EDIBLE MUSHROOM *Agaricus sylvaticus* CAN PREVENT THE ONSET OF ATHEROMA PLAQUES IN HIPERCHOLESTEROLEMIC RABBITS

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Received June 20th, 2008; Accepted September 3rd, 2008; Published November 17th, 2008

**Abstract** – Since the involvement of free radicals in the pathophysiology of atherosclerosis was proposed, antioxidant supplementation arose as a potential strategy for the management of this disease. Thus, we decided to investigate the potential benefit of a natural antioxidant-rich edible mushroom (*Agaricus sylvaticus*) on the prevention of atherosclerosis. New Zealand rabbits underwent atherosclerosis induction by feeding a cholesterol-enriched chow (Group A), while Group B simultaneously received edible mushroom *A. sylvaticus* water solution. Control group received standard rabbit chow only (Group C). At the end of 10 week treatment period serum samples were drawn for lipid profile, uric acid, thiobarbituric acid reactive substances (TBARS), and total antioxidant status (TAS). The area of aorta arteries taken by atheroma plaques was evaluated. Groups A and B presented higher cholesterol levels (p< 0.01) and reduced TAS (p<0.01), when compared to the Group C. However, TBARS and uric acid levels for Group B animals’ were reduced, in comparison to Group A (p<0.05), and equals to group C. Moreover, animals from group A developed extensive atherosclerotic areas (47.0±14.0%), and that was prevented by the supplementation of *A. sylvaticus* (6.6±2.9%, p<0.01). Data suggested that *A. sylvaticus* can prevent the development of atherosclerosis in spite of hypercholesterolemia.

**Key words**: atherosclerosis, free radicals, oxidative stress, antioxidants, reperfusion injury, uric acid

**INTRODUCTION**

Recently, it has been observed that the involvement of free radicals in the formation of an atherosclerotic condition is very complex and multifactorial (8), and can account for endothelial lesion. Once damaged, the endothelium of a blood vessel stops exerting the role of physiological barrier and becomes more permeable to particles present in the blood, among them the low density lipoproteins (LDLs). When LDLs enter the sub-endothelial layer, they are engulfed by macrophages which are transformed into foam cells.

When free radicals production surpasses the physiological defense capacity of antioxidants, oxidative stress takes place, causing morphological and functional disturbance in the attacked cells. From that point on, cell damages...
begin to accumulate, followed by the onset of clinical symptoms of (mainly degenerative) diseases, making it necessary to take therapeutic actions, such as the use of antioxidant supplementation, so as to prevent the development of illnesses.

The use of antioxidants in the prevention of atherosclerosis has been quite studied in experimental models lately, directed primarily to prevent oxidative modifications of LDLs, slowing down the onset of atheroma plaques as well as to reduce cholesterolemia (1,6,12,19). Nevertheless, an adequate antioxidant therapeutic diet has not been established yet. There appears to be a general consensus regarding the use of vitamins C and E as being the best generic antioxidant formulation, as it brings together the great hydroxyl radicals scavenging affinity, with the hydrophobic properties of vitamin E, and the ability of vitamin C to be quickly metabolized without producing important secondary oxidative species, thus preventing the formation of oxidative reactions in cell membranes (8).

On the other hand, several antioxidant activities of mushrooms have been attested. Zhang et al. (31) demonstrated the existence of lipid antioxidants such as ergosterol and carbohydrate derivatives in Agrocybe aegerita mushroom extract, that have inhibited the production of lipid peroxides in vitro through the inhibitory action of the cyclooxygenase enzyme. The same effect was demonstrated with the mycelium of Grifola frondosa (30) mushrooms. The production of superoxide radical was likewise blocked by bioflavonoids obtained from mushroom sporophores (23). Antioxidant activity was observed in derivatives of neogroflolin obtained from the Japanese mushroom Albatrellus ovinus (18). In experimental models of hypercholesterolemic rats or rabbits, Pleurotus ostetatus extract exhibited hypocholesterolemic activity associated with the capacity of boosting the levels of the antioxidant enzymes superoxide dismutase and glutathione peroxidase (2-3).

