LVESP, left ventricular end-systolic
58 pressure; O₂^{•-}, superoxide anion; ROOH, hydroperoxides; TBARs, thiobarbituric acid reactive
59 substances
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1. Introduction

Doxorubicin is an anthracycline anti-neoplastic drug used in the treatment of a wide range of solid tumors and of leukemia in children and adults. Despite its good therapeutic results, the clinical use of doxorubicin during chemotherapy is limited by the development of acute, sub-acute, early chronic or late chronic cardiotoxicity [1-2]. Cardiac alterations become more obvious several years after the end of the treatment and may occur as a dilated cardiomyopathy leading to congestive heart failure [3-6].

Despite a large amount of research on this subject, the molecular mechanisms involved in chronic anthracycline cardiotoxicity remain a major topic of discussion. Several aspects of this phenomenon, such as apoptosis, alteration of iron and calcium homeostasis have been described but the exact mechanism has not yet been fully understood. Indeed, oxidative stress is believed to represent an important pathway involved in the cardiac side-effects induced by anthracycline therapy [7-9]. It is widely known that superoxide anion free radical ($O_2^{\bullet-}$) is generated during the “redox cycling” of anthracycline or after oxido-reduction processes taking place inside the anthracycline-iron complex. Then, $O_2^{\bullet-}$ is driven to hydroxyl radical (HO^{\bullet}) in the presence of transition metals or forms peroxynitrite ($ONOO^-$) in the presence of nitric oxide ($^{\bullet}NO$), both of them being strong oxidants which can induce cellular injury [9-11].

Along with preventive measures implemented to limit anthracycline-induced cardiotoxicity [4, 12], several antioxidants molecules have been tested for their potential cardioprotective effects in different experimental models in the last forty years. Despite some beneficial results of vitamin E, vitamin A, N-acetyl cysteine, probucol [13], carvedilol, glutathione, superoxide dismutase, catalase, none of these antioxidants have proved to be of a clear benefit to humans [14-15]. The only drug presently approved for use in clinical practice to reduce cardiac side-effects of anthracyclines is dexrazoxane (Cardioxane[®]) [15-17], a prodrug of ethylenediamine tetra-acetic acid which chelates intracellular iron [15, 18-19].

In this context, it seems interesting to evaluate the possible cardioprotective effects of alpha-lipoic acid (AL). Alpha-lipoic acid or thioctic acid (6,8-dithio-octanoic) is a thiol compound with antioxidant properties which can be found in plants and animals. AL is able to scavenge free radicals (HO^{\bullet} , $HClO$, 1O_2), chelate transition metals (iron and copper) or regenerate oxidized forms of antioxidants (vitamin E, vitamin C and glutathione) [20-21]. AL is both a water- and lipid-soluble molecule, property which allows it to concentrate in cellular and

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4 extracellular environments. Exogenous AL is rapidly absorbed from the diet, and is reduced
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6 intracellularly to dihydrolipoic acid (DHLA), the most active form of the substance [22-23]. Its
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8 low negative redox potential (0.32 V) gives to AL/DLHA a strong reducing character. A number
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10 of studies have reported the beneficial effects of AL in *diabetes mellitus*, insulin resistance [24],
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12 diabetic neuropathy [25-26], vascular inflammation [27-28], erythrocytes membrane stability [29]
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14 and neurodegenerative diseases [23, 30], all these effects being associated with its antioxidants
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16 properties. However, few recent studies have mentioned the pro-oxidant potential of AL and
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18 DHLA depending on the type of oxidative stress and the physiological conditions [31-32].

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20 In this general context, the aims of our work were: 1) to create a model of chronic
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22 cardiotoxicity induced by doxorubicin in rats, 2) to confirm the physiopathological role of
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24 oxidative stress in cardiac injury and 3) to assess the impact of a treatment with alpha-lipoic acid
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26 (AL) in these experimental conditions.

2. Materials and methods

2.1. *Animals and experimental protocol*

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36 The local ethic committee approved the experimental protocol and the investigators
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38 complied with authorization 6007 from the French government, which agrees with the EC
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40 Directive 86/609:EEC for animal experiments. For the purpose of our study, 72 male Wistar rats
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42 (Charles-River, L'Abresle, France; 300-350 g at the beginning of the experiment) were divided
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44 into 4 groups.

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46 In control group (C, n = 18), rats received saline solution: 1 ml/kg/day intraperitoneally
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48 (i.p.) during 15 days; from day -5 (D-5) to day 9 (D9, Fig. 1).

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50 In the doxorubicin-treated group (DOX, n = 18), rats were injected i.p with 1 mg/kg/day
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52 doxorubicin (Adriamycin®, Pfizer, Paris, France) during 10 days, from day 0 (D0) to D9. A total
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54 dose of 10 mg/kg doxorubicin was administered over a 10-days period (Fig. 1).

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56 In the lipoic acid-treated group (AL), rats were injected i.p. with 50 mg/kg/day alpha-
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58 lipoic acid (Thiogamma 600®, Wörwarg Pharma, Germany) during 15 days, from D-5 to D9. A
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60 total dose of 750 mg/kg lipoic acid was administered over a 15-days period (Fig. 1).

