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BASIC STUDIES

Chemopreventive effect of selenium and Chinese medicinal herbs on *N*-nitrosobis(2-oxopropyl)amine-induced hepatocellular carcinoma in Syrian hamsters

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Keywords
chemoprevention – hepatocellular carcinoma – herbal medicine – reactive oxygen species – selenium

Abbreviations
ALT, alanine aminotransferase; BOP, *N*-nitrosobis(2-oxopropyl)amine; Bs, *Bupleurum scorzonerifolfium* Willd; Cr, creatinine; GST-P, glutathione *S*-transferase placental form; HCC, hepatocellular carcinoma; H–E, haematoxylin and eosin staining; IL-6, interleukin 6; 8-OHdG, 8-hydroxydeoxyguanosine; ROS, reactive oxygen species; Sb, *Scutellaria baicalensis* Georgi; Sb/Bs, *Scutellaria baicalensis* Georgi/*Bupleurum scorzonerifolfium* Willd; Sel, selenium; Sil, silymarin; TGF-β1, transforming growth factor-β1; TNF-α, tumour necrosis factor-α; TUNEL, TdT-mediated dUTP nick end labelling.

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Abstract

Background/Aims: Oxidative DNA damage by reactive oxygen species is involved in the process of liver carcinogenesis. To test the hypothesis that a remedy containing *Scutellaria baicalensis* Georgi (Sb) and *Bupleurum scorzonerifolfium* Willd (Bs) (Sb/Bs remedy) modulates hepatic neoplastic growth, BOP (*N*-nitrosobis(2-oxopropyl)amine)-induced liver cancers in hamsters were established. *Methods:* Parameters such as survival rate, tumour area, tumour foci, 8-hydroxydeoxyguanosine (8-OHdG), caspase-3, transforming growth factor (TGF-β1) and tumour necrosis factor-α (TNF-α) were measured after Sb/Bs remedy treatment during BOP-induced carcinogenesis. *Results:* The results showed that the Sb/Bs remedy and its constituents Sb and Bs suppressed the tumour area in BOP-induced liver tumours. Because selenium (Sel) is toxic at a high dose (10 mg/kg), with a low survival rate (0%), the combination of Sb/Bs remedy and low-dose Sel (1 mg/kg) was found to decrease the tumour area and the number of tumour foci while increasing serum TNF-α and TGF-β1, but not IL-6 levels. Besides, the Sb/Bs remedy, when combined with low-dose Sel, not only decreased the expression of 8-OHdG and increased caspase-3 expression within the glutathione *S*-transferase placental form-positive tumour foci but also increased tumour apoptosis in BOP-induced hamsters. *Conclusions:* We conclude that low-dose Sel has a chemoprevention effect on BOP-induced liver tumours and such an effect was more enhanced when combined with Sb/Bs treatment.

Hepatocellular carcinoma (HCC) is a common primary malignancy of the liver and ranks as the first cause of cancer death in Taiwan (1). HCC is a multistage, multifactorial disease and the risk factors for its occurrence include infection with hepatitis B virus and/or hepatitis C virus, exposure to aflatoxin and heavy alcohol consumption (2–4). Furthermore, it has been hypothesized that oxidative stress and the generation of reactive oxygen species (ROS) cause mutations in cancer-related genes and alter the function of important proteins that regulate DNA repair, the cell cycle and apoptosis (5). Decreasing the oxidative stress by antioxidants or free radical scavengers might have chemopreventive effects during the process of liver carcinogenesis.

The strategies of cancer prevention include immunoprevention using α-interferon and chemoprevention using acyclic retinoid, chemical agents and/or antioxidants. Suppression of hepatic necro-inflammation by these strategies may serve to prevent hepatocarcinogenesis (6). In addition to vaccination against viral hepatitis infection, interferon α, acyclic retinoid, glycyrrhizin and ginseng are currently under clinical investigation for HCC prevention (7). The European herb, silymarin (Sil), is also documented as having a hepatoprotective effect due to its anti-inflammatory, anticarcinogenic as well as free radical-scavenging properties (8).

A recent investigation has shown that selenium (Sel) supplementation is clearly indicated to prevent cancer occurrence in Sel-deficient mice (9). The protective mechanisms of Sel include a significant reduction in the intracellular ROS, the reversal of DNA fragmentation and the suppression of caspase and apoptosis signal-regulating kinase one activity (10). In a Sel-deficient cell model, it was easier to bring about apoptotic cell death by peroxides, but not by superoxide radicals, compared with Sel-supplemented cells (11). Furthermore, flow cytometric analysis showed that Sel-deficient cells were less capable of scavenging intracellular peroxides after exposure to exogenous H_2O_2 than Sel-supplemented ones (11). Nevertheless, high doses of Sel resulted in cytotoxicity and the induction of 8-hydroxydeoxyguanosine (8-OHdG) in DNA of primary human keratinocytes (NHK) (12).

The flavonoids in herbs are well known to be strong antioxidants and possess a variety of anticancer effects such as cell growth arrest, kinase activity inhibition, induction of apoptosis, suppression of matrix metalloproteinases secretion and reduction in tumour-invasive behaviour (13). Individuals who over-generate ROS are at a high risk of developing cancer, cardiovascular disease, cataracts and other degenerative diseases because of oxidative damage to cell constituents (DNA, proteins, lipids, etc.) and cell structures. Besides, an exogenous supplement of antioxidants (vitamins E, C, β-carotene, etc.) is able to protect against cancer and other degenerative diseases in individuals with innate or acquired high levels of ROS. Because of the fact that excessive antioxidants may be dangerous and may interfere with protective functions, particularly in those with a low innate baseline level of ROS (14), the combination of medicinal herbs and Sel treatment may provide a synergistic chemopreventive effect and lessen the side effect of excessive antioxidant supplementation. Based on global gene expression profiles, our previous studies have shown that a remedy containing *Scutellaria baicalensis* Georgi/*Bupleurum scorzonerifolfium* Willd (Sb/Bs remedy) is able to down-regulate the expression of immediate early genes and cell cycle-related genes, and is thus able to inhibit cell growth in proliferating hepatocytes (15). Accordingly, it is interesting and important to elucidate the chemopreventive effect of Sb/Bs remedy on hepatic carcinogenesis. The aim of this study was to elucidate the chemopreventive effect of Sb/Bs remedy with/without a Sel supplement on BOP (*N*-nitrosobis(2-oxopropyl)amine)-induced HCC carcinogenesis.

Materials and methods

Animals

A total of 132 male Syrian hamsters (National Laboratory Animal Center, Taipei, Taiwan, ROC), 15 weeks old and weighing approximately 150 g, were housed in the Institutional Animal Care (National Academy Press, 1996), five per polycarbonate cage, and maintained under standard laboratory conditions: room temperature, $23 \pm 2\,^{\circ}C$; relative humidity, $60 \pm 5\%$; and a 12 h/12 h light/dark cycle. The animals were fed with a standard diet and water *ad libitum*. They were treated under the 'Principles of laboratory animal care' regulations of the Yang-Ming University Committee (NIH publication No. 86–23, revised, 1985). BOP (10 mg/kg) was injected subcutaneously twice per week for 10 weeks (16). BOP (1 g/bottle) was purchased from Nakalai Tesque and diluted in saline to a final concentration of 10 mg/ml before use.