Mushrooms of the Agaricus gender (A. bisporus, A. blazei, A. sylvaticus and others) have been extensively studied due to a variety of medicinal properties they exhibit such as: antitumoral, antiangiogenic, antihyperlipidemic, immunomodulator, bactericide, and nitric oxide releaser, among others (11,17,24). Innumerable antioxidant molecules were identified in the extracts of these mushrooms, such as vitamins E and D, diverse carbohydrates, bioflavonoids and minerals (9,14,17,29). Such context offers great antioxidant potential, since many molecules are combined in a single food. Though Agaricus sylvaticus antioxidant properties have not been studied at all, we verified in our laboratory that the extracts from these mushrooms present high total antioxidant status (data not published). Hence, it is possible that the frequent intake of this naturally antioxidant-rich food may prevent the process of atheroma plaques formation.

This study aimed to verify the potential benefits of a diet with Agaricus sylvaticus in order to prevent atheroma plaques formation in rabbits, as well as to verify the involvement of oxidative stress in the genesis of atherosclerosis.

**MATERIALS AND METHODS**

**Animals and study protocol**

Thirty-one male young adults (2.5 ± 0.5kg) rabbits, New Zealand breed, were divided randomly in three groups undergoing different diets as follow.

- **Group A** (N=11): animals that received a diet enriched with cholesterol during the period of induction of atherosclerosis.
- **Group B** (N=10): animals that received a cholesterol-rich diet together with an aqueous solution of edible mushroom Agaricus sylvaticus during the period of atherosclerosis induction.
- **Group C** (N=10): animals that received only regular rabbit chow diet during the period of atherosclerosis induction.

During the treatment, the animals were kept in a biotery with controlled temperature and 12 hours dark/light cycles. The animals had access to water “ad libitum”. The study was approved by the Animal Experiments Ethics Committee – CEEA, of São José do Rio Preto Medical School – FAMERP, according to document 3041/2004, expedited on September 10, 2004. All experiments were conducted according to the guidelines proposed by the National Research Council.

At the end of a ten-week period, the animals were sedated with 60% chloral hydrate solution (400mg/kg animal weight; i.p.), peripheral blood samples were drawn for total cholesterol (TC), low density lipoproteins cholesterol (LDLc) and high density lipoproteins cholesterol (HDLc), triglycerides (TG), uric acid (UA), thiobarbituric reactive substances (TBARS), and total antioxidant status (TAS). Aorta arteries were removed for atheroma plaques evaluation.

**Preparation of cholesterol-rich chow and atherosclerosis induction**

Cholesterol enriched chow was prepared by vaporizing a cholesterol/chloroform solution over standard rabbit chow (Purina Nutricelcho; Cargill Nutrição Animal – São Paulo, Brazil), at a ratio of 1.5g of cholesterol to 100g of chow. Cholesterol content of standard rabbit chow was 42mg/100g of chow. After vaporization, chow was mixed thoroughly mixed and samples were assayed for the cholesterol content to ensure its homogeneity. Before being consumed, the chow, as such, was placed for 48 hours inside a furnace dome so that the chloroform could completely
防治动脉粥样硬化作用的A. sylvaticus

预防动脉粥样硬化

光谱法测定在600 nm处的光密度。自由基测定，其显示稳定的蓝绿色，产生了一定的吸收值（OD）。

光密度（OD）

结果以百分比（%）形式表示ABTS.

This dose of Agaricus sylvaticus was chosen following dosages established in medical literature, corresponding to the use of a 1.5 g dose of mushroom/day for adult humans.

Each animal was dosed in the mouth of animals with the aid of a disposable syringe (20mg mushroom/kg/day; 7 days/week/10 weeks).

Animals from Groups A and C received an equal daily ration of mushroom prepared in this manner and the leftovers from the previous day were collected and weighed to control the cholesterol ingestion by each animal. This procedure was performed 7 days a week for 10 consecutive weeks.

The animals from group C received an equal daily ration of standard rabbit chow (4).

Administration of Agaricus sylvaticus

A commercially produced aqueous suspension of the mushroom was used, whose formula is standardized and registered as food by Brazil’s State Health Department (register Nº 6.1021.0002.001-7; Cogumelo do Sol Agaricus do Brasil LTDA). Suspension of Agaricus sylvaticus was orally administered to Group B animals by direct application into the mouth of animals with the aid of a disposable syringe (20mg mushroom/kg/day; 7 days/week/10 weeks).