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4 In the last group (DOX-AL, n = 18), rats were treated with both doxorubicin and alpha-
5 lipoic acid at the same dose as the two previous groups. During the firsts 5 days, the rats were
6 injected with alpha-lipoic acid (50 mg/kg/day), then was given a combined therapy: doxorubicin
7 (1 mg/kg/day) and alpha-lipoic acid, over 10 days (Fig. 1).
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11 The rats were sacrificed one month (D40; Series I, n = 36) or two months (D70; Series II,
12 n = 32) after the end of the treatment. Each series was composed of 4 groups of animals (C: n =
13 9; DOX: n = 9; AL: n = 9; DOX-AL: n = 9) (Fig. 1).
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16 The body weight of rats was monitored daily during the period of treatment (D-5 to D9), then
17 every 10 days. Moreover, food and water consumption was determined. The hematocrit (Ht) and
18 plasma hydroperoxides concentration were evaluated over the all period of the study.
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22 At the end of the study (D40; D70) the volume of abdominal ascites was measured and
23 heart functional parameters were evaluated *in vivo* by left ventricular catheterization. Liver tissue
24 samples were withdrawn for further histological analysis.
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28 29 30 2.2. *Heart functional parameters measured in vivo*

31 The rats were anaesthetized with sodium thiopental (60 mg/kg, i.p.) and heparinized (500
32 IU/kg). After thorax depilation, a catheter connected to a pressure transducer, was inserted into
33 the left ventricle through chest wall in order to measure heart rate (HR) and left ventricular
34 pressures (left ventricular end-diastolic pressure: LVEDP, left ventricular systolic pressure:
35 LVSP, left ventricular developed pressure: LVDP = LVSP-LVEDP) during the first minute of
36 cardiac catheterization. Left ventricle contractility was expressed as +dP/dt and left ventricle
37 relaxation as -dP/dt.
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44 At the end of the study the blood was taken by cardiac puncture, centrifuged and the
45 plasma was immediately frozen into liquid nitrogen. Hearts were excised, segmented and frozen.
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49 50 2.3. *Blood/plasma determinations*

51 2.3.1. Hematocrit (Ht)

52 The hematocrit (Ht, or erythrocyte volume fraction) of rats was measured before the injection of
53 doxorubicin or alpha-lipoic acid (D-5), at the end of the treatment (D10) and every 15 days
54 during period of rat's observation. For that purpose, a small quantity of blood was collected from
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4 the tail in a heparinized capillary tube and centrifuged to determine the proportion from blood
5 volume that is occupied by red blood cells.
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9 10 2.3.2. Plasma lipid peroxidation

11 *Determination of plasma hydroperoxides using the “FORT” test.*

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13 The “FORT” test is a colorimetric test based on the ability of transition metals such as iron, to
14 catalyze in the presence of hydroperoxides (ROOH), the formation of free radicals (ex: RO•,
15 ROO•) which are then trapped by a chemically amine derivative (CrNH₂). This amine reacts with
16 free radicals forming a stable colored cation radical (CrNH₂^{•+}) which absorbance is measured at
17 505 nm. The intensity of color is in direct correlation with the amount of free radical compounds
18 (Beer Lambert Law) and, consequently, the oxidative level of the sample analyzed [33].
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24 Hydroperoxides were measured in the plasma at D-5, D0, D10, D40 and D70.
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28 *Determination of Thiobarbituric Acid Reactive Substances (TBARs).*

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30 Measurement of plasma lipid peroxides was done by a colorimetric reaction with
31 thiobarbituric acid. 1.5 ml of trichloroacetic acid/thiobarbituric acid/hydrochloric acid solution
32 was added to 500 µL of plasma. The color of thiobarbituric acid pigment was developed in a
33 water bath at 100°C for 15 mn. After cooling with ice to room temperature, 1 ml of 70%
34 trichloroacetic acid was added. After 1h 30, tubes were centrifuged and color of TBARs layers
35 were measured at 553 nm. The absorbance values were compared with a standard curve. Results
36 were expressed in µmoles/g proteins; plasma protein was determined according to the method of
37 Bradford [29]
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44 Plasma TBARs were assessed only at the end of the study (D40; D70).
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48 2.3.3. Plasma ascorbyl free radical (AFR) determinations by electron spin resonance 49 (ESR) spectroscopy

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51 35 µL of plasma samples were inserted into a quartz capillary tube which was placed in a
52 HS cavity in order to analyze them at room temperature with a Bruker EMX-100 X-band
53 spectrometer (Wissembourg, France). The following parameters were selected for optimal
54 detection of AFR[34]: modulation frequency: 100 kHz, amplitude modulation: 0.8 G, microwave
55 power: 40 mW, microwave frequency: 8.5 GHz, conversion time: 40 ms, time constant: 327 ms,
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4 scan time: 41 s, gain: 5.10^5 , number of scans: 6. The height of AFR signal intensity was
5 measured and expressed in arbitrary units (AU).
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9 10 2.4. *Tissue determinations*

11 2.4.1. Determination of cardiac Thiobarbituric Acid Reactive Substances (TBARs)

