INHIBITORY EFFECT OF *BOSCHNIAKIA ROSSICA* ON DEN-INDUCED PRECANCEROUS HEPATIC FOCI AND ITS ANTIOXIDATIVE ACTIVITIES IN RATS

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ABSTRACT

Objective: To investigate the inhibitory effect of *Boschniakia rossica* (BR) on rat precancerous hepatic foci induced by diethylnitrosamine (DEN) and its antioxidative activities. Methods: The expression of tumor marker—placental form glutathione S-transferase (GST-P), p53 and p21 protein were investigated by immunohistochemistry techniques using ABC method. TNF-α was measured by ELISA and antioxidative activities of SOD, MDA, GSH-Px, GST and CAT were investigated by colorimetric method in rat serum and mitochondria of liver cells. Results: The 500 mg/kg of BR-H_2O extract fraction from BR-methanol extract had inhibitory effect on the formation of DEN-induced GST-P-positive foci in rat liver and the expression of mutant p53 and p21 protein was lower than that of hepatic precancerous lesions. The serum TNF-α was increased by the administration of BR extract in the early stage of chemical hepatocarcinogenesis in rat livers. The serum and liver cells mitochondria activities of SOD and GSH-Px rose again in rats administered with BR-H_2O extract and the increasing activity of GST and content of MDA in the hepatic precancerous were decreased by the BR-H_2O extract. Conclusion: These results indicated that BR-H_2O extract has inhibitory effect on DEN-induced precancerous hepatic foci in rats and induced TNF-α production in rats. The antioxidative action was exhibited by the administration of BR-H_2O extract in the early stage of chemical hepatocarcinogenesis in rat livers.

Key words: *Boschniakia rossica*, Placental form glutathione S-transferase, Hepatocarcinogenesis, Antioxidation, TNF-α, Diethylnitrosamine.

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Boschnikia rossica Fedtsch. et Flerov is a parasitic plant growing on the root of Alnus plants (Betulaceae).[1] It is one of the valuable medicinal plants growing on Changbai Mountain areas, it grows greatly on the Changbai Mountain at 1450–1800 meters above sea level, Jilin, China. It is also grows in the Democratic People's Republic of Korea (DPRK), Japan and Russian. *Boschiakia rossica* was named "Bu Lao Cao" (antisenility plant), because it has the effects of tonifying the kidney and strengthening Yang, and has been used as a tonic in China. We isolated four iridoid compounds from *Boschniakia rosseca* of the Changbai mountain by chromatographic techniques.[2] We found in references that methanol extract of *Boschnikia rossica* exerted inhibitory effect on the formation of diethylnitrosamine (DEN)-induced GST-P-positive foci in liver of F344 rats.[3,4] In the present study, we report the inhibitory effect of BR-water fraction of BR-methanol extract on the formation of precancerous hepatic lesion induced by DEN and the effect of BR on TNF-α production and its antioxidative activities in rats.

MATERIALS AND METHODS

Chemicals

Diethylnitrosamine (DEN), 2-Acetylaminofluorene (AAF) were obtained from Sigma Inc., kits for superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) malondialdehyde (MDA), and glutathione S-transferase (GST) were obtained from the Institute of Biotechnology, Nanjing Jiancheng. Vectastain ABC kit (pk 4001) was obtained from Vector Laboratories Inc. (USA); anti-GST-P antibody was kindly supplied by Professor Shigeki Tsuchida, Second Department of Biochemistry, Hirosaki University School of Medicine, Japan. p53 (DO-1) and pan ras (F-132) monoclonal antibody were purchased from Santa Cruz Biotechnology, Inc. ELISA kit for TNF-α was obtained from Endogen Inc. (USA).

Preparation of the Extract of *Boschniakia Rossica* (BR)

Boschniakia rossica harvested from the Changbai Mountain area was used and the plants were identified by the authors. They were dried, cut, made into powder and extracted overnight with methanol five times. The methanol extract was fractionated with CH_2Cl_2 and H_2O, and H_2O extract was vaccum-concentrated. The extract was dried by speed vacuum.

Animals and Treatments

Male Weistar rats, age 6 weeks and weighing 160–180 g were used in the experiments of hepatocarcinogenesis from the start. Animals were housed in groups of 5 animals in plastic cages with stainless-steel grid tops at room temperature with a 12 h light/dark cycle.