The *N*-nitrosobis(2-oxopropyl)amine-induced hepatocellular carcinoma model

To validate the BOP-induced HCC model, strong nuclear staining indicative of a high level of DNA alkylation was observed in the liver at all time points (17). The animals were divided into eight groups. Control hamsters injected with normal saline were

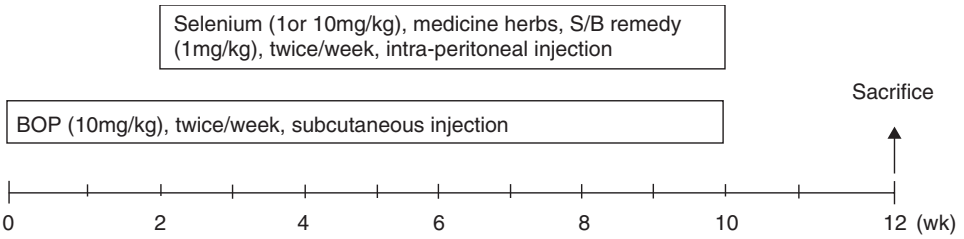

Fig. 1. Study protocol for chemoprevention studies by selenium and/or Chinese herbal medicines using an *N*-nitrosobis(2-oxopropyl)amine (BOP) -induced hepatocellular carcinoma model. S/B, Sb+Bs.

Table 1. Protocols for silymarin, selenium, herbal medicines on *N*-nitrosobis(2-oxopropyl)amine-induced hepatocellular carcinoma in hamsters

Group	Normal	Vehicle	Bs	Sb	Sil	Sel	Sel+Sb/Bs	Sel(H)+Sb/Bs
n	8	10	20	20	20	20	17	17
BOP	–	+	+	+	+	+	+	+
Bs	–	–	1 mg/kg	–	–	–	–	–
Sb	–	–	–	1 mg/kg	–	–	–	–
Sil	–	–	–	–	1 mg/kg	–	–	–
Sel	–	–	–	–	–	1 mg/kg	1 mg/kg	10 mg/kg
Sb/Bs	–	–	–	–	–	–	1 mg/kg	1 mg/kg

BOP, *N*-nitrosobis(2-oxopropyl)amine, 10 mg/kg; Bs, *Bupleurum scorzonerifolfium* Willd; Sb, *Scutellaria baicalensis* Georgi; Sb/Bs, *Scutellaria baicalensis* Georgi+*Bupleurum scorzonerifolfium* Willd; Sel, selenium; Sel(H), selenium high dose (10 mg/kg); Sil, silymarin. The experimental groups were defined in detail in 'Methods'.

defined as the normal group. The BOP-induced HCC was established by a subcutaneous injection of BOP for 10 weeks. They were treated by medicinal herbs or Sil or Sel. The protocol (Fig. 1) for medicinal herbs' treatment was as follows: (i) Normal group, without BOP treatment; (ii) Vehicle group, BOP hamsters receiving normal saline; (iii) Bs group, BOP hamsters receiving *B. scorzonerifolfium* Willd (Bs, 1 mg/kg); (iv) Sb group, BOP hamsters receiving *S. baicalensis* Georgi (Sb, 1 mg/kg); (v) Sil group, BOP hamsters receiving Sil (1 mg/kg); (vi) Sel group, BOP hamsters receiving Sel (1 mg/kg); (vii) Sel+Sb/Bs group, BOP hamsters receiving low-dose Sel (1 mg/kg)+Sb/Bs remedy (1 mg/kg); and (viii) high Sel+Sb/Bs group, BOP hamsters receiving high-dose Sel (10 mg/kg)+Sb/Bs remedy (1 mg/kg) (Table 1).

Preparation of medicinal herbs: extraction procedure

The medicinal herbs of *S. baicalensis* Georgi (Sb) and *B. scorzonerifolfium* Willd (Bs) were purchased from a local wholesale distributor (Taipei, Taiwan, ROC). The experimental herbal preparation was prepared as follows: Sb (36 g) and Bs (84 g) were extracted with 3.6 L of water and boiled at 100 °C until the total volume was reduced to 1000 ml. The extracts were filtered through layers of gauze and the residues were discarded. The filtrates were stored at − 20 °C and then lyophilized. The yield of lyophilized powder (denoted as Sb/Bs remedy) was 16 g.

High-performance liquid chromatography

The compositions and quality of the Sb/Bs remedy were analysed by high-performance liquid chromatography (HPLC). In brief, the HPLC system consisted of a chromatographic pump (PM-80, Bioanalytical System, West Lafayette, IN, USA), an injector (Rheodyne 7125, Cotati, CA, USA) equipped with a 20 µl sample loop and an ultraviolet detector (Varian, Walnut Creek, CA, USA). The herbal extract and its major ingredients were separated using an Alltima reversed phase C18 column (250 × 4.6 mm ID; particle size 5 µm; Deerfield, IL, USA) at ambient temperature. The mobile phase comprised 10 mM monosodium phosphoric acid–acetonitrile (69:31, v/v, pH 3.0), with a flow rate 1 ml/min. The mobile phase was filtered through a Millipore 0.45 µm filter and degassed before use. The UV wavelengths were set at 203 and 277 nm to detect Sb and Bs respectively. Output data from the detector were integrated via an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA). Various controls were involved in the experiments including retention time, spiking with an authentic standard, change of wavelength and change of the composition of the mobile phase, and these were used to examine the content of the samples.

Preparation of selenium and silymarin

Sodium selenite Na_2SeO_3, a preventive antioxidant, was purchased from Sigma (St Louis, MO, USA). The potential pharmacological benefit of Sil, an extract of the seeds of *Silybum marianum* or milk thistle, is inhibition of the inflammatory and cytotoxic cascade and/or of lipid peroxidation. Sil was purchased from Sigma.

Histological examinations for tumour area and tumour foci

Animals were observed and weighed weekly during the drug administration period and monthly thereafter. At the end of the 12th week, all surviving animals were sacrificed under ketamine (YSP, Yung Shin Pharmacy, Taiwan, ROC) anaesthesia, followed by macroscopical examination of the main target organs for BOP-induced tumorigenicity. The organs were fixed in 10% phosphate-buffered formalin and processed for histological examination with haematoxylin and eosin (H–E) staining. All proliferating lesions were diagnosed histopathologically and counted using serial sections. All neoplastic foci in the liver were counted under a light microscope (Olympus BH-2, Tokyo, Japan), while the percentage of tumour area was analysed using an imaging analysis software (SIGMA SCAN PRO 5.0, SPSS Inc., IL, USA).

Immunofluorescence staining for glutathione *S*-transferase placental form/caspase-3 and glutathione *S*-transferase placental form/8-hydroxydeoxyguanosine expression

Glutathione *S*-transferase placental form (GST-P), a phase II detoxifying enzyme, is not expressed in normal liver cells, but is highly and specifically induced during early hepatocarcinogenesis as well as in HCC cells (18). GST-P-positive lesions, being present in both preneoplastic and neoplastic foci, were immunohistochemically demonstrated by the avidin–biotin–peroxidase complex method. In brief, tissue sections were incubated with mouse anti-GST-P monoclonal antibody (1:500 dilution, Abcam, Cambridgeshire, UK) overnight at 4 °C and then with rhodamine-conjugated donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 1 h at 37 °C. All the sections were also subsequently incubated with fluorescein-conjugated anti-caspase-3 antibody (1:500 dilution, Abcam) or anti-8-OH-dG antibody (N45.1 at 1:50 dilution, JaICA, Shizuoka, Japan) for 1 h at 37 °C. All sections were observed under laser confocal microscopy (TCSSP2, Leica, Wetzlar, Germany). The fluorescein–isothiocyanate and rhodamine images were merged using LEICA image analysis software. The double-staining techniques for GST-P/caspase-3 and GST-P/8-OH-dG were adapted from the report of Kitamura and Ninomiya (19). The positive-stained cells were semiquantitatively defined and assessed, namely, (±), <5%; (+), 5–25%; (2+), 25–50%; (3+), 50–75%; and (4+), >75% of cancer cells per high power field (× 200).

