Kidney Function Indices in Mice after Long Intake of *Agaricus brasiliensis* Mycelia (=*Agaricus blazei*, *Agaricus subrufescens*) Produced by Solid State Cultivation

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Abstract: Problem statement: *Agaricus brasiliensis* (=*Agaricus blazei*, *Agaricus subrufescens*) or Sun mushroom has widespread use for potential health benefits such anti-tumor and immunomodulatory effects. Studies detected that others edible mushrooms affected renal metabolism and despite the widespread use of *A. brasiliensis* there are no studies that address biological effects on the renal function indices after their oral administration. Therefore, this study had as objective to verify the effects on kidney function indices after long intake of *A. brasiliensis* mycelium. **Approach:** Wheat grains was cultured during 18 days with *Agaricus brasiliensis* mycelium by solid state culture and used for chown formulation. Groups of female Swiss mice (20 per group) were fed during 14 weeks with 100 and 50% of the formulated feed denominated A100 and A50, respectively. Control group received formulated chown with wheat grains without mycelium. The water intake and excreted urine volume; the physic chemistry analysis of the urine and the serum levels of glucose, proteins, urea, creatinin and uric acid was determined (Meditron Junior-Boehringer, reagent strips Combur 10; microscopy and ADVIA 1650 Bayer). **Results:** A100 and A50 groups ingested 19.1 and 15.8% more water compared to C group, respectively. The urine and serum analysis showed that the verified parameters remained invariables for all groups, including glucose levels, which resulted in a 10% reduction of A100 group, without statistical difference ($p > 0.05$ Vs C). **Conclusion/Recommendations:** The prolonged intake of supplemented feed with *A. brasiliensis* mycelium didn't result in indicative alterations in the kidney function indices. The preventive use of the mushroom did not show any deleterious effects on kidney; however complementary studies are necessary to guarantee complete safety; possible correlation between increase of urinary excretion and hypotensive effect reported in the literature and also studies with diabetics animals to verify an possible hypoglycemic effect of the *A. brasiliensis* mycelium.

Key words: Sun mushroom, renal metabolism, oral ingestion, safety

INTRODUCTION

Mushrooms have been used for hundred of years in oriental cultures as tea and food supplement. *Agaricus blazei* Murrill, renamed as *Agaricus brasiliensis*[1] and more recently as A. *subrufescens*[2]-popularly known as Sun mushroom, Good mushroom or Himematsutake-is native from Piedade, a small town in the mountains of São Paulo state, Brazil. It has been detected that the diseases rate in adults in Piedade has been extremely low since people began to take these mushrooms as a part of their regular diet[3].

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Researches were carried out in Japan demonstrating their effective antitumoral action, awaking the scientific and popular interests around this mushroom and then, in the 90s, the commercial production of *A. brasiliensis* has started in Brazil. This mushroom reaches excellent prices in the market, due their singular flavor, almond fragrance, optimum texture, with innumerous culinary possibilities and nutritional value and mainly because their antitumoral and immune stimulant properties[4]. Between the most important biomolecules, are the proteoglucanas, lectins, ergosterol, steroids, which are detected on the fruiting body, mycelium and in the culture medium. These stimulate the sale of a lot of products derived from the dried whole or milled fruiting body, or in other forms such as tablets, extracts, teas and capsules; mainly in Brazil, China and Japan[5]. Further, the mycelium of *A. brasiliensis* is also used for formulation of nutraceutical and functional foods[6-8].

The significant interest in the use of these mushrooms and their extracts as diet supplements is based both on popular medicine and on modern scientific studies[9]. Despite their widespread use for potential health benefits, there are no studies that address the biological effects of *A. brasiliensis* on the renal function indices after oral administration to animals or humans[10]. Tam *et al.*[11] report that the extract of the mushroom *Pleurotus sajor caju* affects the renal hemodynamics, causing a decrease in the glomerular filtration rate by more than 50% after 120 min. Warner *et al.*[12] reported that a lectin derived from the mushroom *Marasmius oreades* caused a glomerular thrombotic microangiopathic lesion in mice.

