

ANTIOXIDANT ACTIVITY OF BOL D'AIR JACQUIER® BREATHING SESSIONS IN WISTAR RATS — FIRST STUDIES

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Abstract

Objectives: The Bol d'Air Jacquier® is used to create a molecule able to deliver oxygen at the cellular level to manage hypoxia due to environmental pollution, ageing, or inflammatory disease. This study was designed to determine, firstly, whether the device generated oxidative stress and, secondly, whether it might induce an antioxidant effect. **Material and methods:** Over a period of 62 weeks, 10 male Wistar rats were randomized into two groups: the Bol d'Air group (BA) regularly breathed peroxidizing terpenes delivered by the device and the control group breathed water vapour during 9-min sessions, at the frequency of 1–12 per month. Several antioxidant compounds and KRL levels were determined in the blood and major organs. **Results:** The results showed that the two groups did not differ with respect to the organ concentrations of Cu,Zn SOD, GPx, GSH, GSSG and TBARS. The device might have a weak slimming effect over time. The BA group presented a significantly higher GR level in plasma throughout the experiment, and in the muscle at the end of the study. In the BA group, the plasma Cu,Zn SOD level was related to the number of breathing sessions per week before blood collection. The BA group also had a higher KRL antioxidant status compared to the control group at two different time-points: at the onset of the study, in the blood of young rats; and after three breathing sessions per week, in the blood and RBCs of old rats. **Conclusions:** The device did not generate oxidative stress and seemed to produce global antioxidant effect depending on the number of sessions per week, especially in old rats.

Key words:

Global and specific antioxidant approaches, Peroxidizing terpenes

INTRODUCTION

Oxidative stress is induced by a wide range of environmental factors including hyperoxia: the cells growing at low oxygen concentrations exhibit a prolonged life span [1], whereas those growing at high concentrations have a reduced duration of life [2]. More recently, many researchers have pointed out that hypoxia engendered considerable stress, which triggered a survival program by which the cells attempt to adapt to the new environment by activating factors (like hypoxia-inducible factor) to stimulate angiogenesis, glycolysis and erythropoiesis [3].

The aim of the research conducted by René Jacquier was to find a means of providing balanced oxygenation to healthy and sick people, regardless of the degree of environmental and physical pollution. In 1946, he developed the first “Bol d'Air Jacquier®” device [4]. This device generates peroxidizing volatile terpenes of *Pinus pinaster*. The terpenes are the typical constituents of conifer resin synthesized from isoprene [5] and are worth studying because of their bactericidal and fungicidal activity [6,7]. Moreover, the peroxidizing form is supposed to add some more properties to the terpenes [4]: during the peroxidation, the double bonds of α and β pinenes (main components of volatile terpenes) are broken

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and molecular oxygen is added. These new molecules are inhaled and pass through the alveoli into the blood where they fix onto haemoglobin. For René Jacquier, the oxygen was in its tetravalent form at that moment (this specific oxygen molecule is quoted in Grignard [8] and Vincent et al. [9]). The unstable compound comprising haemoglobin, pinenes and oxygen is supposed to deliver oxygen to the cells more easily than does haemoglobin [10].

The damaging or toxic effects of oxygen are related to the formation and release of reactive oxygen species (ROS), such as superoxide, hydroxyl radical or hydrogen peroxide. The importance of oxidative damage in the pathogenesis of many diseases (including those of the brain, neural tissue, and the cardiovascular system) as well as in the degenerative processes of ageing has become increasingly apparent since the mid 1950s [11]. The main cellular components susceptible to ROS-induced damage are lipids (peroxidation of polyunsaturated fatty acids in membranes), carbohydrates, nucleic acids and proteins (denaturation or glycation). The major antioxidant mechanisms used by the cells (superoxide dismutase (SOD), catalase, the glutathione system) regulate the formation of these radicals until the oxygen load overwhelms the enzymes, leading to detrimental effects on the cells [12].

The present study was conducted to evaluate, firstly, whether the Bol d'Air Jacquier® generated oxidative stress, and, secondly, whether it could have a protective antioxidant effect. To accomplish this, we measured the levels of TBARS and of the main antioxidants: SOD and glutathiones.

When evaluating the total antioxidant capability of an organism, it is impossible to perform a complete analysis of all the antioxidants due to the large number of molecules that play, or can play a role. It is also difficult to understand exactly all of the chemical reactions involved in the cellular defence as well as the interactions between them [13]. Moreover, we know that an antioxidant cannot distinguish between the radicals that have a physiological function and those that cause damage [14], and a substance that is generally accepted as an antioxidant may possess a prooxidant activity under certain conditions [15]. For example,

a statistically significant increase in SOD activity represents a general alteration of the oxidative processes characteristic of dementia, which supports the proposal that the enzyme could be used as an early diagnostic marker of Alzheimer's disease [16]. Ciriolo et al. [17] pointed out that the levels of antioxidant enzyme activity differed considerably in various parts of the same organ (brain).

We thus need another, more global point of view on the antioxidant status that would include evaluation of the cell's capability to resist oxidative stress. The Spiral Test (Kirial SA Couternon, France) is a good evaluation method, which makes it possible to assess the ability of total blood and red blood cells (RBCs) to withstand the free-radical-induced haemolysis [18–20]. Indeed, we know that when the cells are subjected to an oxidative-type stress with free radicals, they activate all their enzymatic and molecular capacities to resist this aggression until the cell membrane or wall is modified to the point of allowing the cell contents to escape. During the oxidative challenge, the time of resistance depends on the cell resistance imparted by the enzymatic and molecular properties of the cells [18]. Several studies demonstrated that the higher the KRL level, the greater the antioxidant capability of the organism [18,21–23]. This method may be applied to evaluate the antioxidant status of body organs [24,25].

In our study, it was used in an experiment on Wistar rats to compare those breathing Bol d'Air Jacquier® with a control group breathing water vapour.