Induction of Precancerous Hepatic Foci

Enzyme-altered hepatic foci and hyperplastic nodules were induced by the modified protocol of Solt and Farber.[5,6] The animals were divided into 3 groups and treated as shown in Figure 1. The rats in group B and C were given a single i.p. injection of DEN (200 mg/kg body weight) dissolved in saline to initiate hepatocarcinogenesis. After 2 weeks on a basal diet, the rats received 0.004% 2-AAF in the diet for the following 6 weeks. Group C, after 2 weeks of injection of DEN, were given a diet containing 0.004% 2-AAF+500 mg/kg BR for the following 6 weeks as a BR treatment group. Group A, as a control group, was intraperitoneally injected with saline instead of DEN and then maintained on basal diet for 8 weeks. All rats of the experimental and control groups were subjected to two-thirds partial hepatectomy (PH) at the third week. Rats in each group were killed for examination at the eighth week.

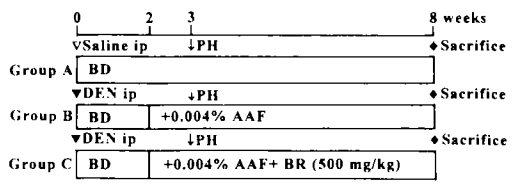

Fig. 1. Experimental protocol

vSaline, 0.85% Nacl ip; ▼Diethylnitrosamine (DEN), 200 mg/kg ip; ↓(PH): Two-third partial hepatectomy; ♦Sacrifice; AFF: 2-Acetylaminofluorene; BR: Boschniakia rossica; BD: Basal diet.

Immunohistochemical Staining for GST-P, p53 and $p21^{ras}$

Rat liver slices were fixed with ice-cold acetone and embedded in paraffin. Immunohistochemical staining for GST-P was performed by the ABC method using anti-GST-P antibody; immunohistochemical staining for p53 and $p21^{ras}$ proteins were performed using p53 (D0–1) and pan ras (F-132) monoclonal antibody, respectively.

Quantitative Analysis

The number and the area of GST-P-positive hepatic foci larger than 0.1 mm in diameter were analyzed using the microscopic quantitative analyzer (OC.M 19 m/m square 10/10×10, Tokyo, Japan).

Determination of TNF-α Content

By method of Endogen TNF-α ELISA using spectrophotometer for ELISA (MR 4100, USA).

Determination of SOD, GSH-Px, MDA, GST and CAT Activities

SOD: by xanthine oxidase colorimetric method; GSH-Px: by DTNB colorimetric method; MDA: by thiobarbitric acid colorimetric method; GST: by spectrophotometric method; and CAT: by colorimetric method.

Statistical Analysis

Statistical analysis was carried out using the x^2-test and the Student's t-test. Values of $P<0.05$ were considered statistically significant.

RESULTS

Effect of BR on DEN-induced Enzyme Altered Hepatic Foci in Rats

Immunohistochemical investigation of expression of GST-P, p53 and p21 proteins in DEN-induced precancerous hepatic foci (group B), in rat cell administration of BR extract according to the Solt-Farber protocol of rats (group C) and control (group A) are summarized in Table 1. GST-P staining was 81% positive in group B and 22% positive in group C, while in group A it was negative. Percentages of expression of oncogene product p53 and p21 proteins in group B were 33% and 22% positive respectively, while in group A and C they were negative. The number (No/cm^2) and area (mm^2/cm^2) of GST-P-positive hepatic foci in group C (given DEN-AAF+BR) was significantly decreased compared to the values of group B (given DEN-AAF), and these quantitative values are shown in Table 1.

Effect of BR Extract on Rat Serum TNF-α in the Early Stage of Chemical Hepatocarcinogenesis

Rat serum TNF-α content in group A (control),

group B (DEN-AAF) and group C (DEN-AAF+BR) were 90.67±3.06, 86.16±4.03 and 102.13±13.56, respectively. TNF-α was increased by the administration of BR-H_2O fraction from BR-methanol extract for 6 weeks in the early stage of rat chemical hepatocarcinogenesis (P<0.05, B vs C, Figure 2).

Table 1. Effect of Boschniakia rossica on the expression of GST-P, p53 and p21 protein in early stages of rat chemical hepatocarcinogenesis

Group	Treatment (8 weeks)	n	GST-P positive (%)	No. of foci (No/cm^2)[a]	Area of foci (mm^2/cm^2)[a]	p53 positive (%)	p21 positive (%)
A	Saline-BD-PH	14	Negative	0	0	Negative	Negative
B	DEN-AAF-PH	16	13 (81.3%)	1.75±1.34[b]	0.20±0.26	3 (33.3%)[c]	2 (22.2%)[c]
C	DEN-AAF-BR-PH	18	4 (22.2%)*	0.35±0.70**	0.03±0.06**	Negative	Negative

*P<0.01, compared with group B (x^2-test); ** P<0.01, compared with group B (t-test).
[a]: Foci more than 0.1 mm in diameter were quantified. [b]: Values represent ($\bar{x}\pm s$). [c]: 9 rats were used.