The base materials and media used in Solid State Culture (SSC) are generally cheaper and simpler than other methods. SSC is characterized by the development of microorganisms in a low-water-activity environment on a nonsoluble material acting as both nutrient source and physical support. SSF offers numerous advantages, this process has lower energy requirements, produce less wastewater and is environmental-friendly as it resolves the problem of solid wastes disposal. In addition, the SSC medium generally contains low water content, which not only reduces the risk of contamination but also offers a favorable condition for fungal growth, because it resembles the natural habitats for fungi[13,14].

Agro-industrial residues are used for several bioprocess, including production of organic acids[15], enzymes and biological detoxification of coffee husks[16], aroma compounds[17-19], entomopathogenic fungi for biological control[20,21], edible mushrooms[22,23]. Further, SSC can be used as a method for produce food, when the used substrates are nutritionally valuable and free of toxic components, such cereals.

The popular use of the preventive use of mushroom *A. brasiliensis* and the absence of studies about the effect of a long-term intake induces to this study. Therefore, to investigate the effect of *A. brasiliensis* mycelium on the kidney function indices in healthy mice it was conducted a two step work. First, to obtain great quantity of mycelium from the mushroom in a short time, without requirement of separation phases it was used wheat grains particles as substrate for solid state cultivation. The cultivated material was used to prepare a chown and given to mice during three months to verify the *A. brasiliensis* mycelium effect on the renal metabolism.

MATERIALS AND METHODS

Microorganism and inoculums: The strain utilized in this study was *Agaricus brasiliensis*-LPB 03[24] and preserved in the collection of Laboratório de Processos Biotecnológicos, UFPR. The strain was maintained at ambient temperature in agar slants and cultivated in Potato Dextrose Agar (PDA) and sub cultured every three months.

The strain was cultivated in Petri dishes with PDA, during 10 days at 30°C, then five pieces of mycelium (1 cm^2) were cut and inoculated in 50 mL of medium containing (g L^{-1}): glucose (20), yeast extract (3.95), $MgSO_4 7H_2O$ (0.3), $K_2HPO_4 3H_2O$ (0.5) and pH 6.0 (± 0.2)[24], previously sterilized (121°C min^{-15}).The incubation was carried out at 30°C, 120 rpm, during 7 days. The mycelium was filtered (mesh of 0.5 mm^2), washed with 50 mL of distilled sterilized water and inoculated in 500 mL of medium (g L^{-1}): glucose (35), yeast extract (2.5), peptone (5), KH_2PO_4 (0.88), $MgSO_4.7H_2O$ (0.5), pH 5.5[25]. The incubation conditions were the same as described before. After filtration (mesh 0.5 mm) and washing with 500 mL of distilled sterilized water, this suspension of mycelium was used as inoculum.

Substrate and solid state cultivation procedures: Wheat grains (particles between 0.8 and 2 mm diameter) were used as substrate, previously washed, wetted during 12 h in clean water, put in polyethylene's tray and sterilized at 121°C for 50 min. The inoculation was realized in chamber of laminar flow and the initial humidity of inoculate substrate was adjusted to 45-50%[26]. The incubation was carried out at 30°C, 90% of relative humidity of air, during 18 days. The fermented material was dried out (55°C), milled (particles <2 mm),

analyzed for centesimal composition[27] and, supplemented with lipids: 2.2 mL g^{-100} of soy oil, proteins: 5.0 of egg albumin, to fill up the nutrition needs[28]. Samples of the cultivated material were analyzed for fungi biomass trough ergosterol content[29].