MATERIAL AND METHODS

Animals and diet

10-week-old male Wistar rats ($n = 10$) weighing $265 \text{ g} \pm 16.5$ were purchased from Dépré (F-18230 Saint Doulchard). All the animals were caged in groups of five per cage and kept in a room with a 12 h light/dark cycle, at 24°C average room temperature and 60% air humidity. The rats were maintained on a standard-diet laboratory rat chow (Lab-blocks, from Scientific Animal Food & Engineering (SAFE) F-89290 AUGY). Food and tap water were available ad libitum. Vitamin and mineral salt supplementation complied with respective recommendations [26]. Animal weights were recorded weekly.

We followed the general guidelines for the care and use of laboratory animals recommended by the Council of European Communities [27].

Treatment

The rats were randomized into two groups of five rats. One group regularly breathed the peroxidizing terpenes delivered by the Bol d'Air Jacquier® device (BA group) and the other breathed water vapour delivered by a modified device (control group). The experiment was conducted over a period of 62 weeks.

Blood was collected from the tips of the tails into 75 µl sodium heparinized haematocrit capillary tubes (Hirschmann Laborgeräte, Germany) under local anaesthesia (EMLA® cream, Astrazeneca Lab.). At the beginning of the experiment, blood was drawn at T0, T15 min (after the first breathing session of 15 min) and T24h (hours). Then, the rats were subjected to 9-min breathing sessions, from 1 to 12 per month, and blood was regularly collected at T3W (weeks), T5W, T12W, T17W, T21W, T26W, T31W, T45W from 3 to 8 days after the previous breathing session.

At the end of the experiment (T62W), the rats were not deprived of food and no Bol d'Air® session had been performed for 21 days. They were anesthetized and killed with Isoflurane (Aerrane®, Baxter SA BP 56 78310 Maurepas Cedex, France -0.1 ml/kg). Blood was collected into 10 ml tubes containing 500 µl of ethylene diamine tetra-acetate 2% (EDTA), and major organs of the rats were preserved by freezing at -20° C.

Plasma was obtained by low-speed centrifugation (1600 g for 20 min). Protein content was assessed with the bicinchoninic acid assay (Sigma, l'Isle d'Abeau Chesnes, France), using serum albumin as the standard.

Lipid peroxidation markers (TBARS)

Lipid peroxidation products were determined as the thiobarbituric acid reactive substances (TBARS) in plasma, as described by Quintanilha et al. [28], and in organs, using the method by Ohkawa et al. [29]. TBARS levels were determined by the spectrophotometric method, using malondialdehyde as the standard. The results are expressed as

nanomoles of TBARS per liter of plasma, and expressed as micromoles per gram of protein in organs.

Measurement of antioxidant enzyme activity (SOD, GPx and GR), GSH (reduced glutathione) and GSSG (total glutathione)

We measured SOD1 activity at 412 nm by testing the degree of inhibition of nitrite formation [30]. Glutathione peroxidase (GPx) was determined after Plagia and Valentine [31], using cumene hydroperoxide as the substrate. Glutathione reductase (GR) was determined after Goldberg and Spooner [32]: GR catalyses the reduction of GSSG in the presence of NADPH which is oxidised to NADP+. The decrease in absorbance is measured at 340 nm. Enzymatic activity in erythrocytes was expressed as units per milligram of haemoglobin and in tissue as units per gram of protein. All enzyme activity was adapted to microplate titration using IEMS microplate titrator (KRL Reader, Kirial SA, F-Couternon).

The GSH in erythrocytes and tissue was measured following the procedure of Anderson [33] and using reduced glutathione as the standard. GSSG concentrations were determined after Jocelyn [34].

Total antioxidant capability of whole blood, red blood cells and plasma

The total antioxidant capability of whole blood was measured by monitoring the rate of cell haemolysis (KRL TM test), using a microplate titrator according to the method by Prost [18,21].

Whole blood and washed red blood cells (RBCs) were diluted (v:v 1:25 and 1:50) with KRL buffer (300 mosm/l), and 50 µl of whole blood or RBC suspension was assayed using a 96-well microplate coated with a free radical generator (GRL 400, Kirial SA, Couternon, France). The kinetics of sample resistance to haemolysis was determined by monitoring the changes at 620-nm absorbance, at 37°C. The value used as the standard and reference was the time of half-hemolysis (T1/2 haemolysis), and was expressed in minutes.

Another application of this method is determining plasma antioxidant status by incubating rat plasma samples to test

their total antioxidative scavenging capability. Control blood from a Wistar rat and plasma from all experimental rats were diluted in the KRL buffer (300 mosm/l, v:v standard blood 1:25 and plasma 1:12). Then 50 μ l of standard blood and 50 μ l of plasma were pooled onto the microplate coated with a free radical generator GRL.

Total antioxidant capability of organs

By determining the total antioxidant status of the organs, it is possible to assess the capability of the system to withstand oxidative stress [25].

Rat organs were defrosted, washed in 150 mmol/l of NaCl, homogenized in a Potter Elvehjem apparatus (VWR International, Fontenay-sous-Bois, France) with 0.1 mol/l of phosphate buffer at pH 7.4 (heart: 100 mg/2 ml — leg striated muscle: 100 mg/ml — liver: 100 mg/5 ml — brain 100 mg/0.5 ml — kidney: 100 mg/10 ml — adipose tissue: 100 mg/0.2 ml) and centrifuged for 10 min at 3000 g. A 96-well microplate coated with a free radical generator (Kirial SA, Couternon, France) was re-hydrated with KRL buffer (120 μ l), and then 50 μ l of supernatant was added to 50 μ l of the control RBCs (100 μ l/5 ml of KRL buffer). Control RBCs were prepared under the same conditions as the experimental RBCs. The kinetics of hemolysis was determined at 540 nm absorbance, at the temperature of 37°C. The final results were converted to EAR (Equivalent Antioxidant Capacity) and expressed as units per milligram of protein [35].