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling staining

The cleavage of genomic DNA during apoptosis yields double-stranded, low-molecular-weight DNA fragments (mononucleosomes and oligonucleosomes) as well as single-strand breaks of the cell's high-molecular-weight DNA. DNA strand breaks could be identified by labelling the free 3′-OH termini with modified nucleotides in an enzymatic reaction involving terminal deoxynucleotidyl transferase (TdT), which catalysed polymerization of nucleotides to free 3′-OH DNA ends in a template-independent manner. A modified TdT-mediated deoxyuridine triphosphate nick end labelling (TUNEL) method was used to label affected nuclei in the histological sections (20). Tissue sections (6 μm) of the fixed tissues were prepared and stained by the TUNEL method using a commercially available kit (1:100 dilution, Calbiochem Biotechnology, Darmstadt, Germany).

Biochemical analysis and cytokine measurement

After the animals were anaesthetized with ketamine for sacrifice, venous blood samples were taken and the livers were removed for further analysis. The blood samples were immediately centrifuged at 1300 *g* at 4 °C and serum was stored at −20 °C until use. The serum biochemical analyses such as aspartate transaminase (AST), alanine transaminase (ALT) and creatinine (Cr) were measured using a colorimetric analyser (Kodak DT System, Johnson-Johnson Co., Rochester, New York, USA). Serum interleukin 6 (IL-6), tumour necrosis factor-α (TNF-α) and transforming growth factor β (TGF-β) level were determined using an EIA kit (R&D system, Minneapolis, MN, USA).

Statistical analysis

Data were expressed as mean ± SEM. Statistical differences were assessed by repeatedly measured one-way analysis of variance (ANOVA). Curves for overall survival were calculated according to the method of Kaplan–Meier (21). A difference between groups with $P < 0.05$ was considered to be statistically significant.

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Table 2. Effects of silymarin, selenium, herbal medicines on *N*-nitrosobis(2-oxopropyl)amine-induced hepatocellular carcinoma in hamsters

Group	Normal	Vehicle	Bs	Sb	Sil	Sel	Sel+Sb/Bs
No	8	10	20	20	20	20	17
BOP	−	+	+	+	+	+	+
Survival rate (%)	100	100	75	50	85	95	82.4
BW (g)	178 ± 6	$109 \pm 2^{\#}$	$97 \pm 4^{*,\#}$	$94 \pm 4^{*,\#}$	$107 \pm 4^{\#}$	$103 \pm 2^{\#}$	$103 \pm 3^{\#}$
ALT	118 ± 18	$165 \pm 21^{\#}$	128 ± 17	$84 \pm 17^{*}$	$91 \pm 14^{*}$	$97 \pm 11^{*}$	$82 \pm 13^{*}$
AST	110 ± 13	143 ± 10	135 ± 13	123 ± 15	123 ± 8	$162 \pm 12^{\#}$	139 ± 14
Creatinine	0.23 ± 0.03	$0.43 \pm 0.03^{\#}$	$0.58 \pm 0.09^{*,\#}$	$0.48 \pm 0.04^{\#}$	$0.47 \pm 0.02^{\#}$	0.32 ± 0.05	$0.40 \pm 0.04^{\#}$

$^{*}P < 0.05$ vs. vehicle group (BOP+normal saline).

$^{\#}P < 0.05$ vs. normal group (without BOP treatment).

ALT, alanine transminase; AST, aspartate transaminase; BOP, *N*-nitrosobis(2-oxopropyl)amine; Bs, *Bupleurum scorzonerifolfium* Willd; BW, body weight; Sb, *Scutellaria baicalensis* Georgi; Sb/Bs, *Bupleurum scorzonerifolfium* Willd+*Scutellaria baicalensis* Georgi; Sel, selenium; Sil, silymarin. The study was designed eight groups as described in 'Methods'.

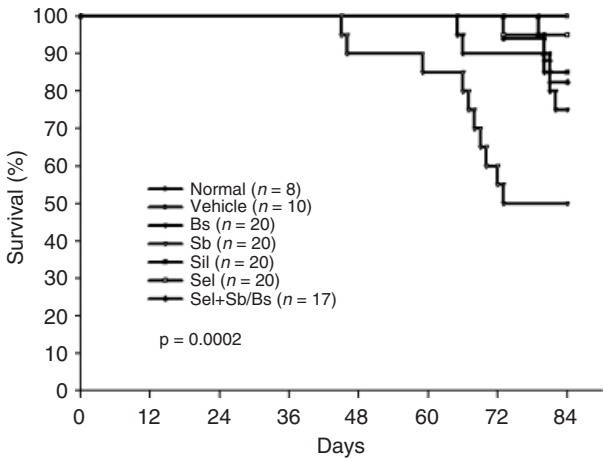

Fig. 2. Overall survival probabilities according to protocol status. BOP administered hamsters with Sb treatment had a significantly lower rate of overall survival than did those with Sil treatment ($P < 0.05$) or Sel treatment ($P < 0.05$). Using Sel combined with Sb and Bs treatment could significantly increase the survival rate than the Sb group alone ($P < 0.05$). ●, Normal ($n = 8$); ○, vehicle ($n = 10$); ▼, Bs group ($n = 20$); ▽, Sb group ($n = 20$); ■, Sil group ($n = 20$); □, Sel group ($n = 20$); ▲, Sel+Sb/Bs group ($n = 17$).

Results

Effects of *Scutellaria baicalensis* Georgi/*Bupleurum scorzonerifolfium* Willd remedy on serum biochemistry and actuarial overall survival of *N*-nitrosobis(2-oxopropyl)amine-induced hepatocellular carcinoma

The serum ALT level of the vehicle group (BOP+normal saline) (165 ± 21 U/dl) was significantly higher compared with the normal group (no BOP treatment) (118 ± 18 U/dl), indicating the hepatoxicity of BOP in the BOP-induced HCC model. In BOP-treated groups, treatment with Sb (84 ± 17 U/dl), Sil (91 ± 14 U/dl) and Sel, with (82 ± 13 U/dl) or without (97 ± 11 U/dl) the combination of Sb/Bs remedy, significantly decreased plasma ALT levels compared with the vehicle group (BOP+normal saline) (165 ± 21 U/dl) (Table 2). These results suggested that the medicinal herbals with or without Sel treatment were able to attenuate the hepatoxicity in BOP-treated hamsters. The serum Cr levels in all the treatment groups were within the normal limit, suggesting minimal renal toxicity in BOP-treated HCC models (Table 2). It is worth noting the low survival rate for the high Sel (10 mg/kg)+Bs/Sb group (0%) compared with the low-dose Sel (1 mg/kg) alone (95%) or with the Sb/Bs remedy combination (82.4%), confirming the Sel toxicity (Table 2). The actuarial survival rates in Bs, Sb, Sil, Sel (1 mg/kg) and Sel+Sb/Bs were 75, 50, 85, 95 and 82.4% respectively (Fig. 2). There was a significant decrease in body weight in BOP-treated groups compared with the normal group, indicating the cachexia effect of BOP in such a chemical-induced HCC model (Table 2).