Study design: The University Federal of Paraná Committee of Animal Welfare approved all procedures involving animals (Project n°70). Eighty swiss female mice (*Mus musculus*) aged 30-35 days weighing 17-24 g were housed in plastic cages and kept in an air-conditioned room (20-25°C), with a 12 h light-dark cycle, at 55±10% humidity. The animals were divided into four groups (20 per group) consisting of two control and two treatment groups. During 14 weeks the A100 group received the formulated chown, which contained 0.289 mg of *A. brasiliensis* mycelium/g of cultivated material. The second animals group was denominated as A50 and received 50% of the mycelium formulated chown mixed with 50% of regular chown. The control groups received regular chow (C). Water and chow were supplied *ad libidum* and the body weight monitored weekly.

Urine and blood samples: The water intake was monitored in intervals of 24 h during a period of 27 days. Urine samples were collected from each group of mice kept in fasting, with water *ad libitum*, during a period of 18h, by spontaneous excretion in metabolic cages. Due to the reduced urinary volume, sample of ten animals were collected in the same flask in order to provide one sample. At the end of the 14th week, blood was obtained via heart puncture in mice with a diethyl ether anesthesia; the samples were centrifuged at 3.500 rpm for 10 min and the serum was kept at -80°C until analyzed.

Measurement of samples: The volume of excreted urine was measured and the following parameters were analyzed: Urinary flow, color, aspect, pH, density, proteins, glucose, bilirubins, urobilinogen, cetonic bodies and nitrites (equipment: Meditron Junior-Boehringer, reagent strips: Combur 10). The urinary sediment was analyzed by microscopy focusing the presence of crystals, cylinders and cells (erythrocytes, leucocytes and epithelial cells). The serum levels of glucose, proteins, urea, creatinine and uric acid were determined by automatic assay (ADVIA 1650 Bayer).

Data and statistical analyses: All values are expressed as media ± SD of the three-fold analyses. For water

intake, an average of 20 samples was analyzed. Data were analyzed by one-way ANOVA (significant difference at $p<0.05$).

RESULTS

The results of the physicochemical analyses from the urine, biochemical serum analyses and water intake and excretion of urine from mice which ingested during a long-term feed supplemented with *A. brasiliensis* are showed at Table 1, 2 and Fig. 1, respectively.

Table 1: Physicochemical analyses of urine collected from mice submitted to long-term treatment with feed supplemented with *Agaricus brasiliensis*

Analyses	A100[a]	A50[b]	Control[c]
Color	Citrine yellow	Citrine yellow	Citrine yellow
Aspect	Limpid	Limpid	Limpid
Volume (mL)	5.30 (±2.69)^z	2.80(±1.41)^z	3.20(±1.67)^z
Density	1.02(±0.02)	1.02(±0.00)	1.02(±0.00)
pH	6.00(±0.05)	6.50(±0.04)	6.00(±0.05)
Glucose (mg dL^{-1})	-	-	-
Protein (mg dL^{-1})	Traces	-	-
Urobilinogen	-	-	-
Biliary pigments	-	Traces	-
Bilirubin (mg dL^{-1})	-	-	-
Cetonic bodies (mg dL^{-1})	15	100	15
Nitrites	Present	Present	Present
Epithelial cells	Rare	Rare	Rare
Erythrocytes (mL^{-1})	2,000	<1,000	<1,000
Leucocytes (mL^{-1})	1,000	2,000	1,000
Cylinders	Absent	Absent	Absent
Crystals	Triple Phosphate Amorphous phosphate	Triple phosphate Amorphous phosphate	Triple phosphate Amorphous phosphate

Note: [a]: A100; [b]: A50: Chown with 29 and 14,5% of mycelium, respectively; [c]: Controls: Chown without mushroom. Data represent the media ± SD from n = 20; [z]: Without statistic difference (p>0.05)
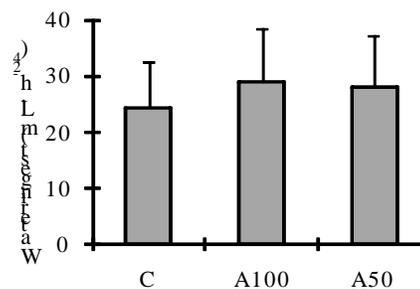