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13 Measurement of heart lipid peroxides was done by a colorimetric reaction with thiobarbituric
14 acid. Hearts were homogenized in ice-cold phosphate buffered saline (0.05 M, pH 7). Then, 1.5
15 ml of trichloroacetic acid/thiobarbituric acid/hydrochloric acid solution was added. The color of
16 thiobarbituric acid pigment was developed in a water bath at 100°C for 15 mn. After cooling to
17 room temperature with ice, 1 ml of 70% trichloroacetic acid was added. After 1h 30, tubes were
18 centrifuged and color of TBARs layers were measured at 553 nm. The absorbance values were
19 compared with a standard curve. Results were expressed in $\mu\text{M/g}$ cardiac tissue.
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27 2.4.2. Superoxide Production by Fluorescence Histology

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29 In the presence of superoxide, ethidine, a fluorescent compound, is formed from
30 dihydroethidium (DHE) and thus allows quantifying superoxide production. Frozen heart tissues
31 were fixed for 10 mn in acetone. Slides were incubated in a light-protected humidified chamber
32 at room temperature with DHE ($5 \mu\text{M}$) for 5 mn. Slides were immediately analyzed with a
33 computer-based digitizing image system (Microvision, France) using a fluorescent microscope
34 (Eclipse 600, Nikon, France) connected to a video camera (TriCCD, Sony, France). Fluorescence
35 was detected at 590 nm and carried forward to the nuclear number. Results were expressed in
36 fluorescence intensity/nuclear number.
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45 46 2.5. *Statistical analysis*

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48 All data are expressed as means \pm S.E.M. To compare the groups, at 1 month or at 2
49 month after the end of treatment, statistical analyses were performed with the one-factor analysis
50 of variance (ANOVA) test (SigmaStat); ANOVA was followed, if necessary, by a Newman
51 Keuls test. To compare the evolution of parameters during the all period of study (period of
52 treatment and period of rat's observation) we used a two-factor repeated measures analysis of
53 variance (ANOVA) test (SigmaStat). Significance was established at a value of $P < 0.05$.
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3. Results

Two rats of DOX-AL group died during the observational period (at day 26 and day 30 after the end of the treatment). A very high volume of abdominal ascites fluid was observed in these dead rats.

3.1. *Body weight and food consumption in rats*

Controls and lipoic acid-treated rats have gained in weight during all period of study (period of treatment and period of rat's observation). Doxorubicin induced a significant loss of rats' body weight starting from the 3rd day of treatment. Lipoic acid associated with doxorubicin treatment was not able to counter this body weight loss. After interruption of treatment, there was a trend towards recovery of body weight in rats from DOX and DOX-AL groups with kinetics identical to these of rats from Control and AL groups (Fig. 2A).

As might be expected, we found in our study that rats' body weight and food consumption followed approximately the same trend (Fig. 2B). Regarding the quantity of water drunk, no significant differences were observed among the four groups of animals except during the 1st and 4th days after the end of the treatment where animals treated with doxorubicin drunk a lower quantity of water than control rats (data not shown).

Two months after the end of the treatment (D70) there was a greater body weight recovery in rats of DOX-AL group compared with DOX group but without increase of food consumption (Fig. 2A, 2B). However, it appears later that body weight gain in DOX-AL group was explained by the presence of a larger volume of abdominal ascites fluid (Table 1).

3.2. *Abdominal ascites fluid and liver histological analysis*

We noted that the volume of ascites fluid accumulated 1 month (D40) and 2 months (D70) after the end of the treatment was significantly higher in rats treated with doxorubicin alone or combined with alpha-lipoic acid (DOX -AL). It was also observed that the abdominal ascites fluid was greater in the DOX-AL group than in the DOX group (Table 1). Histological analysis showed the presence of condensation and sinusoidal dilation in liver tissues of rats treated with doxorubicin alone or in combination with lipoic acid (Table 2).

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4 3.3. *Heart weight, heart weight to body weight ratio*

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6 Two months (D70) after the end of the treatment, doxorubicin alone or associated with
7 alpha-lipoic acid induced a significant decrease of heart weight as compared to this of control or
8 AL groups (Fig. 3A). Heart to body weight ratio, which estimates cardiac hypertrophy, was
9 significantly ($P < 0.05$) increased in DOX group (Fig. 3B).
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15 3.4. *Cardiac parameters evaluated in vivo by left ventricle catheterization*

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17 Heart rate (HR) was not influenced by doxorubicin treatment at D40 and D70. Left
18 ventricular developed pressure (LVDP), +dP/dT and -dP/dT were not modified 1 month after the
19 end of treatment (D40) but at D70, we observed in animals treated with doxorubicin alone (DOX)
20 or in combination with alpha-lipoic acid (DOX-AL) a significant decrease ($P = 0.034$) of +dP/dT
21 (Table 3).
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27 3.5. *Blood/plasma parameters*

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29 3.5.1. Hematocrit (Ht)

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31 As shown in Figure 4, a cumulative dose of 10 mg/kg of doxorubicin over 10 days (D10)
32 induced a decrease in Ht values and the emergence of anemia in rats of DOX and DOX-AL
33 groups. This anemia persisted 15 days (D25) after the end of the treatment. Subsequently during
34 the period of animal's surveillance, Ht values were similar in the four groups of rats.
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40 3.5.2. Plasma hydroperoxides (ROOH) concentration determined by the "FORT" test