Effects of *Scutellaria baicalensis* Georgi/*Bupleurum scorzonerifolfium* Willd remedy on tumour area and tumour foci in *N*-nitrosobis(2-oxopropyl)amine-induced hepatocellular carcinoma

After BOP injection for 10 weeks, there were an increase of the tumour area and the number of tumour foci (Fig. 3A–C) in the livers of the BOP-treated hamsters. The area of tumour foci, calculated by imaging analysis software, was quantifiable (Fig. 3D). Using H–E staining on liver sections, it was found that the tumour foci contained chromogranin-dense nuclei cells (Fig. 4A). The tumour foci were observed in all BOP-treated hamsters (100%). When the BOP hamsters were treated with Sel or the Sb/Bs remedy, there was a decreased hyperchromatism in the tumour foci (Fig. 4B–D). Furthermore, the tumour areas in the Sb ($1.48 \pm 0.09\,mm^2$), Bs ($1.23 \pm 0.05\,mm^2$), Sb/Bs

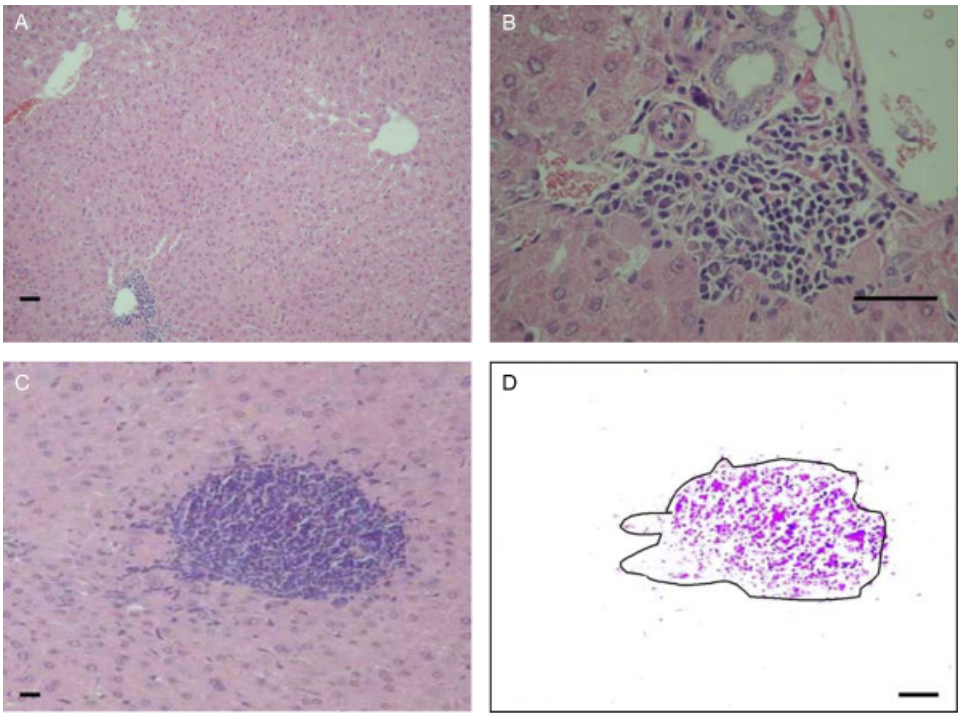

Fig. 3. Morphology of *N*-nitrosobis(2-oxopropyl)amine (BOP)-induced hepatocellular carcinoma. The BOP-induced liver tumours were demonstrated by haematoxylin and eosin stain in light microscopy (A, B, C), which could be quantified by computerized image analysis software (D). Scale bars = 200 μm.

remedy ($0.57 \pm 0.11\,mm^2$) and Sel ($1.11 \pm 0.06\,mm^2$) groups were significantly decreased compared with the vehicle group ($2.90 \pm 0.11\,mm^2$) (Fig. 5). Sil, the positive control of this study, also reduced the tumour area ($1.13 \pm 0.08\,mm^2$) (Fig. 5) and the number of tumour foci (123 ± 7 foci/cm^2) (Fig. 6). Sel, either with (102 ± 3 foci/cm^2) or without (140 ± 8 foci/cm^2) the Sb/Bs remedy combination, also reduced the number of tumour foci compared with the vehicle group (164 ± 9 foci/cm^2) (Fig. 6). It is noteworthy that the Sb/Bs remedy combined with Sel (1 mg/kg) significantly reduced the number of tumour foci (102 ± 3 foci/cm^2) compared with Sel (140 ± 8 foci/cm^2) or Sil alone (123 ± 7 foci/cm^2). These results suggest that the combination therapy might have a synergistic effect on chemoprevention in a BOP-induced HCC model.

Effects of *Scutellaria baicalensis* Georgi/*Bupleurum scorzonerifolfium* Willd remedy on glutathione *S*-transferase placental form expression in *N*-nitrosobis(2-oxopropyl)amine-induced hepatocellular carcinoma

Glutathione *S*-transferase placental form is strongly expressed not only in transformed tumour foci but also in initiated cells that occur at a very early stage of chemical hepatocarcinogenesis. It is regarded as one of the most reliable markers for preneoplastic lesions but not in normal liver cells in BOP-treated hamster livers. The foci areas were reduced by Sil, Sb, Bs, Sel without or with the Sb/Bs remedy combination compared with the vehicle group (Fig. 7).

Effects of *Scutellaria baicalensis* Georgi/*Bupleurum scorzonerifolfium* Willd remedy on glutathione *S*-transferase placental form/caspase-3 and glutathione *S*-transferase placental form/8-hydroxydeoxyguanosine expression in *N*-nitrosobis(2-oxopropyl)amine-induced hepatocellular carcinoma

To monitor BOP-induced free radical peroxidant product damage to DNA in tumour foci, 8-OHdG (red fluorescence) and GST-P (green fluorescence) were colocalized by immunofluorescence double staining (orange fluorescence) (Fig. 8). Similarly, co-localization of caspase-3 (red fluorescence) and GST-P-positive foci (green fluorescence) was demonstrated simultaneously (Fig. 9). Compared with the vehicle group, Sil, Sb, Bs, Sel and the Sel combined Sb/Bs remedy treatment decreased the amount of 8-OHdG

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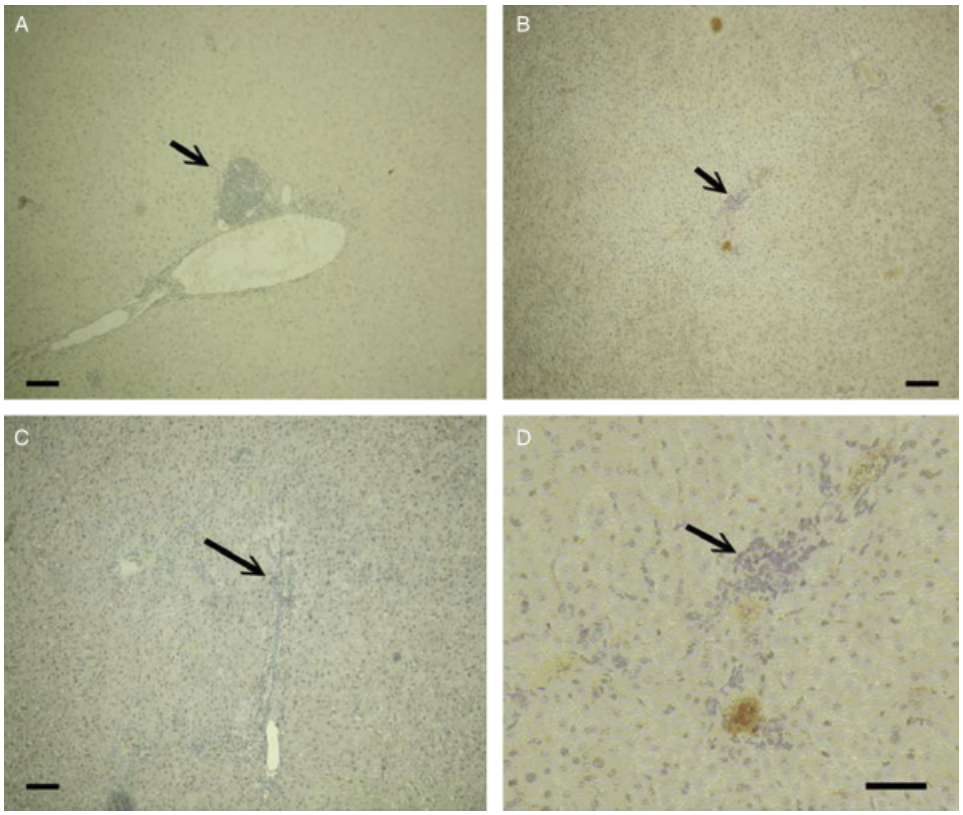