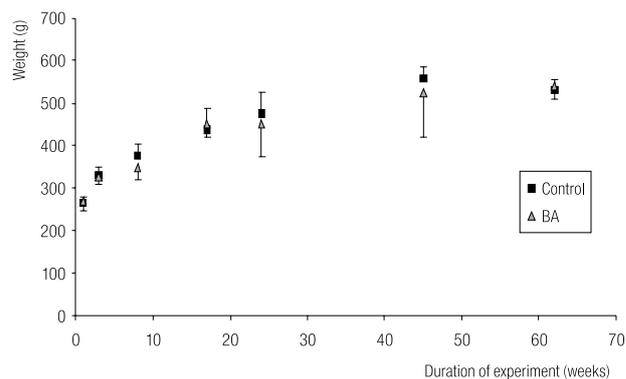
Statistical analysis

Statistical analysis of data was carried out using Statistica 5® (Statsoft, Tulsa, OK, USA) software. All data are presented as means \pm standard deviation (\pm SD). The data were tested by one-way and/or two-way analysis of variance (Anova, Manova). The difference was considered statistically significant at $p < 0.05$.

RESULTS

Average body and organ weight

As shown in Figure 1, there was no difference in body weight between the two groups: control group: 451.73 g \pm 67.6 and BA group: 444.26 g \pm 55.21 ($p = 0.80$), but the two-



Over a total period of 62 weeks, 10 male Wistar rats were randomized into two groups: the Bol d'Air® group (BA — grey triangle) regularly breathed peroxidizing terpenes delivered by the Bol d'Air® device, and the control group (Control — dark square) breathed water vapour, during 9-min sessions, at the frequency of 1–12 sessions per month. Animal weights were recorded weekly. Values are means \pm SD; $n = 5$ /group from T0 to T45W (week 45 of experiment), and $n = 4$ /group from T45W to end of experiment.

Fig. 1. Evolution of rat body weight throughout the experiment (week 1-62). Influence of BA treatment.

-way analysis of variance showed that the Bol d'Air® may have had a weak slimming effect throughout the experiment ($p = 0.04$ for both variables, group and time).

No difference was found between the two groups (control group vs. BA group) with respect to organ weight: brain — 1.98 g \pm 0.1/1.88 g \pm 0.9; heart — 1.25 g \pm 0.2/1.34 g \pm 0.1; kidney — 1.45 g \pm 0.2/1.49 g \pm 0.1 and liver 15.80 g \pm 1.7/17.27 g \pm 0.97.

Antioxidant level in major organs

As shown in Table 1, organ levels of Cu/Zn SOD did not differ between the BA and control group, even though a slightly higher level was found in the BA group at T45W. There was no statistical difference between the BA and control group with respect to the RBC Cu, Zn-SOD during the experiment.

As shown in Table 2, no statistically significant difference in RBC Cu,Zn-SOD was noted in the control group when the number of breathing sessions before blood collection was analyzed (Table 2). In the BA group, the greater was the number of breathing sessions, the higher the level of SOD.

Table 1. Superoxide dismutase (SOD) levels in organs at the end of experiment and in RBC during the experiment. Influence of BA treatment

| Organs | SOD for organs (U/g protein) at the end of experiment (week 62) | |
|----------------|---|------------------------------|
| | Control | BA |
| Liver | 19.47±15.2 | 12.91±5.4 ^{NS} |
| Heart | 0.15±0.01 | 0.17±0.03 ^{NS} |
| Brain | 7.36±3.13 | 8.72±2.93 ^{NS} |
| Kidney | 754.73±550.2 | 987.16±532.5 ^{NS} |
| Adipose tissue | 0.11±0.03 | 0.11±0.001 ^{NS} |
| Muscles | 1.44±0.09 | 1.82±0.52 ^{NS} |
| Times | SOD for RBC (U/mg Hb) during the experiment | |
| | Control | BA |
| T21W | 1160.23±654.8 | 530.52±394.5 ^{NS} |
| T27W | 522.28±247.9 | 494.33±158.7 ^{NS} |
| T31W | 574.20±524.3 | 304.51±252.2 ^{NS} |
| T45W | 690.51±481.8 | 1494.55±1076.7 ^{NS} |
| T62W | 797.5±540.8 | 604.1±355.95 ^{NS} |

From T0 to T62W (week 62 of experiment), 10 male Wistar rats were randomized into two groups: the Bol d’Air® group (BA) regularly breathed peroxidizing terpens delivered by the Bol d’Air® device, and the control group (Control) breathed water vapour, during 9-min sessions. Blood was regularly collected at T21W (week 21 of experiment) to T45W from 3 to 8 days after, and 21 days after the previous breathing session at the end of the experiment. We measured SOD1 activity at 412 nm by testing the degree of inhibition of nitrite formation [27]. SOD activity in tissue is expressed as units per gram of protein and in erythrocytes as units per milligram of hemoglobin. Values are means ±SD; n = 5/group from T0 to T45W (week 45 of experiment) and n = 4/group from T45W to end of experiment. NS — non-significant, S — significant.

Table 3. Glutathione peroxidase (GPx) levels in major organs at the end of experiment and in RBC during the experiment. Influence of BA treatment

| Organs | GPx (U/g tissue at the end of experiment (T62W)) | |
|----------------|--|------------------------------|
| | Control | BA |
| Liver | 1432.29±267.3 | 1377.29±235.9 ^{NS} |
| Heart | 313.34±42.5 | 362.88±57.1 ^{NS} |
| Brain | 74.80±9.7 | 86.41±21.6 ^{NS} |
| Kidney | 4262.99±213.6 | 4579.02±275.5 ^{NS} |
| Adipose tissue | 133.53±22.4 | 221.57±89.2 ^{NS} |
| Muscles | 5389.00±4778.4 | 7166.49±3250.4 ^{NS} |
| Times | GPx for RBC (U/mg Hb) during the experiment | |
| | Control | BA |
| T21W | 1559.47±65.4 | 1496.31±132.6 ^{NS} |
| T27W | 1815.36±148.2 | 1884.43±113.1 ^{NS} |
| T31W | 1920.07±57.7 | 1962.81±91.2 ^{NS} |
| T45W | 4056.30±3014.3 | 3005.12±768.4 ^{NS} |
| T62W | 6025.25±940.8 | 5391.04±979.4 ^{NS} |

For description of experiment see legend to Table 1. Blood was regularly collected at T21W (week 21 of experiment) to T45W from 3 to 8 days after, and 21 days after the previous breathing session at the end of experiment. Glutathione peroxidase (GPx) was determined after Plagia and Valentine [31], using cumene hydroperoxide as the substrate. GPx activity in tissue is expressed as units per gram of protein, and in erythrocytes as units per milligram of hemoglobin. Values are means ±SD; n = 5/group from T0 to T45W (week 45 of experiment) and n = 4/group from T45W to end of experiment. NS — non-significant, S — significant.