This dose of Agaricus sylvaticus was chosen following dosages established in medical literature, corresponding to the use of a 1.5 g dose of mushroom/day for adult humans (7). Animals from Groups A and C received an equal volume of potable water.

Thiobarbituric acid reactive substances (TBARS)

In order to access free radical–mediated oxidative stress, TBARS dosage was performed with the use of the Yagi method slightly modified by Percário et al. (21) which is based on the reaction of two thiobarbituric acid molecules (TBA) with one molecule of malondialdehyde (MDA) to form a TBA-MDA-TBA adduct giving a maximal absorbance at 535 nm.

Total antioxidant status (TAS)

The total antioxidant status assessment was conducted by exposing samples to a free radical produced at controlled quantities. The incubation of (2,2-Azino-bis-3-ethylbenzothiazoline sulfonic acid; ABTS.)

Dosage of total cholesterol and fractions, triglycerides and uric acid

Estimations were performed by photometric methods with the use of commercial kits (Labtest; São Paulo, SP, Brazil).

All biochemical measurements were performed in duplicates using an E225D spectrophotometer (CELM medical equipments, São Paulo - SP, Brazil).

Area of aorta taken by atheroma plaques (ATHEROMA)

The aorta was removed in complete extension from the aortic arch to the iliac bifurcation. Arteries were then dissected to remove annexed structures, opened to expose the luminal faces, washed in xylol to remove adhered lipids, and colored by Sudan Red to evidence the atheroma plaques that became red. They were thenfixed in 10% buffered-formalin, and pressed between two glass blades allowing exposition of atheroma plaques at the luminal side, which were pictured. Pictures were digitalized (Genius, Mousepen 8x6), making it possible to measure the area with atheroma plaques and the total area of aorta’s luminal-face. The percentage of atheroma plaques was determined by the ratio of plaques areas to the total artery area, multiplied by 100.

All analytical processes were carried out double-blinded, in which technicians had no information concerning animals’ group names while processing samples or arteries.

Statistical Analysis

All variables were expressed as mean ± standard deviation (SD). For each analyzed parameter a Multiple Variance test was performed, with the application of software Biostat 5.0 (Instituto de Desenvolvimento Sustentável Mamirauá, Belém – PA, Brazil). When identified, significant differences were explored in matched-pair comparisons among groups by Tukey test. In all tests, a significance level was considered to be p < 0.05.

RESULTS

The average values and standard deviations evaluated for the lipid profile (TC, HDLc, LDLc and TG) are presented in Table 1. Though Groups A and B do not differ between themselves, significant higher levels of TC, LDLc and TG were identified when compared to Group C (p<0.01). On the other hand, HDLc values were higher in group B than those presented by Groups A and C (p<0.01). There were no significant differences found between Groups A and C for HDLc values.

The average values and standard deviations for TBARS, TAS, AU and Atheroma are presented in Table 2. Group A showed significant higher TBARS levels when compared to Groups C and B (p<0.05), which did not differ between each other. TAS values were statistically higher for Group C than for the other two groups (p<0.01), which did not differ between each other. In relation to UA, Group A presented statistically higher levels than the other two groups (p<0.01), which did not differ between each other. Atheroma was significantly higher in Group A than in Group B (p<0.01), but undetectable in Group C. Furthermore, Group B did not present significant differences when compared to Group C. Fig. 1 presented luminal aspects of animals’ aortas from all groups.

DISCUSSION

As for the laboratorial indicators of the lipid profile, the values found were previously expected, that is, Groups A and B presented TC, LDLc and TG values much higher when compared to Group C, but did not present differences among themselves. Nevertheless,
Table 1. Total cholesterol values (TC), fraction of lipoprotein of low (LDLc) and high density (HDLc) and triglycerides (TG) for the three groups studied.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>TC (mg/dl)</th>
<th>LDLc (mg/dl)</th>
<th>HDLc (mg/dl)</th>
<th>TG (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>11</td>
<td>2,139.8 ± 178.3*</td>
<td>2,108.3 ± 170.4*</td>
<td>10.7 ± 10.5 ns</td>
<td>255.5 ± 35.1*</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>2,112.5 ± 242.7*</td>
<td>1,950.4 ± 348.1*</td>
<td>25.4 ± 14.9†</td>
<td>240.5 ± 123.5*</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>65.3 ± 26.6</td>
<td>53.2 ± 17.3</td>
<td>2.3 ± 0.8</td>
<td>11.0 ± 14.4</td>
</tr>
</tbody>
</table>