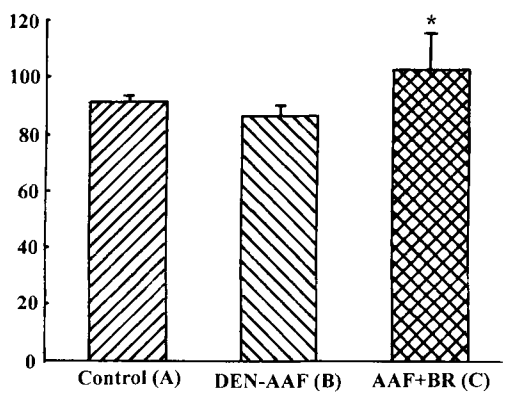

Fig. 2. Effect of BR extract on serum TNF-α content in the early stage of rat chemical hepatocarcinogenesis.

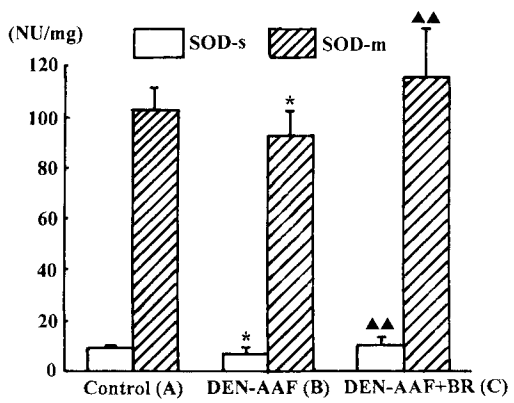

Fig. 3. Effect of BR extract on serum and liver mitochondria SOD activities in the early stage of rat chemical hepatocarcinogenesis.

Effect of BR Extract on Antioxidative Activities in the Early Stage of Rat Chemical Hepatocarcinogenesis

Effect of BR-H_2O extract on rat serum and liver mitochondria SOD, MDA, GSH-Px and GST activities in the early stage of chemical hepatocarcinogenesis are shown in Figure 3–6. Both serum and mitochondria activities of SOD and GSH-Px were decreased in group B (given DEN-AAF) compared with group A (control), but risen again in group C (given DEN-AAF+BR) by administering BR. The increased activity of GST and content of MDA in group B due to formation of precancerous hepatic foci were decreased in group C by the administration of BR extract for 6 weeks.

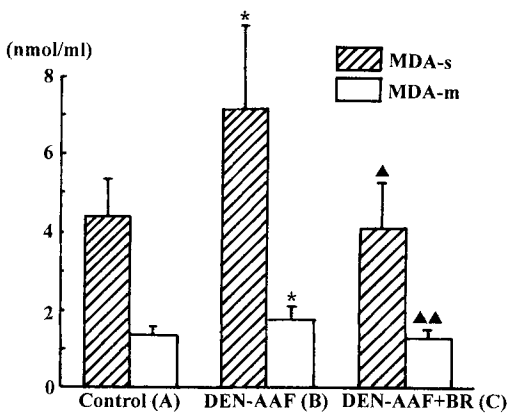

Fig. 4. Effect of BR extract on serum and liver mitochondria MDA content in the early stage of rat chemical hepatocarcinogenesis.

DISCUSSION

Effect of *Boschniakia Rossica* on DEN-induced Precancerous Hepatic Foci during Chemical Hepatocarcinogenesis in Rats

Placental form of glutathione S-transferase (GST-P) was first isolated from rat placenta as a sensitive marker enzyme in early stage of rat chemical hepatocarcinogenesis by Sato K et al. in 1984.[7] Thereafter, using DEN as an initiator and AAF as a promoter, a modified system based on the Solt-Farber method was designed to screen the medium-term bioassay of