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42 Ten days of treatment with alpha-lipoic acid (50 mg/kg/day) induced a significant
43 decrease of plasma hydroperoxides in AL group as compared to control group: (1.36 ± 0.08 vs.
44 2.44 ± 0.41 mmol/l H_2O_2 , $P < 0.05$), which was noticeable only after 5 days of treatment in both
45 AL-treated groups. A cumulative dose of 10 mg/kg of doxorubicin lead to an increase of ROOH
46 in both DOX and DOX-AL groups in comparison to AL group. One month after the end of the
47 treatment (D40), the rate of plasma hydroperoxides remained higher in animals treated with
48 doxorubicin, but not 2 months after the end of the treatment (Fig. 5).
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4 3.5.3. Plasma concentration of Thiobarbituric Acid Reactive Substances (TBARs)
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6 Two months after the end of the treatment, the plasma TBARs was significantly higher in
7 the DOX-AL group as compared to Control and AL groups; while only a tendency ($P = 0.059$) to
8 increase TBARS values in DOX group was observed (Table 4).
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13 3.5.4. Ascorbate plasma concentrations
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15 One month after the end of the treatment, the plasma concentration of ascorbate was
16 significantly reduced in DOX-AL group as compared to AL and Control groups. Two months
17 after the end of the treatment (D70) the decrease of plasma concentrations of ascorbate was more
18 evident in both groups of rats treated by doxorubicin: DOX and DOX-AL and associated with a
19 significant increase of AFR to ascorbate ratio (Table 4).
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26 3.6. *Tissue parameters*
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28 3.6.1. Heart tissue TBARs
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30 Two months after the end of the treatment, there was a significant (+50%, $P < 0.01$)
31 increase of TBARs the heart of rats from DOX and DOX-AL groups (Table 3) compared with the
32 values of C or LA heart tissue samples.
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37 3.6.2. Heart tissue superoxide anion production
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39 The production of superoxide anion ($O_2^{\bullet-}$) assessed with DHE, revealed a higher oxidative
40 stress in cardiac tissues of rats treated with doxorubicin alone (DOX) 1 month after the end of the
41 treatment. Two months after the end of the treatment, the heart tissue production of $O_2^{\bullet-}$ was
42 significantly higher in both groups of rats treated with doxorubicin (DOX, 9.10 ± 0.18 and DOX-
43 AL, 9.40 ± 0.19) as compared to Control (4.6 ± 0.10) or AL (4.7 ± 0.1) groups (Table 4).
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4. Discussion

The administration of a cumulative dose of 10 mg/kg doxorubicin induced a decrease in rats' body weight, associated with reduced food consumption. After the end of the treatment, during the two months of experimental design, a body weight and ingested food recovery was observed but did not reach values of Control group. Treatment with alpha-lipoic acid had no effect on the doxorubicin-induced anorexia. It is noteworthy that for rats treated with doxorubicin in combination with alpha-lipoic acid the higher weight recovery was not due to food ingestion but was secondary to a larger accumulation of ascites fluid, revealed and measured during rats' euthanasia. The presence of ascites fluid could be the consequence of cardiac, renal or liver impairment [35-36]. In our situation the hepatic and cardiac alterations were more obvious, as revealed by a higher degree of histological alterations in the liver of rats treated with doxorubicin and an alteration of heart contractility.

Besides anorexia, doxorubicin induced an anemia that persisted 15 days after the end of the treatment; subsequently, the hematocrit values returned to normal. The anemia is a frequent side-effect reported after chemotherapy.

Doxorubicin alone or combined with alpha-lipoic acid induced a decrease in heart weight 2 months after the end of treatment, happening sooner in rats treated with doxorubicin alone. This phenomenon has been described in other different studies [37-38] and could be explained by cardiomyocytes' apoptosis [39-41]. The heart to body weight ratio, a cardiac hypertrophy index, was increased 2 months after treatment in DOX group. In DOX-AL group this ratio was not significant, probably because of "artificial" increase of body weight due to ascites accumulation.

Cardiac functional parameters, measured in vivo in our experimental conditions, were affected 2 month after the treatment by a cumulative dose of 10 mg/kg doxorubicin, with a lower left ventricular contractility (+dP/dt) in DOX and DOW-AL hearts, inducing a non significant reduction of PDVG and of left ventricular relaxation index (-dP/dt). As was already observed in our laboratory[42], the impairment of contractility is a very late event and is only at its beginnings two months after a cumulative dose of 10 mg/kg while the general condition of rats is so dramatically deteriorating that it threatens life and leads to high mortality. Sacco et al [37] have shown that a total cumulative dose of 7.5 mg doxorubicin does not affect PDVG but decreases +dP/dt 13 weeks after the end of treatment. In fact, the heart contractility deterioration could become more evident after cardiac β -adrenergic stimulation with isoprenaline [37].