Group A = rabbits submitted to the induction of atherosclerosis by ingestion of hypercholesterolemic diet; Group B = animals supplemented with mushroom A. sylvaticus and submitted to the induction of atherosclerosis; Group C = control animals; N= number of animals; ns= non significant x group C; * p≤0.01 x group C; † p<0.01 x group A. Data is presented as mean ± SD.

Table 2. Percentage of the aorta covered by atheroma plaques (Atheroma) and serum levels of thiobarbituric acid reactive substances (TBARS), total antioxidant status (TAS) and uric acid (UA) for the three groups studied.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Atheroma (%)</th>
<th>TBARS (ng/ml)</th>
<th>TAS (mmol/l)</th>
<th>AU (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>11</td>
<td>47.0 ± 14.0 *</td>
<td>71.3 ± 38.2 †</td>
<td>0.47 ± 0.13 *</td>
<td>24.53 ± 17.94*</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>6.6 ± 2.9 *†</td>
<td>61.0 ± 36.6 ns§</td>
<td>0.53 ± 0.39 *</td>
<td>2.69 ± 0.95 ns†</td>
</tr>
<tr>
<td>C</td>
<td>10</td>
<td>undetectable</td>
<td>56.5 ± 35.2</td>
<td>0.96 ± 0.16</td>
<td>0.02 ± 0.04</td>
</tr>
</tbody>
</table>

Group A = rabbits submitted to the induction of atherosclerosis by ingestion of hypercholesterolemic diet; Group B = animals supplemented with mushroom A. sylvaticus and submitted to the induction of atherosclerosis; Group C = control animals; N= number of animals; ns= non significant x group C; * p≤0.01 x group C; † p<0.01 x group A; ‡ p<0.05 x group C; § p<0.05 x group A. Data is presented as mean ± SD.

Figure 1. Luminal view of the aortas. Red stained areas represent atheroma plaques. A – rabbits fed cholesterol-enriched chow, only; B - animals fed cholesterol-enriched chow associated with antioxidant vitamins E and C; C - animals fed cholesterol-enriched chow associated with Agaricus sylvaticus; D - animals fed regular chow only.
Group C, whose animals were supplemented with *A. sylvaticus*, presented statistically higher HDLc values than those of the other two groups. However, this data should be analyzed with skepticism, as TC levels are around 2,000 mg/dL, overmatching the HDLc increase.

On the other hand the true benefits perceived through *A. sylvaticus* supplementation could have been the great reduction of the superficial area of the aorta covered by atheroma plaques (Fig. 1), considering that the use of *A. sylvaticus* (Group B) – a naturally antioxidant-rich edible mushroom - have partially prevented the atherosclerosis plaque onset, when compared to animals that received cholesterol-rich chow only (Group A). It should also be considered that this protection was obtained despite of elevated values of TC (Tables 1 and 2 and Fig. 1).

The use of antioxidant vitamins in the prevention of atherosclerosis has been tested in recent epidemiologic studies, though with conflicting results (10,15,22,26,27,32). In fact, while some of these studies have failed to demonstrate any protective effect as a result of vitamin supplementation (15,27,32), one demonstrated discreet effect (26) and the rest found positive effects in the prevention of the disease (10,22). As for the diversity of effects, Weimberg (28) clarifies that, in studies with animal models, almost all of the same presented positive effects in preventing the development of atherosclerosis. In studies with human beings, a wide variety of factors can contribute to masquerade the benefits of an antioxidant vitamin supplementation. Apparently these benefits are dose-dependent (28) or related to specific genetic aspects, such as the expression of allele 1 or 2 for the haptoglobin gene (13).

