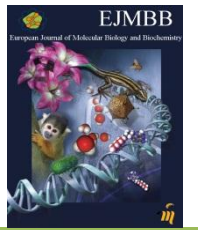
e - ISSN - 2348 - 2206
Print ISSN - 2348 - 2192

European Journal of Molecular Biology and Biochemistry

Journal homepage: www.mcmed.us/journal/ejmbb



EFFECTS OF TREATING OLD RATS WITH AN *Agaricus blazei* EXTRACT ON THE OXIDATIVE STATE OF THE LIVER

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Article Info

Received 23/01/2014
Revised 16/02/2014
Accepted 19/02/2014

Key words: Aging, Oxidative stress, Protection by *Agaricus blazei*.

ABSTRACT

Increased oxidative stress during aging is a striking phenomenon in several tissues including the liver. For this reason there has been a constant search for drugs and natural products able to prevent or at least to mitigate this problem. In the present study we have conducted an investigation on the effects of an aqueous extract of *Agaricus blazei*, a medicinal mushroom, on the oxidative state of the liver of old rats (21 months). The extract was administered intragastrically during 21 days at doses of 200 mg/kg. The classical indicators for the oxidative state were measured as well as several antioxidant enzyme activities. The administration of the *A. blazei* extract was protective to the liver of old rats against oxidative stress by decreasing the lipid peroxidation levels and by increasing the protein reduced thiol groups content. These results are similar, though less pronounced, to those reported in a previous study with the brain of old rats. It remains to be demonstrated if these effects also occur in humans during aging. If confirmed, an extract of this mushroom could be incorporated into the diet of old people as a supplement able to stimulate the body defense against oxidative stress.

INTRODUCTION

Agaricus blazei is a medicinal mushroom which has become the subject of great interest, due to its nutritional value and pharmacological properties [1, 2]. In a preceding work we have reported the effects of treating old rats with an *A. blazei* aqueous extract on oxidative and functional parameters of the brain tissue and brain mitochondria [3, 4]. The daily intragastric administration of 200 mg/kg of an aqueous *A. blazei* extract during 21 days was effective in improving the oxidative state of the brain tissue of 21 months old rats. The aqueous extract of *A. blazei*, for example, considerably increased the total antioxidant capacity of the brain tissue and diminished lipid peroxidation and the levels of reactive oxygen species [3, 4]. Treatment with the extract also improved the impaired energy metabolism of the brain mitochondria of old rats, especially the coupled respiration driven by succinate [3, 4]. These actions were attributed, partly at least, to the antioxidant activity of the *A. blazei* extract, which is quite pronounced [5, 6]. The *A. blazei* extract contains in fact several phenolics such as gallic acid, syringic acid and pyrogallol, which have been demonstrated to possess high antioxidant activities [7]. The *A. blazei* extract is also rich in polysaccharides [6], a fact that can be significant if one remembers that fungal polysaccharides have been recently demonstrated to exert hepatoprotective actions [8,9]. Furthermore, *A. blazei* is also rich in nucleotides and nucleosides [10], a group of substances whose actions on the central nervous system involve, among others, neuroprotection under pathological conditions [11, 12].

The positive effects of the *A. blazei* extract on the brain tissue [3, 4] raise the question about the response of other tissues in aged rats. To get at least a preliminary answer to this question we decided to investigate the influence of an extract of the mushroom on the oxidative

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state of the liver tissue of 21 months old rats. The classical indicators for the oxidative state were measured as well as several antioxidant enzyme activities. This study should answer the question if the *A. blazei* extracts are or not able to influence in a positive way the oxidative state of organs other than the brain in old rats.

MATERIALS AND METHODS

Preparation of the *Agaricus blazei* extract

The previously grounded dehydrated basidioma of *Agaricus blazei* were submitted to an aqueous extraction [5, 6]. Fruiting bodies (basidiocarps) of *A. blazei* were obtained from a local producer in Maringá, PR, Brazil, in Spring 2009. The dried basidiocarps were milled until obtaining a fine powder. The samples (10 g) were extracted by stirring with 100 mL of water (28°C) at 130 rpm for 3 hours and filtered through Whatman paper n° 1. The extraction was repeated three times. The filtrates (yield 50%) were lyophilized and stored in freezer until use.