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4 In our experimental conditions, the addition of alpha-lipoic acid to doxorubicin treatment
5 did not prevent neither cardiac alteration nor degradation of general physical condition. Al-Majed
6 et al [43] noted a cardioprotective effect of alpha-lipoic acid 48 hours after doxorubicin
7 administration. While alpha-lipoic acid combined with doxorubicin might provide some
8 protection few days after doxorubicin treatment, there are no studies to confirm or reject this
9 cardioprotective effect at long term after the end of chemotherapy.
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15 Regarding the assessment of plasma and cardiac oxidative stress, the methods used in this
16 work were various and complex, each of them having some characteristics of sensitivity and
17 specificity. Plasma lipid peroxidation was assessed as 1) plasma hydroperoxides concentration
18 evaluated during the treatment and in the moment of rats' euthanasia and 2) plasma
19 malonedialdehyde (MDA) concentration measured at the time of rats euthanasia (1 and 2 months
20 after the end of treatment). We noted a significant decrease of hydroperoxides plasma levels after
21 a cumulative dose of 250 mg/kg alpha-lipoic acid (5 days of treatment). This decrease was
22 maintained until the end of treatment (15 days of treatment with a cumulative dose of 750 mg/kg
23 alpha-lipoic acid) without evidence of a dose-effect relationship. In addition, a cumulative dose
24 of 10 mg/kg doxorubicin (10 days of treatment) induced an increase in plasma hydroperoxides;
25 the values were 2.5 times higher as compared to those obtained in AL group. These results are in
26 favor of an early free radical circulating stress during doxorubicin treatment. On the other hand, 2
27 months after the end of treatment, plasma TBARs were significantly higher in rats treated with
28 doxorubicin in combination with alpha-lipoic acid (DOX-AL group).
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41 To get a better evaluation of plasma oxidative stress it was necessary to evaluate the
42 levels of some antioxidant systems. Thus, we determined the plasma concentration of vitamin C
43 by HPLC and the plasma ascorbyl radical level by electron paramagnetic resonance (EPR)
44 spectroscopy. Two months after treatment, the plasma concentration of ascorbate was
45 significantly lowered in rats treated with doxorubicin alone or in combination with alpha-lipoic
46 acid. This phenomenon can be explained by a decrease in hepatic vitamin C synthesis and by an
47 excess of free radical species production as we previously reported [38].
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54 Evaluation of cardiac oxidative stress by TBARs or by ethidine (DHE) fluorescence
55 showed a significant increase of $O_2^{\bullet-}$ production and of the malonedialdehyde levels in DOX and
56 DOX-AL groups, 2 months after doxorubicin administration. In a similar study carried out in our
57 laboratory, the cardiac oxidative stress was not evident 8 days after treatment with a commutative
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4 dose of 10 mg/kg doxorubicin [38]. This could confirm that tissue oxidative stress is a late event
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6 in the onset of doxorubicin cardiotoxicity.

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8 It is important to note that, in our experimental conditions, the plasma or cardiac oxidative
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10 stress was not improved by the addition of alpha-lipoic acid to the doxorubicin treatment. Despite
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12 this, it is important to remember that alpha-lipoic acid alone significantly decreases the plasma
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14 hydroperoxides concentration but in combination with doxorubicin it had no beneficial effect.
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16 Contrary to our initial hypothesis, the results obtained regarding the mortality, the presence of
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18 high quantities of ascites and the hepatic alterations (morphologically and metabolically),
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20 indicate that the combination of doxorubicin with alpha-lipoic acid is rather deleterious than
21
22 beneficial on the parameters evaluated.