Fig. 4. Effects of medicinal herbs and selenium on *N*-nitrosobis (2-oxopropyl)amine (BOP)-induced hepatocellular carcinoma (HCC). (A) BOP+vehicle; (B) BOP+selenium (× 200); (C) BOP+Sb/Bs; (D) BOP+selenium (× 400). Arrows indicate tumour foci. Scale bars = 200 μm.

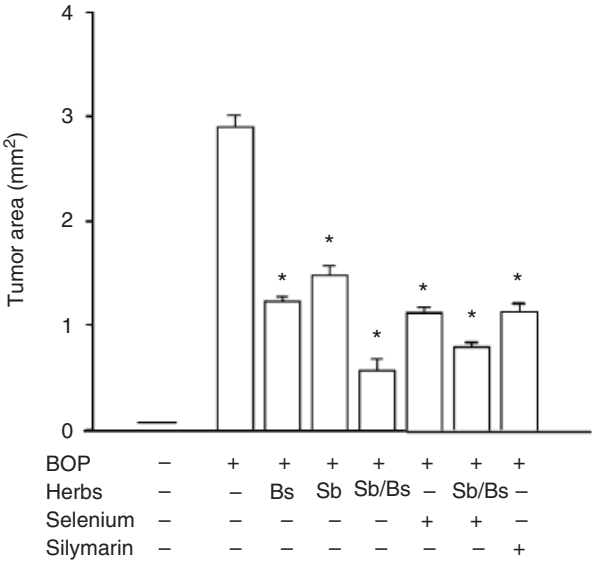


Fig. 5. Effect of medicinal herbs, silymarin (Sil) and selenium (Sel) on tumour area in *N*-nitrosobis(2-oxopropyl)amine (BOP)-induced hepatocellular carcinoma. The percentage of tumour area was measured using an imaging analysis software. *$P < 0.05$ vs. vehicle group (BOP+normal saline).

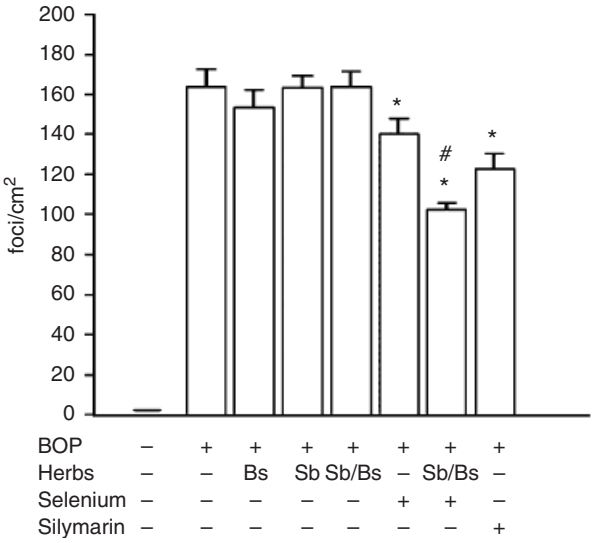


Fig. 6. Effects of medicinal herbs, silymarin (Sil) and selenium (Sel) on foci number in *N*-nitrosobis(2-oxopropyl)amine(BOP)-induced hepatocellular carcinoma. All neoplastic foci in the liver were observed under a light microscope. (Sb, *Scutellaria baicalensis* Georgi; Bs, *Bupleurum scorzonerifolfium* Willd; Sb/Bs, *S. baicalensis* Georgi/*B. scorzonerifolfium* Willd). *$P < 0.05$ vs. vehicle group (BOP+normal saline), #$P < 0.05$ vs. Sel group (BOP+selenium).