Animals and treatment

Male Wistar rats kept in laboratory cages received water ad libitum and a standard chow diet (Nuvilab®). The rats were maintained in automatically timed light and dark cycles of 12 hours. Experiments were done with young adult rats (3 months old, weighing 250 to 300 g) and old rats (21 months old, weighing 450 to 500 g). The *A. blazei* extract was administered intragastrically to the old rats at a daily dose of 200 mg/kg during 21 days. All experiments were done in accordance with the internationally accepted recommendations in the care and use of animals.

Preparation of the liver homogenates

Rats were starved for 18 hours and then anesthetized by intraperitoneal injection of thiopental (50 mg/kg). The criterion of anesthesia was the lack of body or limb movement in response to a standardized tail clamping stimulus. The liver of each rat was surgically removed with scissors, clamped with liquid nitrogen and stored at temperatures under –150 °C. The tissue suspensions (10% w/v in 0.1 M phosphate buffer, pH 7.4) were homogenized by means of a van Potter-Elvejhem homogenizer. Protein contents were determined with the Folin phenol reagent [13] using bovine-serum albumin as standard.

Reactive oxygen species (ROS) determination

The levels of reactive oxygen species were estimated in the liver homogenate using the reaction with 2', 7'-dichlorofluorescein diacetate [14]. The samples were incubated with 100 mM 2',7'-dichlorofluorescein diacetate (DCFH-DA) during 30 minutes a 37°C. The formation of oxidized 2',7'-diclorofluorescein (DCF) was measured fluorimetrically with excitation at 504 nm and emission at 529 nm.

Determination of lipid peroxidation, reduced glutathione and protein reduced thiol contents

The levels of lipid peroxidation were measured in the liver homogenates by means of the TBARS method (thiobarbituric reactive substances). The concentration of the lipoperoxides was determined spectrophotometrically at 532 nm using an extinction coefficient (ε_{532nm}) of $1.56\times10^5\ M^{-1}\,cm^{-1}$ [15].

The reduced glutathione (GSH) levels of the liver homogenate were determined spectrofluorimetrically [16]. The fluorescence intensity at 420 nm with excitation at 350 nm was measured. The reduced protein thiol groups in the liver homogenate were determined using the compound 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) [17]. The concentration of reduced thiols was calculated using a molar extinction coefficient of $1.36 \times 10^4\ M^{-1}\,cm^{-1}$.

Determination of antioxidant enzymes

All enzyme determinations were done using the supernatant of the 10000*g* centrifugation of the liver homogenate. The activity of catalase (CAT) was evaluated by measuring spectrophotometrically the decomposition of H_2O_2 at 240 nm [18]. The activity of the superoxide dismutase (SOD) was assayed by its capacity to inhibit the auto-oxidation of pyrogalol in alkaline medium which was monitored spectrophotometrically at 420 nm [19]. One unit of SOD is defined as the amount of enzyme promoting 50% inhibition of pyrogalol auto-oxidation. The activity of glutathione peroxidase was determined as the decrease in absorbance at 340 nm due to NADPH oxidation dependent on H_2O_2 at 25 ºC [20]. The molar extinction coefficient of NADPH ($6.22\times10^3\ M^{-1}\,cm^{-1}$) was used in the calculations. The activity of glutathione reductase (GR) was determined as the decrease in absorbance at 340 nm due to the NADPH oxidation [21]. The molar extinction coefficient of NADPH ($6.22 \times 10^3\ M^{-1}\ cm^{-1}$) was used in the calculations.

Statistics

All results are presented as means ± mean standard errors. Evaluation of the statistical significance was done by means of the variance analysis (ANOVA) followed by post-hoc Student-Newman-Keuls testing. The 5% level ($p < 0.05$) was adopted as the significance criterion.