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24 In contrast to our results, some experimental studies showed that alpha-lipoic acid
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26 administered 24 h before the injection of adriamycin, once a week over a period of 10 to 12
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28 weeks, had a beneficial effect in renal [44-45] and testicular toxicity [46], without mentioning
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30 majors side-effects. The lack of alpha-lipoic acid efficiency in preventing doxorubicin
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32 cardiotoxicity found in our study could be due to an inhibition of compensatory mechanisms
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34 activated at the start of chemotherapy. Indeed, there is a long period of time where no
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36 modifications in oxidative stress status and in cardiac contractile parameters appear. This latent
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38 period without symptoms might be secondary to the involvement of compensatory mechanisms
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40 that are quite early triggered [35, 47]. It is not easy to evaluate the specific pharmacologic actions
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42 of an antioxidant. For instance, it is known that antioxidants can prevent the cardioprotective
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44 mechanisms triggered by ischemic preconditioning, whereas administration of free radical-
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46 generating systems may mimic ischemic preconditioning [48-50]. It is worth mentioning, though,
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48 that in our protocol, the administration of alpha-lipoic acid was made 5 days prior to the
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50 introduction of doxorubicin. The reactive oxygen or nitrogen species formed after the first doses
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52 of anthracyclines might induce cellular protective pathways that are unfortunately overwhelmed
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54 lately by the following administrations of anthracycline. Then, pre-treatment with an antioxidant
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56 such as alpha-lipoic acid might paradoxically reinforce the toxic effects of doxorubicin, not only
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58 on the oxidative stress levels but also on cellular injury. In addition, a possible negative
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60 interaction between the two substances could not be excluded. Further work is obviously needed
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62 to confirm one of these hypotheses, exploring more thoroughly the timing of doxorubicin and
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64 alpha-lipoic acid administration or the dosages used.
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4 **5. Conclusion**
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6 In conclusion, this study confirmed that the general state of animals is early altered by
7 doxorubicin treatment while the cardiac problems and the plasma and tissue oxidative stress are
8 more obvious two months after completion of the treatment. Contrary to our initial hypothesis,
9 the administration of alpha-lipoic acid in combination with doxorubicin was not followed by a
10 beneficial effect in the heart function or in the general oxidative stress, and led to a higher
11 degradation of the hepatic function (ascites fluid accumulation). The antioxidant “beneficial”
12 properties of alpha-lipoic acid appear clearly depending on the experimental conditions, and the
13 cardioprotective potential of some antioxidant molecules in the context of chemotherapy should
14 be carefully approached.
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4 **Legends to figures:**
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9 **Fig. 1:** Experimental protocol. In control group (C, n = 18), rats received saline solution: 1
10 ml/kg/day intraperitoneally (i.p.) during 15 days; from day -5 (D-5) to day 9. In the doxorubicin-
11 treated group (DOX, n = 18), rats were injected i.p with 1 mg/kg/day doxorubicin during 10 days,
12 from day D0 to D9. In the lipoic acid-treated group (AL), rats were injected i.p. with 50
13 mg/kg/day alpha-lipoic acid during 15 days, from D-5 to D9. In the last group (DOX-AL, n =
14 18), rats were treated with both doxorubicin and alpha-lipoic acid at the same dose as the two
15 previous groups. Rats were sacrificed one month (D40) or two months (D70) after the end of the
16 treatment with doxorubicin or saline, and hematocrit (Ht) was periodically measured.
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31 **Fig. 2:** Evolution of body weight [A] and food consumption [B] in control group (C), alpha-lipoic
32 acid group (AL), doxorubicin group (DOX) and doxorubicin + alpha-lipoic acid group (DOX-
33 AL) during all period of study (*P<0.05, **P<0.01, ***P<0.001: DOX and DOX-AL vs. C and AL;
34 &&P<0.01: DOX vs. C and AL; †P<0.05: DOX-AL vs. C and AL; ‡P<0.05 ††P<0.01: DOX-AL vs.
35 DOX).
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45 **Fig. 3:** Heart weight [A] and heart to body weight ratio [B] in control group (C), alpha-lipoic acid
46 group (AL), doxorubicin group (DOX) and doxorubicin + alpha-lipoic acid group (DOX-AL), 2
47 months (D70) after the end of the treatment.
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55 **Fig. 4:** Hematocrit evolution during all period of study, in control group (C), alpha-lipoic acid
56 group (AL), doxorubicin group (DOX) and doxorubicin + alpha-lipoic acid group (DOX-AL)
57 (*P<0.05, **P<0.01: DOX and DOX-AL vs. C and AL).
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Fig. 5: Plasma hydroperoxides (ROOH) evolution during all period of study in control group (C), alpha-lipoic acid group (AL), doxorubicin group (DOX) and doxorubicin + alpha-lipoic acid group (DOX-AL).

Table 1: Abdominal ascites fluid determined 1 month (D40) and 2 months (D70) after the end of the treatment in control group (C), alpha-lipoic acid group (AL), doxorubicin group (DOX) and doxorubicin + alpha-lipoic acid group (DOX-AL) (**P<0.01, ***P<0.001: DOX and DOX-AL vs. C and AL).

Number of rats which have developed abdominal ascites in each group and volume of ascites (ml).		
	1 month	2 months
C	0/9 0.32 ± 0.08	0/9 0.23 ± 0.02
AL	0/9 0.31 ± 0.04	0/9 0.30 ± 0.03
DOX	8/9 6.52 ± 2.67 ^{***}	9/9 1.01 ± 0.23 ^{**}
DOX-AL	7/7 19.16 ± 3.87 ^{***}	9/9 57.47 ± 24.6 ^{***}

Table 2: Histological analysis of liver tissues collected 2 months (D70) after the end of the treatment in control group (C), alpha-lipoic acid group (AL), doxorubicin group (DOX) and doxorubicin + alpha-lipoic acid group (DOX-AL)

	Histological tissue modifications					
	Condensation	Necrosis	Sinusoidal dilation	Inflammation	Neo-ductules	Thrombosis
C	0/2	0/2	0/2	1/2	0/2	0/2
AL	0/2	0/2	0/2	1/2	0/2	0/2
DOX	2/2	1/2	2/2	0/2	0/2	0/2
DOX-AL	2/2	0/2	2/2	0/2	0/2	0/2

Table 3: Heart parameters evaluated *in vivo* by left ventricle catheterization in control group (C), alpha-lipoic acid group (AL), doxorubicin group (DOX) and doxorubicin + alpha-lipoic acid group (DOX-AL), 1 month (D40) and 2 months (D70) after the end of the treatment (*P<0.05: DOX and DOX-AL vs. C)

	1 month				2 months			
	C	AL	DOX	DOX-AL	C	AL	DOX	DOX-AL
HR (beats/mn)	350 ± 13	357 ± 12	361 ± 20	376 ± 14	343 ± 7	326 ± 7	302 ± 26	357 ± 23.29
LVDP (mmHg)	119 ± 5	123 ± 9	128 ± 9	108 ± 13	116 ± 7	106 ± 6	94 ± 7	86 ± 15
+dP/dt	3823 ± 149	3756 ± 344	3855 ± 300	3413 ± 435	3643 ± 266	3302 ± 163	2674 ± 277*	2583 ± 487*
-dP/dt	3419 ± 173	3483 ± 447	3705 ± 435	3231 ± 502	3403 ± 305	3113 ± 191	2475 ± 315	2330 ± 530