Table 2. Superoxide dismutase (SOD) levels in blood from T12W to T62W in the control and BA groups, by the number of breathing sessions before blood collection. Influence of BA treatment

| Number of sessions before blood collection | SOD for RBC (U/mg HB during the experiment) | | | |
|--|---|---------------|-----------------|-------------|
| | Control | | BA | |
| 0.5 | | 797.46±540.80 | 604.07±355.90 | |
| | | p = 0.88 | | p = 0.60 |
| 1 | p = 0.77 | 752.23±555.20 | 443.12±283.50 | p = 0.02* |
| | | p = 0.83 | | p = 0.0011* |
| 3 | | 690.51±481.80 | 1494.55±1076.70 | |

For description of experiment see legend to Table 1. Blood was regularly collected after 0 to 3 sessions per week from T21W (week 21 of experiment) to T62W. SOD1 activity was measured at 412 nm by testing the degree of inhibition of nitrite formation [30]. SOD1 activity in erythrocytes is expressed as units per milligram of hemoglobin. Values are means ±SD, n = 4 per group for S0.5 [0.5 session/week], n = 15 per group for S1 [1 session/week] and n = 4 per group for S3 [3 sessions/week] — * p < 0.05 was considered statistically significant. NS — non-significant, S — significant.

Table 4. Glutathione reductase (GR) levels in major organs at the end of experiment and in RBC during the experiment. Influence of BA treatment

| GR (U/g tissue at the end of experiment (T62W)) | | |
|---|------------------|--------------------------------|
| Organs | Control | BA |
| Liver | 3658.00±121.7 | 3476.38±389.7 ^{NS} |
| Heart | 6720.46±3859.7 | 3475.31±741.0 ^{NS} |
| Brain | 77.86±15.6 | 91.08±20.5 ^{NS} |
| Kidney | 40489.10±33155.3 | 64954.02±29232.5 ^{NS} |
| Adipose tissue | 1049.57±325.4 | 1942.73±688.7 ^{NS} |
| Muscles | 212.21±743.5 | 5942.12±1899.3 ^{S*} |
| GR for RBC (U/mg Hb) during the experiment | | |
| Time | Control | BA |
| T21W | 237.81±23.5 | 218.69±12 ^{NS} |
| T27W | 255.91±60.9 | 298.78±120.4 ^{NS} |
| T31W | 247.21±17.0 | 253.99±16.6 ^{NS} |
| T45W | 624.87±97.7 | 818.77±197.9 ^{S*} |
| T62W | 125.6±36.9 | 106.16±32.8 ^{NS} |

For description of experiment see legend to Table 1. Blood was regularly collected at T21W (week 21 of experiment) to T45W from 3 to 8 days after, and 21 days after the previous breathing session at the end of the experiment. Glutathione reductase (GR) was determined after Goldberg and Spooner [32]. GR activity in tissue is expressed as units per gram of protein, and in erythrocytes as units per milligram of hemoglobin. Values are means ±SD; n = 5/group from T0 to T45W (week 45 of experiment) and n = 4/group from T45W to end of experiment.

* p < 0.05 BA vs. Control.

NS — non-significant, S — significant.

Table 5. Reduced glutathione (gsh) and oxidized glutathione (GSSG) levels in major organs at the end of experiment (T62W). Influence of BA treatment

| Organs | GSH (U/g protein) | | GSSG (U/g protein) | | GSH/GSSG | |
|----------------|-------------------|---------------------------|--------------------|---------------------------|-------------|---------------------------|
| | Control | BA | Control | BA | Control | BA |
| Liver | 8.20±1.3 | 8.40±0.5 ^{NS} | 4.17±0.6 | 4.73±0.3 ^{NS} | 1.96±0.1 | 1.77±0.1 ^{NS} |
| Heart | 3.29±0.5 | 2.75±0.6 ^{NS} | 1.78±0.05 | 2.01±0.3 ^{NS} | 1.85±0.3 | 1.43±0.5 ^{NS} |
| Brain | 0.62±0.1 | 0.59±0.09 ^{NS} | 0.06±0.07 | 0.06±0.07 ^{NS} | 18.16±11.4 | 15.07±8.2 ^{NS} |
| Kidney | 7.37±0.5 | 7.83±0.3 ^{NS} | 2.70±0.2 | 2.74±0.1 ^{NS} | 2.72±0.1 | 2.86±0.1 ^{NS} |
| Adipose tissue | 0.05±0.005 | 0.11±0.08 ^{NS} | 0.14±0.01 | 0.29±0.2 ^{NS} | 0.38±0.009 | 0.39±0.004 ^{NS} |
| Muscle | 1.19±0.06 | 1.59±0.6 ^{NS} | 1.13±0.08 | 1.48±0.6 ^{NS} | 1.06±0.05 | 1.069±0.02 ^{NS} |
| RBC | 693.51±126.4 | 614.37±48.3 ^{NS} | 1080.30±150.8 | 959.42±88.2 ^{NS} | 639.84±57.2 | 641.07±19.3 ^{NS} |

For description of experiment see legend to Table 1. GSH in erythrocytes and tissues was measured after Anderson [33], using reduced glutathione as the standard. GSSG concentrations were determined after Jocelyn [34]. GSSG and GSH activity in tissue is expressed as units per gram of protein, and in erythrocytes, as units per gram of hemoglobin. Values are means ±SD; n = 4/group.

NS — non-significant, S — significant.

The data in Table 3 indicate that the GPx level in the main organs of BA rats was higher than in those of control rats, but not at a statistically significant level. The two-way analysis of variance revealed statistical significance for the duration effect (p = 0.0024), but not for the Bol d'Air® effect, or for both the effects combined.