RESULTS AND DISCUSSION

The lipid peroxidation levels of young, old and old *A. Blazei*-treated rats are shown in Figure 1. Lipid peroxidation was clearly higher in the liver homogenate of old rats (+47%) when compared to that of young rats. The *A. blazei* extract treatment, however, restored almost completely the lipid peroxidation level of the liver tissue of old rats to that found for young rats. This is practically the same that was observed in the brain in our previous study [3, 4].

The levels of the reactive oxygen species can be seen in Figure 2. Old rats presented an enormous increase relative to young rats, namely +143%. Unlike the lipid

peroxidation levels, however, treatment of the old rats with the *A. blazei*-extract was only partially successful. Here again the response of the liver was similar to the response of the brain except that in the latter the effect of the *A. blazei*-extract was much better defined in terms of statistical significance [3,4].

Figure 3 summarizes the results obtained when the GSH levels were measured. Aging did not significantly affect the GSH concentration of the liver tissue nor had the *A. blazei* treatment any influence. This is almost exactly what was found in the brain total homogenate. It should be mentioned, however, that in brain mitochondria, aging caused a drop in the GSH content that was overcome by the *A. blazei* treatment [3,4]. The reduced protein thiol levels, which are shown in Figure 4, on the other hand, were substantially smaller in the liver of old rats (−23%). Furthermore, in this case, the *A. blazei*- extract treatment was highly effective: the thiol levels in treated old rats were even superior to the levels found in young rats. These observations contrast sharply with those made in the brain. In the latter, aging did not affect the protein reduced thiol content nor did the *A. blazei*-extract treatment have any influence [3, 4].

The activities of the four antioxidant enzymes that were measured are summarized in Table 1. The catalase activity of the 10000*g* supernatant of the liver homogenate was not affected by aging. This is also what happened in the brain in our previous study [3, 4]. The *A. blazei*-extract treatment was also without effect on the catalase activity, contrary to the brain where the same treatment caused some increase in the catalase activity [3, 4]. The superoxide dismutase activity was higher in old rats and remained so upon treatment with the *A. blazei* extract. This differs from the superoxide dismutase in the brain of old rats which did not increase upon aging but that was increased by the *A.blazei*-treatment [3, 4]. The hepatic glutathione reductase activity was smaller in old rats when compared to young rats (−32%). This contrasts with the brain where this enzyme was not affected by the aging process [3, 4]. Treatment with the *A. blazei*-extract produced no changes. The enzyme that changed most significantly in the liver was the glutathione peroxidase for which a 41% decrease was found upon aging. This is the opposite of what was found in the brain, i.e., in the latter an increased glutathione peroxidase activity was found [3, 4]. The effect of the *A. blazei*-extract treatment was also different in both tissues: whereas no changes were found in the liver (Table 1), the brain enzyme was further increased by the treatment [3, 4].

The *A. blazei* extract was particularly able to bring back the levels of lipid peroxidation (TBARS) of old rats to those found in the liver of young rats. This action could be reflecting the free-radical scavenging ability of several constituents of *A. blazei*, as for example the phenolics. The three phenolics that have been identified in *A. blazei*, gallic acid, syringic acid and pyrogallol, have also demonstrated to possess pronounced antioxidant activities [7]. They are probably present in the extract used in the present study if one takes into account their pronounced hydrophilic character. Phenolic antioxidants act as scavengers of radicals, and sometimes as metal chelators, acting both in the initiation step and propagation of oxidation [22]. Antioxidant action can also be expected from the polysaccharides that are present in *A. blazei* (non-reducing sugars; [5, 10, 23, and 24]). In this respect it must be mentioned that the hepatoprotective action of partially purified fungal polysaccharides (including antioxidant effects) has been recently demonstrated [8, 9].