Table 4: Plasma and cardiac oxidative stress parameters evaluated 1 month (D40) and 2 months (D70) after the end of the treatment in control group (C), alpha-lipoic acid group (AL), doxorubicin group (DOX) and doxorubicin + alpha-lipoic acid group (DOX-AL) (*P<0.05, **P<0.01: DOX and DOX-AL vs. C; †P<0.05, ††P<0.01: DOX and DOX-AL vs. AL)

	1 month				2 months			
	C	AL	DOX	DOX-AL	C	AL	DOX	DOX-AL
Plasma TBARs (μmoles/g protein)	0.020 ± 0.001	0.022 ± 0.001	0.024 ± 0.002	0.027 ± 0.003	0.022 ± 0.001	0.023 ± 0.001	0.027 ± 0.001	0.034 ± 0.003*
Ascorbate (μM)	43.7 ± 4.1	42.8 ± 2.8	38.1 ± 4.7	27.0 ± 4.6*	42.4 ± 3.6	39.3 ± 4.9	22.6 ± 2.1***††	22.9 ± 5.0***††
AFR/Ascorbate (AU)/(μM)	0.063 ± 0.003	0.065 ± 0.007	0.080 ± 0.019	0.084 ± 0.006	0.051 ± 0.004	0.054 ± 0.004	0.073 ± 0.006*	0.071 ± 0.008*
Tissular TBARs (μM/g cardiac tissue)	19.6 ± 1.9	16.1 ± 1.4	19.4 ± 1.8	21.9 ± 2.4	13.9 ± 1.4	15.9 ± 1.6	21.7 ± 3.3***††	26.1 ± 2.2***††
DHE 10⁴ (% fluorescence/nucleus)	3.10 ± 0.74	2.70 ± 0.74	7.90 ± 0.25*	4.90 ± 0.68	4.60 ± 0.10	4.70 ± 0.11	9.10 ± 0.18*†	9.40 ± 0.19*†