As for GR, the Bol d'Air® significantly increased its level in the muscles (p = 0.005), but not in other organs (Table 4). For each blood analysis, there was no difference between the two groups, except at T45W (p = 0.037), and the two-way analysis of variance showed that the Bol d'Air® increased the GR level in blood with time throughout the experiment (p = 0.01).

The two groups did not differ with respect to GR/SOD, GPx/SOD and GPx/GR ratios in RBC or major organs during the experiment, except for the GR/SOD ratio in the muscles: control — 201.90±59.9; BA — 461.42±198.1 (p = 0.04). Likewise, there was no between-group difference in the GSH and GSSG levels and ratios in the organs (Table 5), or in the organ TBARS levels (Table 6).

Total antioxidative capability

When assessed with the KRL test, the total antioxidative capability of whole blood did not differ significantly between the two groups (p = 0.72), but the two-way analysis of variance showed that the Bol d'Air® may have

Table 6. TBARS levels in organs at the end of experiment (T62W). Influence of BA treatment

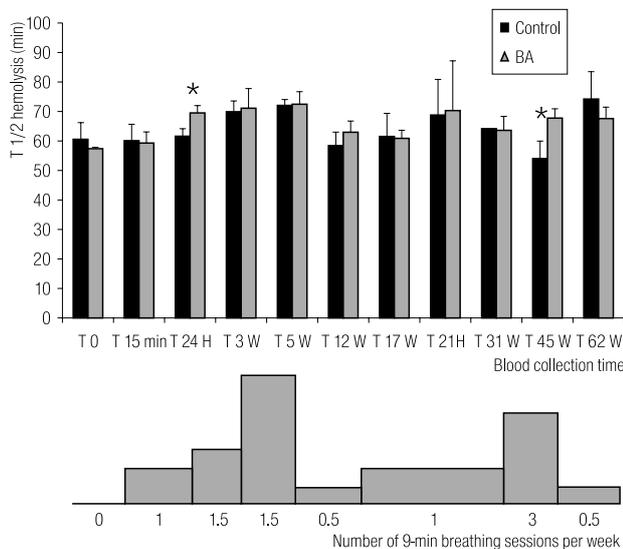
| Organs | TBARS (U/g protein) | |
|----------------|---------------------|----------------------------|
| | Control | BA |
| Liver | 118.99±103.9 | 34.41±11.4 ^{NS} |
| Heart | 98.04±11.9 | 130.94±46.1 ^{NS} |
| Brain | 324.63±69.3 | 347.83±53.4 ^{NS} |
| Kidney | 8.11±6.2 | 16.74±19.8 ^{NS} |
| Adipose tissue | 108.39±78.4 | 247.92±141.3 ^{NS} |
| Muscle | 10.58±3.9 | 10.16±1.9 ^{NS} |

For description of experiment see legend to Table 1. At the end of experiment, lipid peroxidation products in plasma and organs were determined as TBARS by reaction with thiobarbituric acid, after Ohkawa et al. [29]. TBARS activity in tissue is expressed as units per gram of protein.

Values are means ±SD; n = 4/group.

NS — non-significant, S — significant.

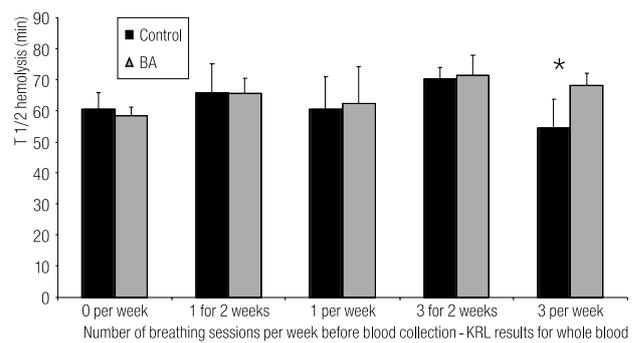
an antioxidative effect with time (p = 0.04) (Figure 2). Figure 2 also shows a significant antioxidative effect with the Bol d’Air Jacquier® at T24h and T45W.



For description of experiment see legend to Figure 1. Blood was regularly collected at T0, T15 min (after the first 15-min breathing session), T24h (hours), T3W (weeks), T5W, T12W, T17W, T21W, T26W, T31W, T45W from 3 to 8 days after, and 21 days after the previous breathing session. The total antioxidant capability of whole blood was measured using KRL test after Prost [18] and Blache and Prost [21]. Values are means ±SD; n = 5/group from T0 to T45W (week 45 of experiment) and n = 4/group from T45W to end of experiment.

*p < 0.05 BA vs. Control.

Fig. 2. Evolution of blood KRL antioxidant status in the control and BA groups by blood collection time. Influence of BA treatment.

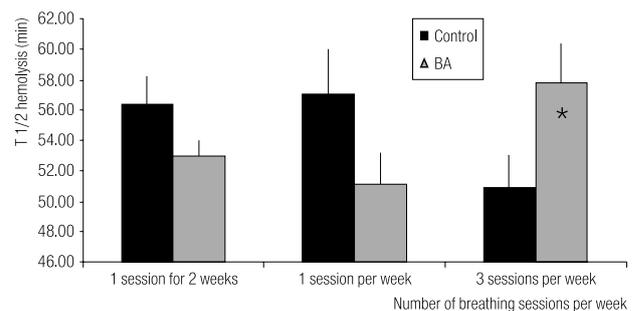


For description of experiment see legend to Figure 1. Blood was regularly collected at the beginning of experiment (no session), 1 session for 2 weeks (= 0.5 session/week), 1 session per week, 3 sessions for 2 weeks (= 1.5 sessions/week) and 3 sessions per week. The total antioxidant capability of whole blood was measured using KRL TM test after Prost [18] and Blache and Prost [21]. Values are means ±SD; n = 10 for S0, n = 9 for S0.5, n = 25 for S1, n = 5 for S1.5 and n = 4 for S3 and for each group.

* p < 0.05 BA vs. Control.

Fig. 3. Evolution of blood KRL antioxidant status in the control and BA groups by the number of breathing sessions per week before blood collection. Influence of BA treatment.