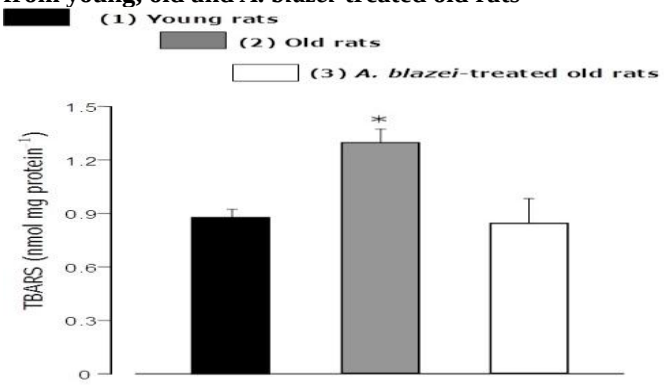
Important constituents of *A. blazei* are adenosine and other nucleosides and nucleotides which are quite abundant in this mushroom [10]. Nucleosides and nucleotides are purinergic agents and purinergic effects of an *A. blazei* extract have been recently demonstrated to occur in the rat liver [10]. Adenosine, but possibly also other activators of A_1 purinergic receptors, confers cytoprotection in the cardiovascular and central nervous systems by activating cell surface adenosine receptors [25]. Activation of these receptors, in turn, is postulated to activate antioxidant enzymes via protein kinase C phosphorylation of the enzymes or of intermediates that promote activation [25]. It is thus possible that adenosine and other nucleosides could have contributed to the overall effects of the *A. blazei* extract. Finally, it is also possible that oligopeptides could be contributing to some degree. This possibility is suggested by the antioxidant activity of an *A. blazei* oligopeptide that was recently described [26].

Table 1. Effects of the *A. blazei* extract treatment on the activity of the antioxidant enzymes in the liver tissue of old rats.

Parameters	Experimental groups		
	(1)Young rats	(2) Old rats	(3) *A. blazei*-treated
Catalase	1.10±0.02	1.03±0.05	0.97±0.05
Superoxide dismutase	2.39±0.09	3.24±0.23[a]	3.12±0.13[b]
Glutathione peroxidase	173.35±8.20	102.31±18.80[a]	115.84±17.83[b]
Glutathione reductase	46.50±1.7	31.76±4.61[a]	30.09±2.03[b]

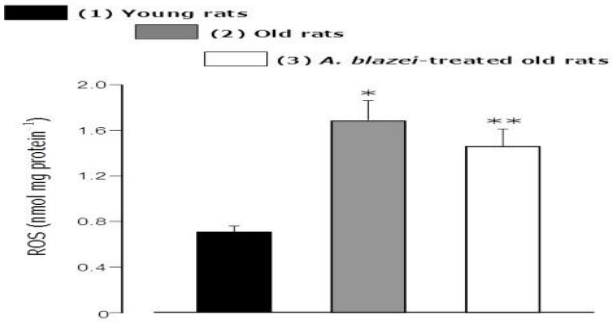
[a]$p < 0.05$ for (2) versus (1) (ANOVA and Student-Newman-Keuls post hoc testing).
[b]$p < 0.05$ for (3) versus (1) (ANOVA and Student-Newman-Keuls post hoc testing).

Figure 1. Lipid peroxidation levels of the liver homogenate from young, old and *A. blazei*-treated old rats