chemical-induced carcinogenesis by Ito et al..[8] This screening system was used in this study and successfully induced the precancerous GST-P-positive hepatic foci and nodules. The results of the present study demonstrated that BR-H_2O fractionated from BR-methanol extract with CH_2Cl_2 and H_2O have an inhibitory effect on DEN-induced GST-P positive precancerous hepatic foci in rats. We also studied the relationship between the expression of GST-P and oncogene product $p21^{ras}$ protein and antioncogene product p53 protein in precancerous hepatic foci of rat livers. The results suggest that one third (33%) of GST-P positive foci were positive for p53 protein and approximately one fourth (22%) of GST-P positive foci were also positive for p21 protein in group B, while in rat livers of group C treated with DEN and AAF plus BR for 6 weeks, p53 protein and $p21^{ras}$ were not immunohistochemically detectable. The result of our study is similar to that reported by Suzuki et al.,[9] that GST-P appeared at an early stage of chemical hepato-carcinogenesis, when oncogene product c-jun was not immunohistochemically detectable. These results indicate that BR-H_2O extract fractionated from BR-methanol extract exhibited inhibitory effect on DEN-induced precancerous hepatic foci in rats administered with BR for 6 weeks during chemical hepato-carcinogenesis.

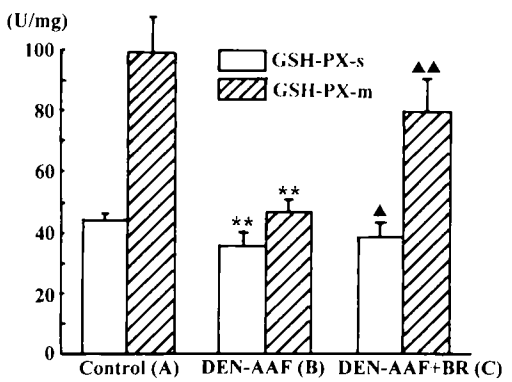

Fig. 5. Effect of BR extract on serum and liver mitochondria GSH-Px activities in the early stage of rat chemical hepatocarcinogenesis.

Immunoactivating Properties of *Boschniakia Rossica*

Macrophases are activated in a non-specific manner when antigens or infectious agents invade the body, and may secrete various cytokines (IL-1 α/β, IL-6, TNF-α, IFN-α/β, etc.) and chemicals (NO, O^-_2, prostaglandins, etc.) for communication to other immune cells, killing the invading pathogens and inducing fundamental host defense.[10] In our study, we observed the serum TNF-α was increased by administration of BR-H_2O extract in the early stage of rat chemical hepatocarcinogenesis. These observations suggest that the antihepatotoxic effect of BR probably is due to its immunomodulation. It may be related to that BR contains polysaccharides. Adachi et al. reported that polysaccharides isolated from plants have immunoactivating properties, including induction of IL-1 and TNF-α.[11] Our results indicate that BR has immunoactivating properties. It may be one of anti-carcinogenic mechanisms of BR.

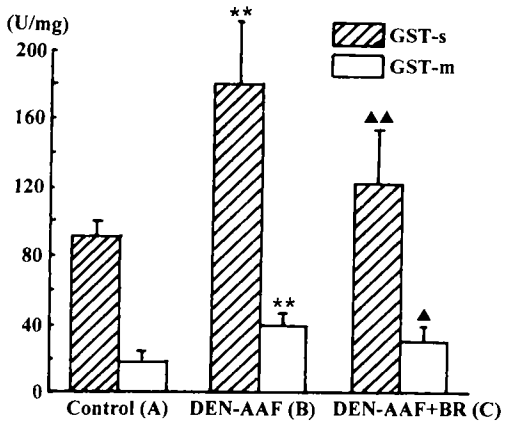

Fig. 6. Effect of BR extract on serum and liver mitochondria GST activities in the early stage of rat chemical hepatocarcinogenesis.

Antioxidative Activities of *Boschniakia Rossica*

Superoxides and free radicals that are produced during utilization of oxygen in living tissues have a high reactivity, and overproduction of superoxide has harmful actions and causes serious derangements, such as coronary arteriosclerosis and diabetes mellitus, as well as being associated with aging and carcinogenesis. SOD, GSH-Px and GST are free radical scavengers and detoxication enzymes and MDA is a lipid peroxidation product. SOD may be a major intracellular enzyme against O_2 toxicity through catalysis of the removal of O_2. Recently, Tsuda et al. reported that BR has a free radical scavenging activity.[12] Kitahara et al. reported that activities of GSH-Px were markedly decreased at an early stage of the DEN-induced hepatocarcinogenesis in rats.[13] Our present study showed that both serum and mitochondria SOD and GSH-Px activities rose again in rats administered BR-H_2O extract, and the increased activity of GST and content of MDA due to formation of precancerous hepatic foci were decreased by the BR-H_2O extract. Therefore, our present study suggests that BR has a strong effect on the disorders caused by free radical production in living tissue and the inhibitory activity on senility. In short, BR extract has an antioxidative effect and it may be one of anticarcinogenesis mechanisms of *Boschniakia rossica*.

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