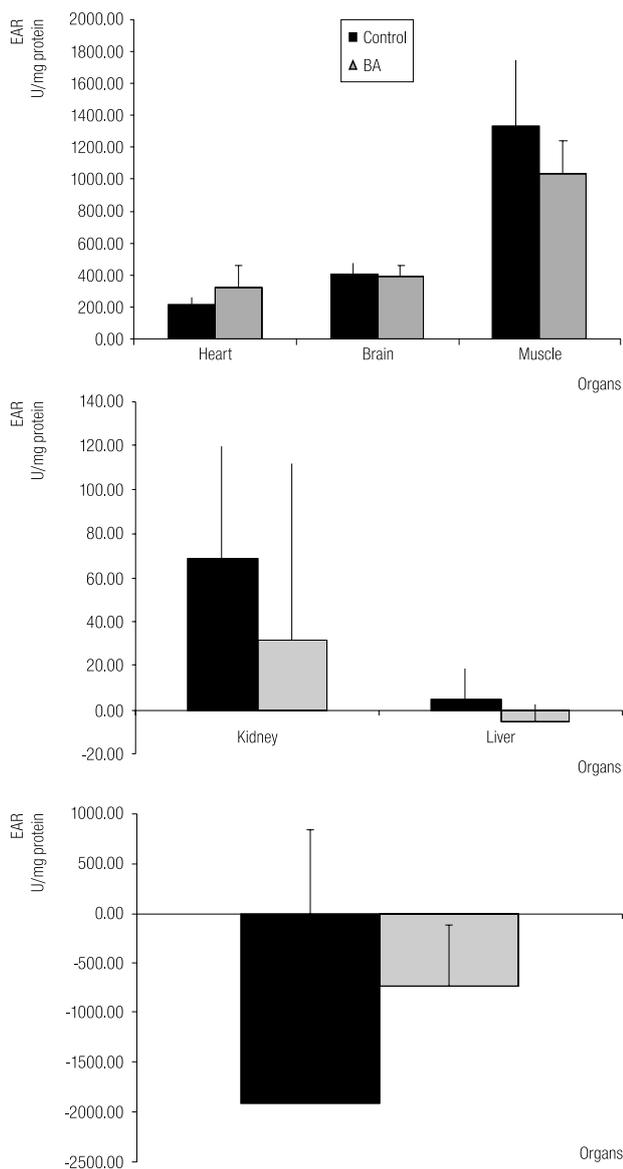
The analysis of the antioxidant status by the frequency of breathing sessions showed that the Bol d’Air Jacquier® might have a significant dose-response effect, depending on the number of BA sessions per week: group effect (BA vs. control): p = 0.0345; number of sessions per week effect: p = 4.8 10⁻⁹; combined group and session effect:



For description of experiment see legend to Figure 1. RBCs were collected after 1 session for 2 weeks (= 0.5 session/week), 1 session per week and 3 sessions per week at T27W (week 27 of experiment) to T52W (week 52 of experiment). Their total antioxidant capability was measured using KRL TM test after Prost [18] and Blache and Prost [21]. Values are means ±SD; n = 4/group from T27W to end of experiment.

* p < 0.05 BA vs. Control.

Fig. 4. Evolution of KRL antioxidant status for RBC in the control and BA groups by blood collection time. Influence of BA treatment.



For description of experiment see legend to Figure 1. At the end of the experiment, the rats were not deprived of food, and no Bol d'Air® session had been performed for 21 days. Total antioxidant capability of organ extracts and control RBCs was measured using KRL TM test after Prost [18] and Blache and Prost [21]. The final results were converted to EAR (Equivalent Antioxidant Capacity) and expressed as units per milligram of protein. Values are expressed as EAR units per milligram of protein. Values are means \pm SD; $n = 4$ /group.

Fig. 5. Organ antioxidant status in the control and BA groups. Influence of BA treatment.

$p = 0.0013$. More precisely, a high number of sessions per week had no significant influence among young and adult rats ($p = 0.53$) but, as shown in Figure 3, the frequency of three sessions a week had a significant effect on older rats ($p = 0.015$).

As regards the antioxidative capability of RBCs (see Figure 4), the global comparison between the two groups did not reveal any statistically significant difference ($p = 0.72$), but the two-way analysis of variance implied that the Bol d'Air Jacquier® may have an antioxidant effect depending on the duration of the experiment ($p = 0.04$). More precisely, the Bol d'Air Jacquier® had significant antioxidative effects at T45W, after 3 sessions per week over two months ($p = 0.03$).

Table 7 presents a synthesis of the evolution of data obtained in the study.

DISCUSSION

Average body and organ weight

In mammals, and especially in Wistar rats, ageing is associated with an increase in body weight due to the accumulation of adipose tissue [36]. The Bol d'Air Jacquier® might have had a weak effect on body weight, but not on organ weight. This finding could be related to the two following hypotheses:

- as suggested by some authors [37], the peroxidation of LDL lipoproteins is reduced by conifer resin terpenes;
- de Cristofaro's study [38] demonstrated that the improvement in oxygen availability at the tissue level due to the Bol d'Air Jacquier® fosters better use of the body's fatty mass. Unfortunately, it was impossible to evaluate the total fatty mass of the rats, because it was generally distributed throughout the body and difficult to isolate.

Antioxidant level in major organs

SOD

The enzymatic dismutation of superoxide $O_2^{\cdot-}$ radicals into H_2O_2 by the ubiquitous enzyme SOD was characterized by McCord and Fridovich in 1969 [39]. There are three forms of SOD. Among these, the copper- and zinc-containing superoxide dismutase (Cu,Zn-SOD) is widely distributed and accounts for 90% of the total SOD. This enzyme has great physiological significance and therapeutic potential [40]. In animal cells, the principal form, Cu,Zn-SOD, is localized primarily in cytoplasmic and nuclear compartments

Table 7. Evolution of all the measured antioxidant factors from T21W to T62W of the experiment. Influence of BA treatment

| Time | Data evolution during the experiment | | | | | | | | | |
|-------------|--------------------------------------|-----|---------|----|---------|-----|---------|----|---------|-----|
| | T21W | | T27W | | T31W | | T45W | | T62W | |
| Group | Control | BA | Control | BA | Control | BA | Control | BA | Control | BA |
| SOD | <-> | <-> | . | . | . | . | . | . | . | . |
| | | > | | > | | > | | < | | > |
| GPx | <-> | <-> | . | . | . | . | . | . | . | . |
| | | > | | < | | < | | < | | > |
| GR | <-> | <-> | . | . | <-> | . | . | . | . | . |
| | | > | | < | | < | | <* | | > |
| RBC KRL | <-> | <-> | . | . | . | . | . | . | . | . |
| | | > | | | | > | | <* | | > |
| Blood KRL | . | . | . | . | . | . | . | . | . | <-> |
| | | <-> | | | | <-> | | <* | | > |
| BA sessions | | | | | | | | | | |

For description of experiment see legend to Table 1. SOD1, GPx and GR activities, and the total antioxidant capability of RBCs and whole blood were evaluated using KRL TM test during the period from T21W to the end of experiment.