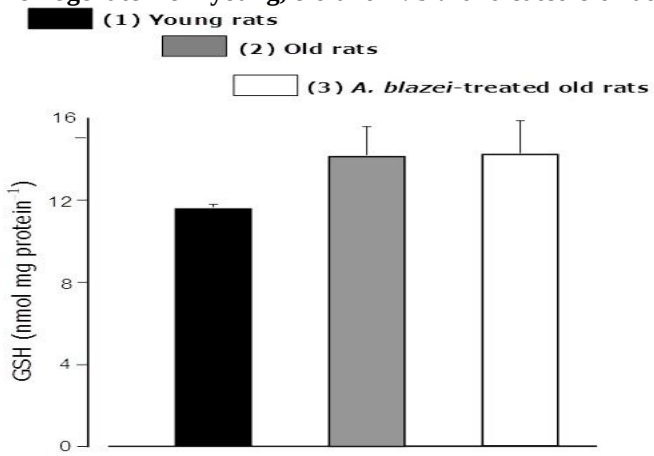
The liver homogenate was prepared as described in Materials and Methods. The lipid peroxidation levels were evaluated as the thiobarbituric acid reactive substances (TBARS). The bars represent the means ± mean standard errors of 5 (young), 5 (old) and 3 (old *A. blazei*-treated) rats. The asterisk (*) indicates $p \leq 0.05$ for the comparison between (1) and (2) and (2) and (3), according to ANOVA followed by Student-Newman-Keuls post hoc testing.

Figure 2. Reactive oxygen species (ROS) levels of the liver homogenate from young, old and *A. blazei*-treated old rats



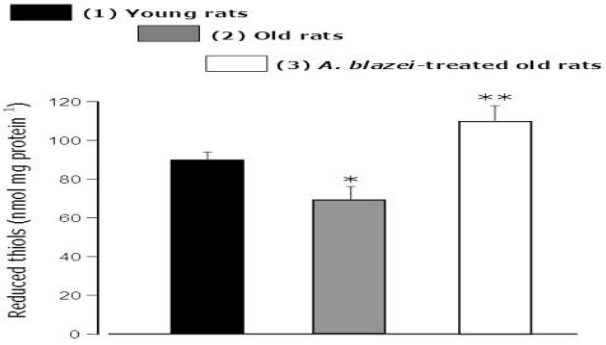
The liver homogenate was prepared as described in Materials and Methods. The ROS levels were evaluated by means of the dichlorofluorescein diacetate method. The bars represent the means ± mean standard errors of 7 (young), 5 (old) and 4 (old *A. blazei*-treated) rats. Symbols: *, $p \leq 0.05$ for the comparison between (1) and (2), according to ANOVA followed by Student-Newman-Keuls post hoc testing; **, $p \leq 0.05$ for the comparison between (3) and (1).

Figure 3. Reduced glutathione (GSH) levels of the liver homogenate from young, old and *A. blazei*-treated old rats



The liver homogenate was prepared as described in Materials and Methods. The GSH levels were evaluated spectrofluorimetrically by the o-phtalaldehyde method. The bars represent the means ± mean standard errors of 5 (young), 5 (old) and 5 (old *A. blazei*-treated) rats.

Figure 4. Reduced protein thiol groups of of the liver homogenate from young, old and *A. blazei*-treated old rats



The liver homogenate was prepared as described in Materials and Methods. The reduced thiol groups were measured with 5,5-dithiobis 2-nitrobenzoic acid (DTNB) method. The bars represent the means ± mean standard errors of 5 (young), 5 (old) and 4 (old *A. blazei*-treated) rats. Symbols: *, $p \leq 0.05$ for the comparison between (1) and (2) and (2) and (3), according to ANOVA followed by Student-Newman-Keuls post hoc testing. **, $p \leq 0.05$ for the comparison between (3) and (1).

CONCLUSION

In conclusion, the administration of the *A. blazei* extract seems to provide some protection against oxidative stress to the liver of old rats by decreasing the intensity of lipidic peroxidation. Administration of the *A. blazei* extract also increased the protein reduced thiol groups, an indication that the oxidative state of liver was at least partly improved. These actions are similar to those found in the brain, but taken as a whole, they were less pronounced [3, 4], especially with reference to the antioxidant enzymes that are generally not affected. It remains to be demonstrated if these effects also occur in humans during aging. If confirmed an extract of this mushroom could be incorporated into the diet as a supplement able to stimulate the body defense against oxidative stress.

ACKNOWLEDGEMENTS

This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação Araucária. Anacharis Babeto de Sá-Nakanishi was a fellowship holder of the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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