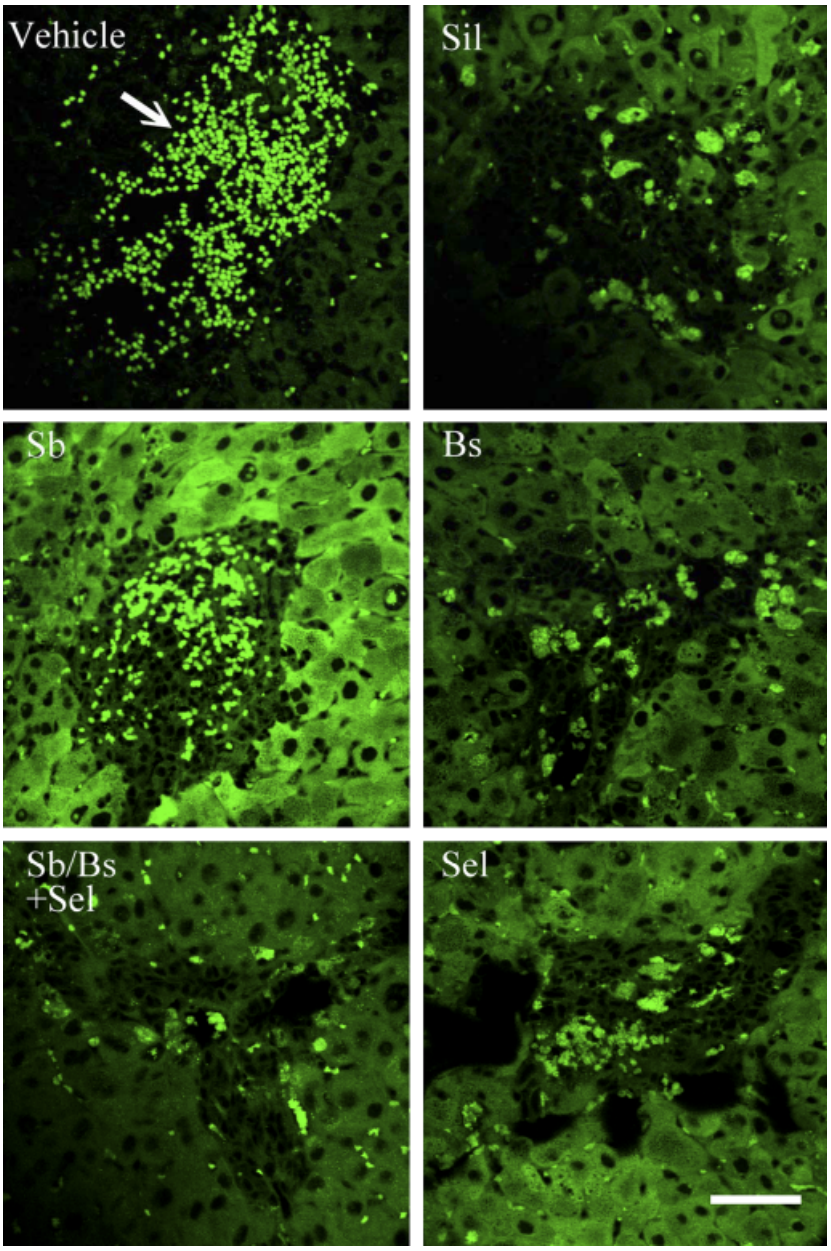
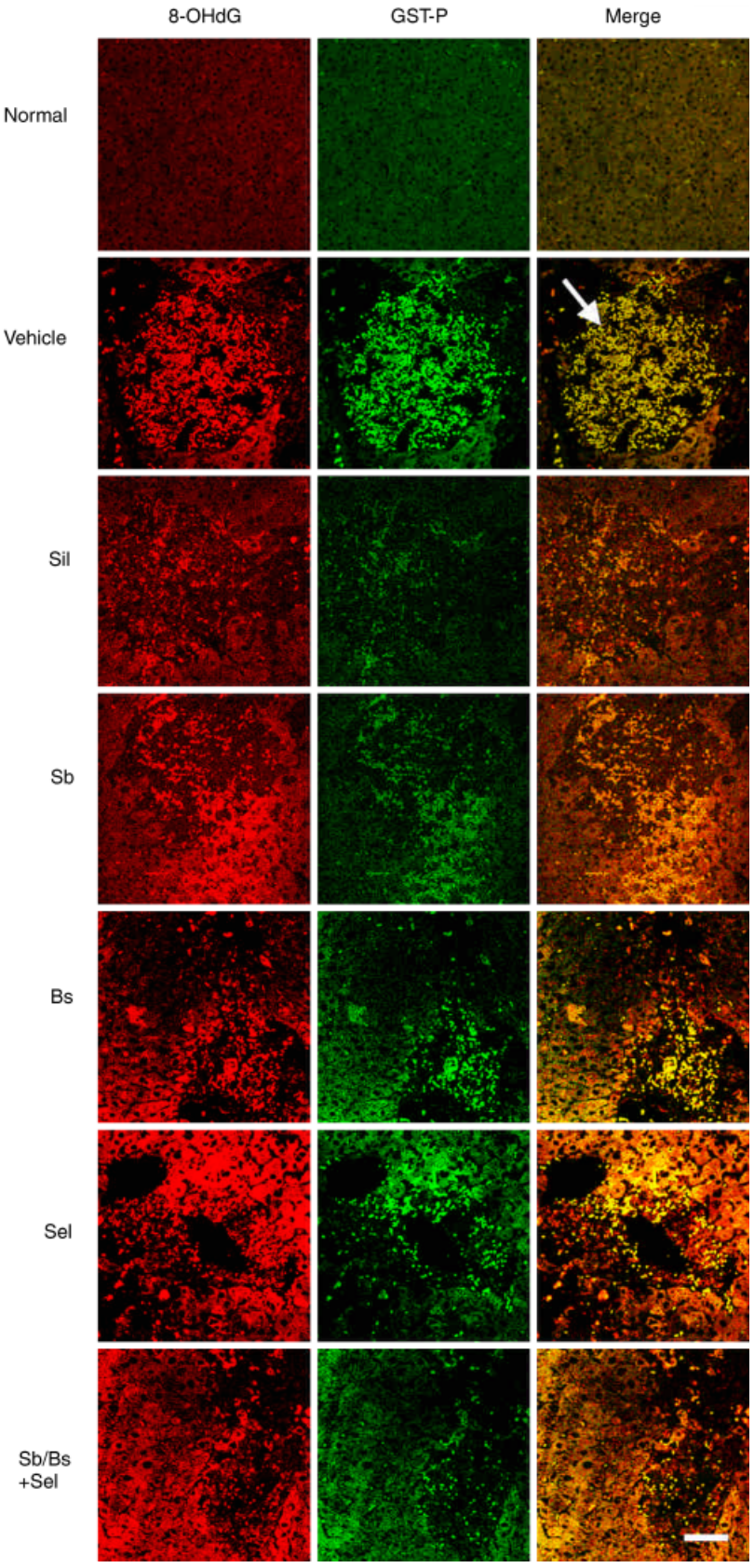


Fig. 7. Effect of medicinal herbs, silymarin (Sil) and selenium (Sel) on placental glutathione *S*-transferase (GST-P) expression in *N*-nitrosobis (2-oxopropyl)amine (BOP)-induced hepatocellular carcinoma. Histological examination of livers from BOP-induced hamsters revealed an increased number of GST-P (+) lesions (Arrow, green fluorescence). Representative liver sections from BOP-treated hamsters were compared between groups, such as vehicle, silymarin, selenium and medicinal herbs' treatment. The GST-P (+) lesions were immunohistochemically assessed using fluorescein by the avidin–biotin–peroxidase complex method. Scale bar = 200 μm. Vehicle, BOP+normal saline; Sil, silymarin; Sel, selenium; Sb, *Scutellaria baicalensis* Georgi; Bs, *Bupleurum scorzonerifolfium* Willd; Sb/Bs, *S. baicalensis* Georgi/*B. scorzonerifolfium* Willd.

Fig. 8. Effects of medicinal herbs, silymarin (Sil) and selenium (Sel) on placental glutathione *S*-transferase (GST-P) (+) foci and 8-hydroxydeoxyguanosine (8-OHdG) expression in *N*-nitrosobis(2-oxopropyl)amine (BOP)-treated hamsters. Histological examination of the livers from vehicle (BOP+normal saline)-treated hamsters revealed an increase in 8-OHdG in the GST-P (+) lesions. Representative liver sections such as normal livers were compared with different groups such as vehicle, silymarin (Sil), *Scutellaria baicalensis* Georgi (Sb), *Bupleurum scorzonerifolfium* Willd (Bs), selenium (Sel) and combined *S. baicalensis* Georgi/*B. scorzonerifolfium* Willd (Sb/Bs) remedy treatment. Animals without BOP treatment were defined as the normal group. All sections were immunofluorescence double stained and observed under laser confocal microscopy. The arrow indicates the colocalization of GST-P (green fluorescence) and 8-OHdG (orange fluorescence). Scale bar = 50 μm.