* $p < 0.05$ BA vs. Control.

[41], and to a lesser extent in lysosomes and the inter membrane space of the mitochondria [42].

The interpretation of SOD activity varies. For Kondo et al. [43], an increased Cu,Zn-SOD level might reflect increased generation of superoxide anions in the cytoplasm rather than in the mitochondria. This level is also higher for people exposed to stress such as in professional aerobic endurance training, which produces an increase in SOD levels in erythrocytes [44]. In mice, overexpression of Cu,Zn-SOD levels reduces lifespan, although probably by mimicking one of the effects of the Down's syndrome [45]. But sometimes, especially in the development of pulmonary diseases, the antioxidant responses are different: asbestosis and sarcoidosis lead to an increase in SOD, whereas no changes can be found in silicosis or hyperoxic lung injury [46]. As regards other aspects, Singh et al. [47] demonstrated that the brains of a group of rats treated with a herbomineral tonic Geriforte, which is used in the treatment of health problems in the elderly in India, showed significantly higher levels of SOD activity than those recorded in the control group.

In our study, the levels of Cu,Zn-SOD in the BA and control groups made it impossible to find out whether the Bol d'Air Jacquier® engendered oxidative stress or not, and a definitive conclusion might be related to other parameters.

Among the several types of SOD, the evaluation of Mn-SOD could be carried out in further studies: since the transcription factors involved in Mn-SOD induction are redox-sensitive, the oxidative stress may be an important factor in determining Mn-SOD expression at the transcriptional level [48,49]. Mn-SOD is a mitochondrial protein that is involved in the detoxification of superoxide radicals produced by the mitochondrial electron transport chains during the normal metabolic processes of the cell [50–52]. A reduction or a deficiency in Mn-SOD activity as well as Cu,Zn-SOD enzyme has been associated with several diseases, such as familial amyotrophic lateral sclerosis [53], ageing [54] and cancer [55,56].

The induction of Mn-SOD in animals appears to be a physiological process in response to environmental or pathological stress [57]. Ionizing radiation induced Mn-SOD gene expression as well as Mn-SOD activity in mouse heart tissues

and in human fibroblast cells [58,59]. It has also been demonstrated that many cytokines and chemical reagents can induce Mn-SOD expression, these including TNF (Tumor Necrosis Factor), IFN- γ (Interferon gamma), and IL-1 (Interleukin-1), in a variety of cell types [60,61]. Sometimes, increased Mn-SOD activity was not accompanied by an increase in other antioxidant defences, H₂O₂-scavenging enzymes [62] in particular, and even some drugs like cisplatin, decreased Cu,Zn-SOD while enhancing Mn-SOD activity [63]. According to Liu et al. [64], the induction of Mn-SOD expression by messengers like TNF- α is partially mediated by intracellular formation of oxygen free radicals, and superoxide is most likely the initiating species involved in the mediation of Mn-SOD gene expression. However, it also appears that IL-1 increasing Mn-SOD via NF- κ B is oxidant-independent [65], suggesting that the intracellular signalling pathways that lead to the induction of Mn-SOD transcription are regulated in a different way.

A more detailed research has to be conducted, and the knowledge of the possible effect of Bol d'Air Jacquier® on Mn-SOD regulation could lead to a better understanding of its impact on the cell regulation.

THE GLUTATHIONE SYSTEM

It is the central mechanism to reduce H₂O₂ and eliminate additional varieties of toxic peroxides [66]. Its deficiency contributes to oxidative stress [67], and the non-stressed cells maintain a high intracellular GSH/GSSG ratio to ensure the availability of GSH. The key enzyme in the redox cycle responsible for the reduction of H₂O₂ is GPx. This reaction specifically requires GSH to serve as the electron donor. The GSSG formed in the course of the reaction is subsequently reduced back to GSH by GR, which uses NADPH generated from the hexose monophosphate shunt system as an electron donor [68]. GSH, reacting with free radicals and organic peroxides serves as an antioxidant in amino acids transport and as a substrate for GPx and GSSG [69].

GPx and GR

Significant results were found for GR but not for GPx, and the GR rate was higher than that of GPx. This situation has already been reported by Bazzichi et al. [70] for humans.

Glutathione reductase levels were three times as high in patients with rheumatoid arthritis as in patients with osteoarthritis, but, according to the authors, this increased activity during the inflammatory process might lead to a protective, though insufficient, effect at the joint level in rheumatoid arthritis. Other authors observed a clear stimulation of GR activity in the skeletal muscles of *T. spiralis*-infected mice [71]. As reported by Dudley et al. [72], non-dystrophic mouse muscles subjected to an ischemia/reperfusion stress and the dystrophic mouse muscles showed a greater activity of the GSH-metabolizing enzymes GR (and also GPx). Thus, according to these findings, stress induces increased GR levels.

Yang et al. [73] pointed out that the GPx and GR activities are important in predicting the tissue redox state. They evaluated the activities of these enzymes in two cell lines with different susceptibilities to oxidative stress. The results suggested that low enzyme activity related to the low reducing power of NADPH/NADP(+) induced increased susceptibility to oxidative stress.