Figure 1

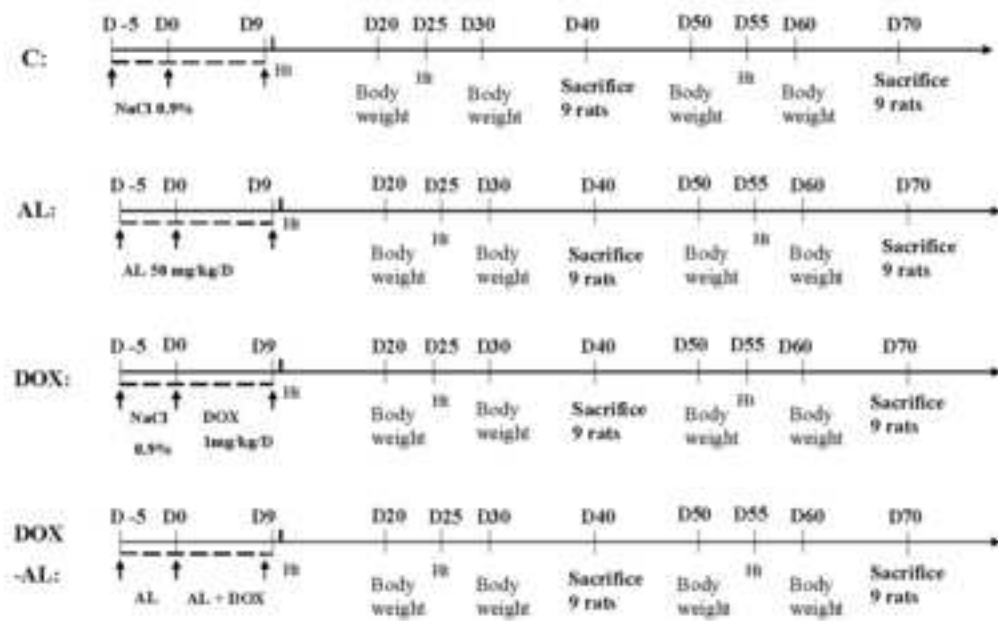


Figure 2

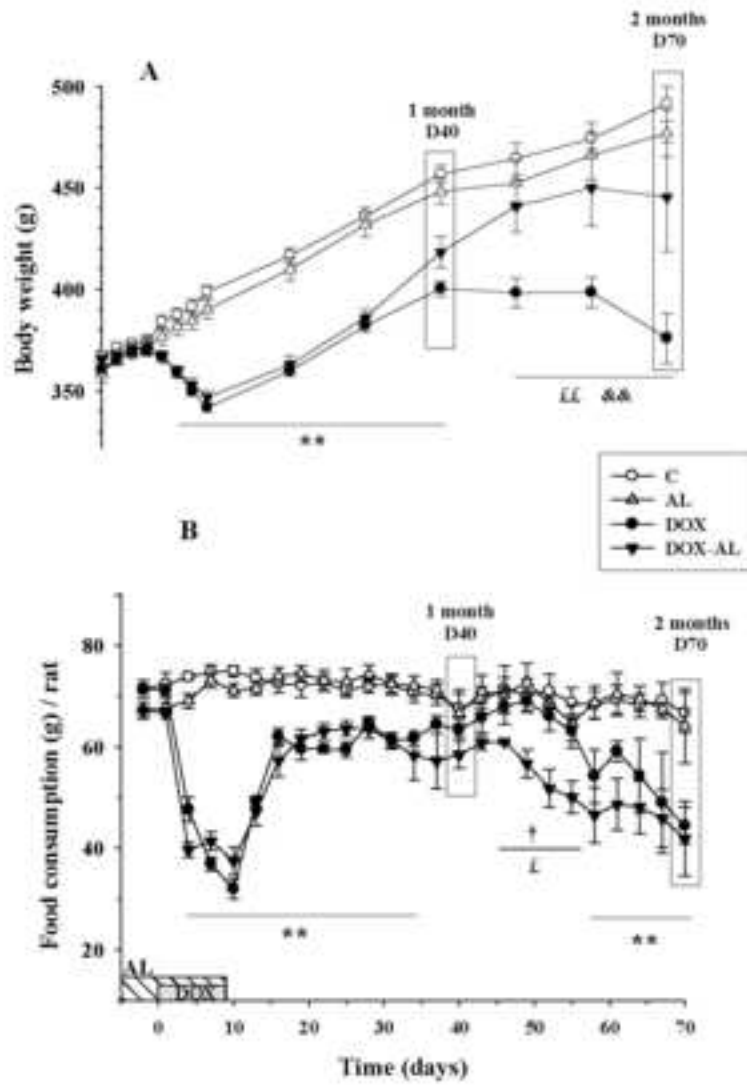


Figure 3

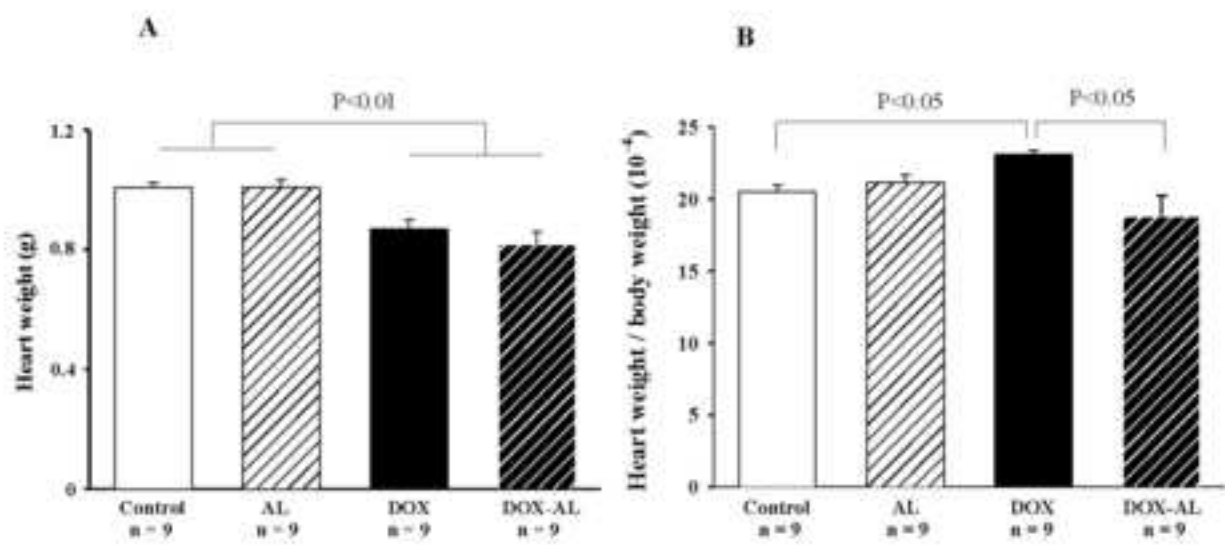


Figure 4

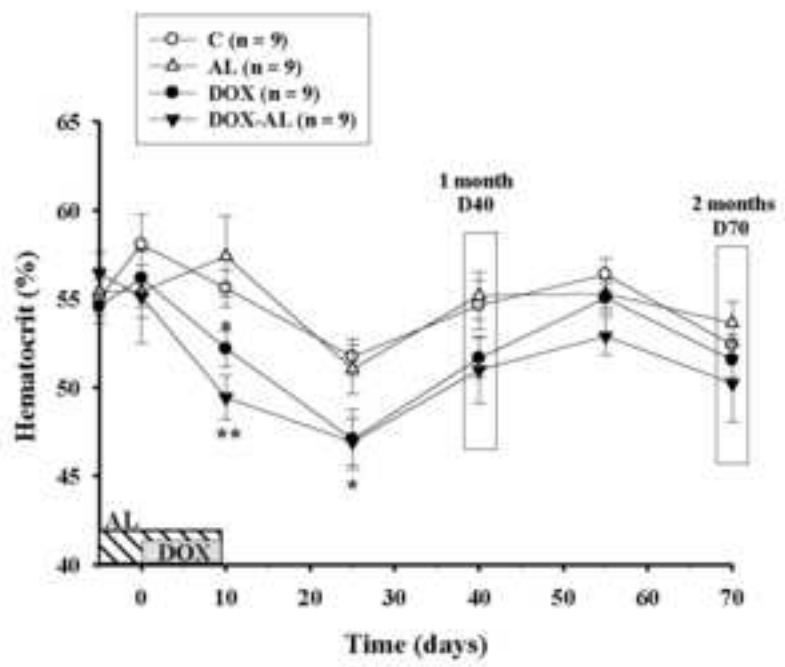


Figure 5