Another important finding is the fact that animals from Group A presented higher TBARS values when compared to the Control Group (Group C), which were similar to those presented by the animals supplemented with *A. sylvaticus* (Group B). This reinforces the suggestion that a hypercholesterolemic diet may indeed be an inductor of free radical production (8), bearing in mind that animals from Groups A and B presented cholesterol values at the order of 2,000 mg/dL. As expected, at experimental conditions during this study, the supplementation with *A. sylvaticus* may have minimized free radical production, thus reflecting on TBARS values statistically similar to those presented by animals of Group C (standard diet).

Parallel to this, there was no difference in protein content of the diet consumed by all animals. Therefore, the differences found for acid uric values do not reflect the metabolic alterations related to protein intake by animals, but rather, endogenous alterations. On the other hand, the presence of vast areas taken by atheroma in Group A animals’ aortas, associated to alterations in the arterial muscular tonus related to physiological sympathetic stimulation, could cause repetitive episodes of low blood flow followed by tissue hypoxia, alternated with flow maintenance at physiological conditions. Such oscillation in tissue oxygenation can trigger a well studied phenomenon called ischemia-reperfusion syndrome, responsible for the production of free radicals and potentially causing oxidative stress. It is known that uric acid is a side-product of this syndrome, thus the elevated UA values presented by animals from Group A might indicate the presence of the syndrome and its involvement in oxidative stress (8,20). Moreover, the intensity of UA levels found for Group A animals indicate that ischemia-reperfusion syndrome might be the an important source of free radicals in this animal model, responsible for the accelerated worsening of the disease. Nevertheless, more studies are needed to establish the real importance of this syndrome in the pathophysiology of atherosclerosis and the potential use of UA measurement in predicting risk.

In relation to TAS, it was expected that Groups B would present higher values, since the use of mushroom supplementation might have maintained high levels of antioxidant molecules in these animals. However, the unexpected similarity between TAS values of Groups A and B, given that only Group B received antioxidant supplementation can be explained by taking into consideration that upon stimulating the production of free radicals (8), hypercholesterolemia triggered the depletion of large reserves of antioxidants, leading to a decrease in TAS values in both groups. Furthermore, only Group B presented TBARS levels similar to the control group (C), suggesting greater control of oxidative stress, with consequent reduction of atheroma plaques. Another possibility is that the TAS method is only capable of detecting soluble antioxidant molecules present in plasma and, owing to the chronic character of this experimental model, other antioxidant defenses not detectable by this
method may be involved, such as the enzyme superoxide dismutase and reduced glutathione (GSH). The latter two are essentially intracellular molecules and, though they might have an important role in oxidative phenomena, do not produce significant alterations on TAS values, since they would not appear in meaningful concentrations in plasma samples obtained for laboratorial analysis, considering that this method evaluates only soluble antioxidant molecules.

Even it has been suggested that the use of antioxidants could prevent atherosclerosis onset (25), a few studies have been successful in establishing causal nexus between the incidence of atherosclerosis and the existence of oxidative stress. The present results suggest that oxidative stress might in fact be associated to the surge of atherosclerosis, since: a)- the antioxidant supplemented group (B) presented statistically lower values for areas covered by atheroma plaques despite the high levels of TC, LDLC and TG; b)- TBARS values were reduced in animals of Group B, supplemented with the mushroom, rich in substances with antioxidant status; c)- significantly reduced values of total antioxidant capacity were found in the two groups that received a diet rich in cholesterol and presented atheroma plaques in the aortas, and d)- the elevated levels of uric acid found in the animals of Group A point out the involvement of ischemia-reperfusion syndrome, known as responsible for free radical production.

The doses of mushrooms utilized were selected from dosages administered in clinical practice, the equivalent of 1.5 g/day of A. sylvaticus mushroom (28). The effects observed would probably be different with other dosages. Therefore, the establishment of a dose/effect study is recommended to evaluate the ideal dosage for obtaining the maximum protective effect with this supplement.

To conclude, supplementation based on A. sylvaticus mushroom, in this experimental model, is outstanding for the prevention of atheroma plaques, even though with dyslipidemia, suggesting that oxidative stress is an important factor in atherosclerosis, whose development can trigger ischemia-reperfusion syndrome, leading to large scale free radical production and accelerated pathogenesis of the disease.

REFERENCES

Prevention of atherosclerosis by *A. sylvaticus*