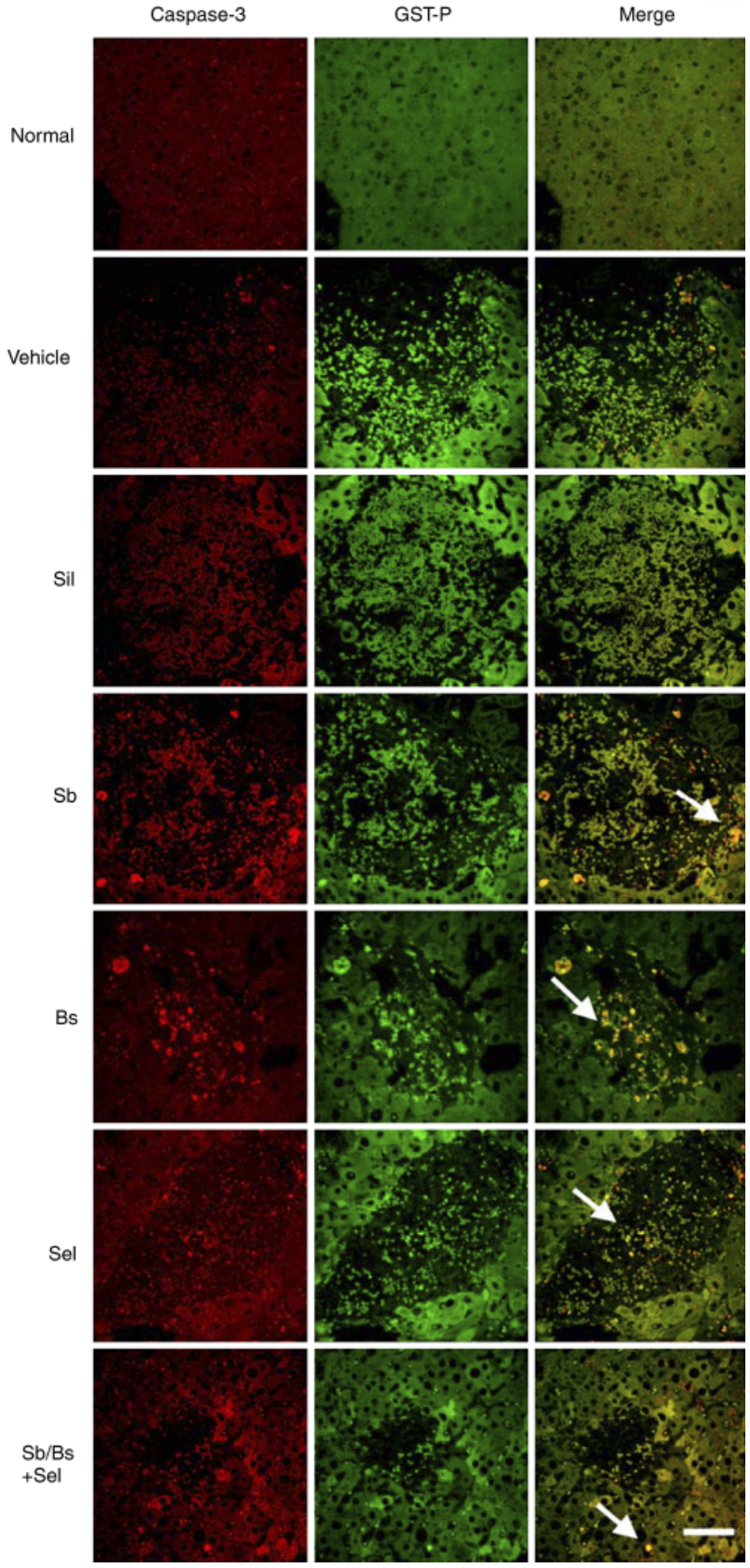


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Table 3. Semiquantification of glutathione *S*-transferase placental form+8-hydroxydeoxyguanosine and glutathione *S*-transferase placental form+caspase 3 positive-stained cells in the livers of silymarin, selenium or herbal medicines treated *N*-nitrosobis(2-oxopropyl)amine-administrated hamsters

Group	Normal	Vehicle	Bs	Sb	Sil	Sel	Sel+Sb/Bs
GST-P+8-OHdG	–	++++	++	+++	++	++	+
GST-P+caspase 3	–	–	++++	+++	+	++	+++

BOP, *N*-nitrosobis(2-oxopropyl)amine, 10 mg/kg; Bs, *Bupleurum scorzonerifolfium* Willd; Sb, *Scutellaria baicalensis* Georgi; Sb/Bs, *Scutellaria baicalensis* Georgi+*Bupleurum scorzonerifolfium* Willd; Sel, selenium; Sel(H), selenium high dose (10 mg/kg); Sil, silymarin. The comparisons of immunohistological expressions between experimental groups were defined in detail in 'Methods'. Semiquantitatively assessed, namely, (±), < 5%; (+), 5–25%; (2+), 25–50%; (3+), 50–75%; and (4+), > 75% of cancer cells per high-power field (× 200).

present in the GST-P-positive foci (Fig. 8). Furthermore, caspase-3 expression was increased after treatment with Sb, Bs, Sel and the combined Sb/Bs remedy in GST-P-positive foci in BOP-induced liver tumours (Fig. 9). We have further evaluated the differences in the co-localization of GST-P and caspase 3 or 8-OHdG among different groups semiquantitatively (Table 3).

Effects of *Scutellaria baicalensis* Georgi/*Bupleurum scorzonerifolfium* Willd remedy on cell apoptosis in *N*-nitrosobis(2-oxopropyl)amine-induced hepatocellular carcinoma

The DNA fragments accumulated during apoptosis in affected nuclei of the histological foci sections were labelled and demonstrated by TUNEL assay (deep green). When treated with Bs, Sb and the Sb/Bs remedy combined with Sel, there was an increased percentage of apoptosis in BOP-induced tumour foci (Fig. 10).

Effects of *Scutellaria baicalensis* Georgi/*Bupleurum scorzonerifolfium* Willd remedy on cytokine expression in *N*-nitrosobis(2-oxopropyl)amine-induced hepatocellular carcinoma

The combination of Sb/Bs remedy and Sel significantly elevated the serum levels of TGF-β1 (Fig. 11A) and TNF-α (Fig. 11B), but not IL6 (Fig. 11C) in BOP-treated HCC. Furthermore, it was of note that the serum TGF-β1 level in the Sel+Sb/Bs group (71 ± 12 ng/ml) was significantly higher than that in both the normal group (45 ± 8 ng/ml) and the vehicle group (50 ± 6 ng/ml) (Fig. 11A). Nonetheless, the Sb group (63 ± 15 ng/ml) and the Bs group (65 ± 19 ng/ml) had a clearly higher serum TGF-β1 level than that of the normal group (45 ± 8 ng/ml) (Fig. 11A). Such an elevation of serum TGF-β1 was also found in the Sb (63 ± 15 ng/ml) and Bs (65 ± 19 ng/ml) groups. However, the Sil group (61 ± 20 ng/ml) and the Sel-alone group (56 ± 9 ng/ml) did not show such an increase in the serum TGF-β1 level.

The Bs group (143 ± 12 pg/ml), Sb group (153 ± 17 pg/ml), Sil group (160 ± 28 pg/ml) as well as the vehicle group (162 ± 27 pg/ml) had clearly lower serum TNF-α levels than that in the normal group (260 ± 26 pg/ml) (Fig. 11B). However, only the Sb/Bs remedy combined with Sel (265 ± 35 pg/ml) group showed a significant increase in serum TNF-α compared with the vehicle group, while Sel alone showed no difference compared with the normal group and the vehicle group.

When treated with herbs or Sel, the results showed that Sel, either with (103 ± 12 pg/ml) or without (144 ± 79 pg/ml) the Sb/Bs remedy combination, was able to reduce IL6 compared with the vehicle group (215 ± 13 pg/ml) (Fig. 11C). Sil was not able to reduce the serum IL6 level (165 ± 70 pg/ml) compared with the vehicle group. There was no significant change in the serum IL-6 level among the Sb group (256 ± 18 pg/ml), the Bs group (204 ± 40 pg/ml) and the vehicle group (215 ± 13 pg/ml).