Fig. 1: Water intake during 27 days from mice submitted to long-term treatment with feed supplemented with *Agaricus brasiliensis*. (Note: A100 and A50: Chown with 29% and 14,5% of mycelium, respectively; controls: Chown without mushroom. Data represent the media (±SD) from n= 20)

Table 2: Biochemical analyses of serum collected from mice submitted to a long-term treatment with feed supplemented with *Agaricus brasiliensis*

Parameters (mg dL^{-1})	Groups		
	A100[a]	A50[b]	Control[c]
Glucose	214.00 (±9.89)[z]	223.00 (±21.21)[z]	238.00 (±1.41)[z]
Total protein	5.20 (±0.28)	5.40 (±0.14)	5.70 (±0.14)
Albumin	2.40 (±0.00)	2.50 (±0.07)	2.60 (±0.14)
Globulin	2.80 (±0.28)	2.95 (±0.07)	3.10 (±0.00)
Creatinine	0.55 (±0.07)	0.50 (±0.0)	0.45 (±0.07)
Urea	38.00(±2.83)	33.50 (±4.95)	28.50 (±0.7)
Uric acid	2.10 (±0.28)	3.30 (±0.14)	2.50 (±0.42)

Note: [a]: A100; [b]: A50: Chown with 29 and 14.5% of mycelium, respectively; [c]: Control: Chown without mushroom. Data represent the media ± SD from n = 20; [z]: Without statistic difference (p>0.05)

DISCUSSION

The results of the physicochemical analyses from the urine did not reveal important alterations (Table 1). In group A100 only protein traces were detected, whereas they were absent in A50, indicating an efficient glomerular filtration and tubular reabsorption of the proteins (Table 1)[30]. Additionally, it was possible to observe a normal level of total proteins (albumins and globulins) in the blood serum of the studied groups-as shown in Table 2- which confirms the sign of normality of the renal function. The negative values for glucose in all groups imply the absence of any renal damage.

Bilirubins and urobilinogen are eliminated through the urine when there is hemolysis or in hepatopathies[31] and the absence of these metabolites was detected in the group A100. The presence of rare epithelial cells is within normal parameters and so are the leukocytes values. Macrohematuria is characterized by the presence of high values of erythrocytes and the values found in the present study are within the normal standard. The absence of cylinders confirms the signs of normality of the renal function that were found. The long period of urine sample collection led to the precipitation of phosphate crystals, from food intake, but with no clinic interest. During this sampling period, the animals were kept in a fast, taking only water, what resulted in the appearance of cetonic bodies in the urine, the presence of which is due to the complete oxidation of fats[32].

The serum levels of glucose, proteins, uric acid, urea and creatinine are listed on Table 2. There are not significant statistical differences between the treatment groups and the control group (p>0.05). The serum creatinine is one of the most widespread ways of evaluating the glomerular filtration and represents an excellent means of measuring the renal function[33]. The serum concentrations of urea, creatinine and uric acid

remain within the standard of normality in the groups under study, implying an absence of kidney damage.

The decrease in serum values of glucose found for groups A100 and A50 were 10 and 6%, respectively. Although they do not represent any significant difference between groups (p>0.05), the reduction up to 10% in the levels of serum glycemia is an important datum when related to the occurrence of diabetes. This is the first report on a glycemia reducing activity using *Agaricus brasiliensis* through a chronic oral ingest of mycelium from this mushroom. These data suggest a dose-dependent hipoglycemic activity of that mushroom. The *Agaricus brasiliensis* has been commonly used in the treatment of many diseases, including diabetes[34] and the present results suggest that there is a support for this health claim. Others studies with diabetics animals are necessary to verify an possible hypoglycemic effect of the *A. brasiliensis* mycelium. Diabetes, characterized by the increase in the levels of glucose in the bloodstream, causes a number of deleterious effects on the organs of the body and according to World Health Organization's projections, diabetes may become a widespread epidemics before 2030[35]. Therefore, preventing, stopping or decreasing the progression rate of diabetes is truly a worthwhile goal. Other mushrooms proved to have a similar effect. The extracts of *Agaricus bisporus*, *Agrocybe aegerita*, *Cordyceps sinenesis*, *Auricularia auricula-judae*, *Lentinus edodes*, *Pleurotus ostreatus*, *Phellinus linteus*, *Ganoderma lucidum* and *Tremella aurantia* have proved to be able to lower blood glucose. For the first three species mentioned above, the lowering glycemia was due to the polysaccharide, with confirmed action in insulin-dependent-diabetes[36-40].