Thus, we might conclude that the enhanced GR levels in old Wistar rats after the Bol d'Air Jacquier® sessions are suggestive of an anti-oxidant effect by acting like a positive stress. In addition, given the close relationship between GR, NADPH/NADP(+) and G6PD, further research should be carried out to evaluate the impact of the device on glucose metabolism.

GSH/GSSG AND TBARS

Since there was no difference between the two groups with respect to GSH and GSSG levels and ratios in organs as well as in organ TBARS levels, the stress induced during the experiment may have had a truly beneficial effect. This could be proved by the evolution of the antioxidant status or in another series of experiments on lipid peroxidation. Lipid peroxidation products are formed with the abstraction of a hydrogen atom from an unsaturated fatty acid. Double bonds are rearranged to form dienes. An attack by molecular oxygen produces a lipid peroxyradical that can abstract a hydrogen atom from an adjacent lipid to form a lipid hydroperoxide. Some of these are

relatively transient, such as hydroxynonenol, while others appear later and accumulate, such as malondialdehyde [74–76]. Malondialdehyde (MDA) has been causatively implicated in the ageing process [77–80], as well as in atherosclerosis [81–86], Alzheimer's disease [87–88] and cancer [89].

Plasma MDA has thus been used as a biomarker of lipid peroxidation and has served as an indicator of free radical damage. When heated under acidic conditions, thiobarbituric acid (TBA) reacts with a number of chemical species (nucleic acids, amino acids, proteins, phospholipids, and aldehydes [90]) to produce a pink chromophore that can be measured by UV or fluorescence detection. These substances are termed TBARS (thiobarbituric acid reacting substances). Since Yagi [91] applied the TBA assay to estimate lipoperoxide concentrations in human serum, it has been a popular method for TBARS detection.

However, in recent years, this method has been subject to pervasive criticism on several grounds, due to the existence of a large range of non-lipid oxidation products in this system that also react with TBA to form colored species that can interfere with the assay. According to Draper et al., except for urine, spectrophotometric analysis overestimated MDA content. Purification of the TBA-MDA complex obtained from liver and fish meal on reverse phase cartridges was found to entail a loss of complex bound to residual peptides in the TCA (trichloroacetic acid) extract [92]. The question that arises is whether TBARS represent a lipid peroxidation product, or the final metabolites of numerous pathways, including lipid peroxidation and PUFAs metabolism [93].

Nevertheless, many researchers still consider TBARS the lipid peroxidation biomarkers, both in the animal experiments (fish [94] rats [95–96], dogs [97]), cells [98] and human studies [99].

Total antioxidative capability

The variations in KRL antioxidant status observed in the course of the experiment, that were mostly identical for both groups, could be related to the seasonal rhythms. Indeed, the circadian and seasonal rhythms in rodents are known to be related to an oscillating system supported

by the suprachiasmatic nuclei [100]. Prost et al. [101] and Narce et al. [102] reported such circadian and seasonal activities in mice and Wistar rats. Our results also pointed out a significant antioxidative effect with the Bol d'Air Jacquier® at T24h and T45W.

At a quiescent state, there is a balance between ROS production and cellular antioxidant capability. The disturbance of this balance, either by a lowered antioxidant protection status or by an increase in oxidizing stress, generates modifications in the redox state of the cells, relayed by modifications in the intracellular redox state. A light stress generally induces a physiological temporary imbalance, which is compensated in a very short time [103]. In this case, the organism meets the challenge with high, but short-lived increases in the antioxidant level.

In our study, the first BA breathing sessions acted like a one-off stimulus able to protect the organism by stimulating the antiradical defence for a very short time. In the follow-up of the experiment, the blood KRL antioxidant states in both the groups were much closer. At T45W, all the rats had been having three sessions a week for two months, and in the Bol d'Air Jacquier® group, the KRL antioxidant status for blood and RBC was significantly higher than in the control group. At that moment, the rats were old, and the protective effect was all the more interesting since some research has shown that old rats were more sensitive to oxidant attack, such as that induced by exposure to radicalizing ozone, than the young and adult ones [104].

The results obtained in the present experiment suggest the presence of a regulatory system for the total antioxidant defence capability, probably ensured by the antioxidant reserve components in the tissue. Considering what we already know on the regulation of glycaemia, this homeostasis of the global and circulating antioxidant level due to the formation of reserves has already been reported by Prost [105]. This system makes it possible to maintain a normal level of the total antioxidant capability. According to the quality of their adaptive system, and when the antioxidant defence level is high, the subjects store antioxidant compounds to maintain this homeostasis, or, on the contrary, release them when necessary.

The RBCs, whose life is around 100 days in mammals, are protected by this plasmatic regulation throughout their life, even though they directly undergo a negative impact when the total antioxidative capability is lower than what is normal for the species. From this point of view, the high KRL level for RBC, after three BA breathing sessions per week (T45W) might prove that the device really had a dose-dependent protective antioxidant effect.

In the case of pathologies (or ageing), the regulatory system can be affected and/or the reserves can fail, which may account for an abnormally high or abnormally low level of global and circulating antioxidants. This would explain the observed differences in the results for the young rats (T5W) and the old ones (T45W) after a high number of BA breathing sessions (see Figure 2): the possible protection generated by the Bol d'Air Jacquier® referred only to the ageing control rats. For young rats, the homeostasis system may act as a buffer against the possible impact of the device. Further research should be carried out in order to confirm these hypotheses.

From T45W to the end of the experiment, the rats had fewer breathing sessions per week and thus fewer activity disturbances like weighing etc. Three different effects were expected: a collapse of immune defence in BA rats (BA generated stress), or the maintenance of a high antioxidant rate, or, on the contrary, a reduction in the antioxidant capability towards the level in the control group. Among these three assumptions, only the last one came true, which appeared at the end of the experiment (T62W).

All these results suggest that the rats in the BA group were subjected to positive stress, leading to adaptive changes that protected them from acute oxidative stress, especially among the older animals. This adaptive change could be related to the regulatory system of the total antioxidative capability created by the formation of antioxidant reserves, but further research needs to be carried out in order to confirm this hypothesis.

The device had a positive dose-dependent effect and a short-term protective antioxidant effect, except for the reduced glutathione level, which appeared as a long-term effect.

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