Group A100 ingested 19.1% more water and excreted a urine volume 65.6% bigger than the control group; A50 ingested 15.8% more water than the control group, however, the results shown a decrease in the urine volume (Fig. 1 and Table 1); all results without significant difference between groups (p>0.05). The concentration and dilution of the urine are fundamental for the maintenance of osmotic balance in mammals[45]. This mechanism is related to the concentration of salts in the serum. Further studies must be carried out in order to verify a possible correlation between the increase of urinary excretion, shown in the present work for A100 group (Table 1), the increase of water intake (Fig. 1) and with a hypotensive effect reported by Watanabe *et al.* in their researches[41,42]. In 2002, these researchers related the hypotensive effect of *Agaricus blazei* on spontaneously hypertensive rats and

in 2003, in subsequent studies, these mushroom proved to have similar effects on mild hypertensive humans[41,42].

Sorimachi *et al.*[43] drew the conclusion that *Agaricus blazei* Murill has some components that activate macrophages resulting in the induction of cytokines secretion and NO *in vitro*. NO, being a free-radical, binds to oxygen, producing two main products: the nitrites (NO_2) and nitrates (NO_3)[6]. NO plays a basic role in function regulation of various systems-cardiac, nervous, muscular, immune and arterial-where it acts over the rate of glomerular filtration (GRF). A decrease in NO produces a greater reabsorption of sodium and that induces an increase in the bood flow, promoting the appearance of the basic arterial hypertension-HTA-[44,45]. Experiments in mice have shown that a reduced production of O_2^- resulted in high renal blood flow, with urinary excretion of nitrate/nitrite, indicating an increase in NO bioavailability[46]. The presence of nitrites in the analyzed samples of urine in the A100 and A50 groups can be related to this mechanism.

Fungi have traditionally been one of the single most abundant sources of lead compounds for the development of therapeutics by the biopharmaceutical industry. It is estimated that one third of adults in the Western world use alternative natural products, including mushrooms. Contrary to chemical drugs, these medications have sometimes been claimed to be non-toxic, because of their natural origin and long-term use as folk medicines. However, problems may arise due to intrinsic toxicity, adulteration, substitution, contamination, misidentification, drug-fungi interactions and lack of standardization[47]. The medicinal qualities of *A. brasiliensis* have been widely spread by the media, so much so that its use nowadays is totally popularized. There are few reports on possible side effects that the use of this mushroom may have over various organic functions. The data presented in the present work indicate that a long-term ingestion of *A. brasiliensis* proved to reduce the glucose levels in up to 10% and did not cause any alterations indicating renal lesion or any functioning abnormality. Further researches must be carried out in order to guarantee safety and effectiveness in the medicinal and nutraceutic uses of *A. brasiliensis*.

CONCLUSION

The physicochemical analyses did not reveal any significant alteration of renal lesion or any functional alteration. All physical, chemical and cellular parameters varied discretely, within the range of normality, for each studied group. The long-term intake of feed supplemented with *A. brasiliensis* did not result in visible alterations in the kidney function indices, but decreased in 10% the blood glucose (p>0.05). Future studies are necessary to uncover a possible correlation between the increase of urinary excretion and the hypotensive effect reported in the literature; to verify an possible hypoglycemic effect diabetics animals and to guarantee complete safety of the ingest form A. brasiliensis mycelium cultured on wheat grains.

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