Fig. 9. Effects of medicinal herbs, silymarin (Sil) and selenium (Sel) on placental glutathione *S*-transferase GST-P (+) foci and caspase-3 expression in *N*-nitrosobis(2-oxopropyl)amine (BOP)-treated hamsters. Histological examination of the livers from BOP treated with normal saline (vehicle group) did not reveal any changes in caspase-3 expression in the GST-P (+) lesions. Representative liver sections such as normal livers were compared with BOP-treated livers receiving different treatments such as vehicle (BOP+normal saline), silymarin (Sil), *Scutellaria baicalensis* Georgi (Sb), *Bupleurum scorzonerifolfium* Willd (Bs), selenium (Sel) and combined *S. baicalensis* Georgi/*B. scorzonerifolfium* Willd (Sb/Bs) remedy treatment. Animals without BOP treatment were defined as the normal group. These BOP-treated livers showed an increase in the expression of caspase-3 in the GST-P (+) lesions, compared with normal- and vehicle-treated livers. All sections were immunofluorescence double stained and observed under laser confocal microscopy. Arrows indicate a merged double staining for GST-P (green fluorescence) and caspase-3 immunofluorescence (orange fluorescence). Scale bar = 50 μm.

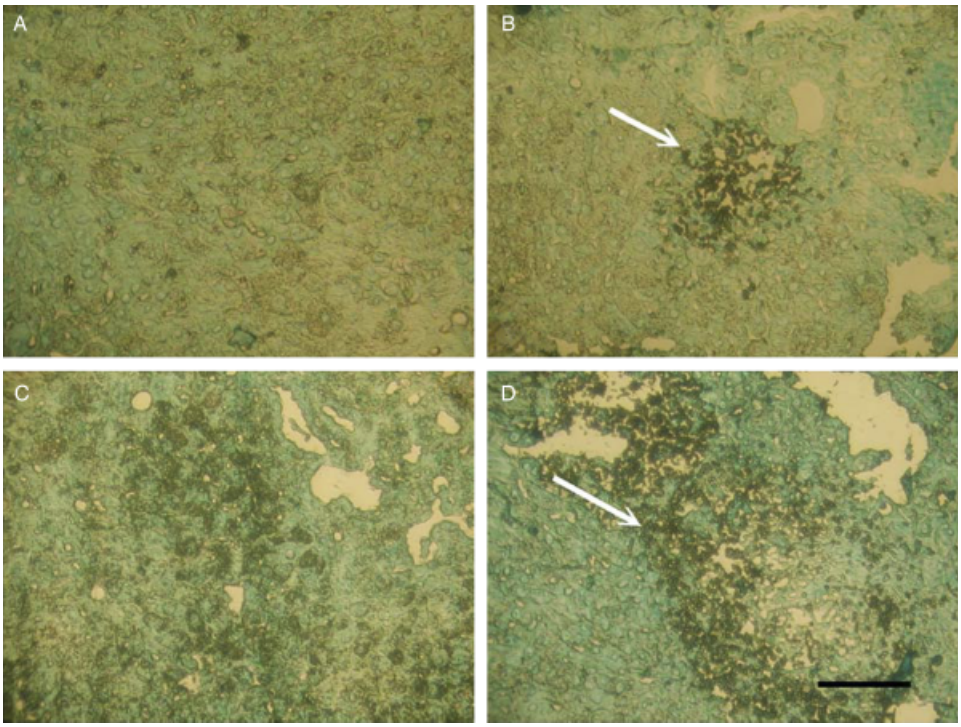


Fig. 10. TdT-mediated dUTP nick end labelling assay for the pro-apoptotic effects of medicinal herbs, selenium (Sel) on tumour foci in *N*-nitrosobis(2-oxopropyl)amine (BOP)-induced hepatocellular carcinoma hamsters. (A) BOP + vehicle; (B) BOP + *Bupleurum scorzonerifolfium* Willd (Bs); (C) BOP + *Scutellaria baicalensis* Georgi (Sb); (D) BOP + *S. baicalensis* Georgi/*B. scorzonerifolfium* Willd (Sb/Bs) + Sel. Arrows indicate apoptosis (+) lesions (brown colour). Scale bars = 200 μm.

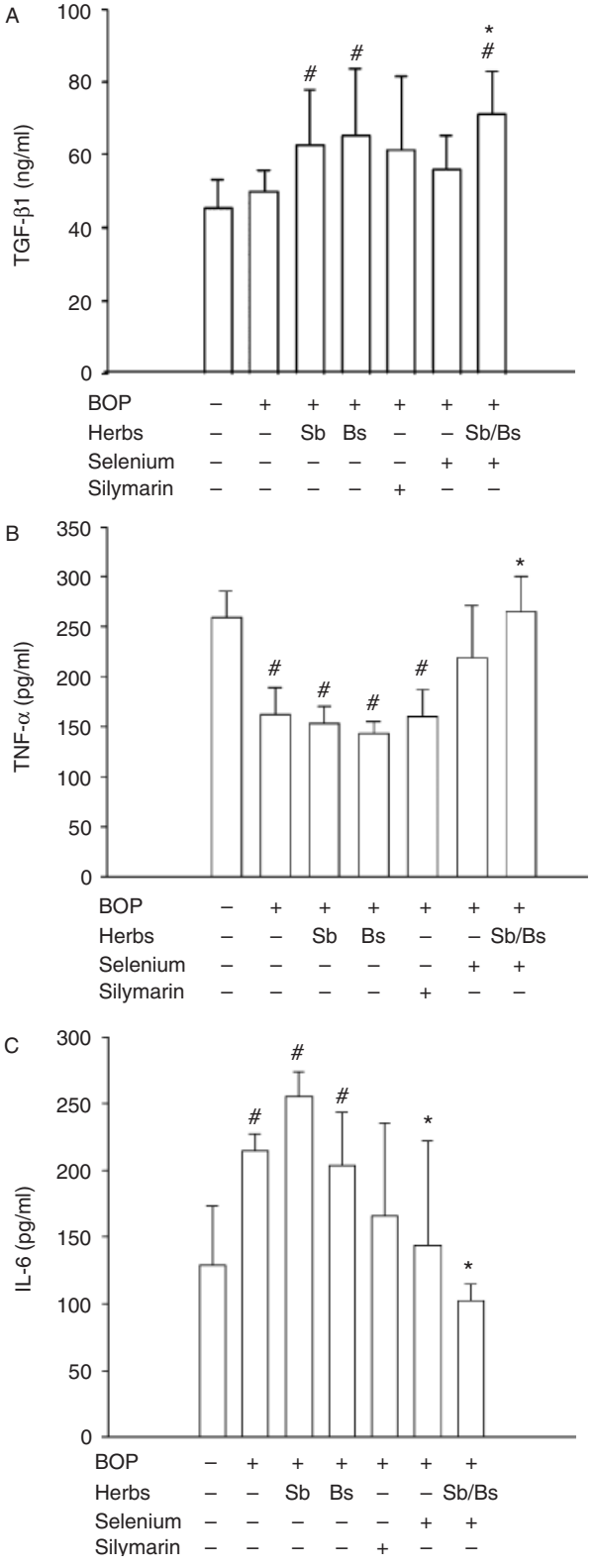
Discussion

In this study, we have demonstrated that BOP induced multiple tumours in the liver. The incidence (100%) and pattern of tumours in male BOP-treated livers were consistent with those described previously (22). The survival rate of the BOP-alone group was 100%. We attribute this phenomenon to the fact that because the BOP-induced HCC is designed to study the early stage, not the late stage of carcinogenesis, all animals in the BOP-alone group lived with the disease (foci) until sacrifice. Sil, Sel as well as the Sb/Bs remedy reduced the number of tumour foci and tumour areas in such a model. To our knowledge, this is the first evidence indicating that the herbal medicine with or without Sel has chemopreventive effects by reducing tumour foci and inducing cell apoptosis. Although the incidence of HBV-related HCC is high in Taiwan, the *in vivo* HBV-related HCC model needs 1 year or longer to produce liver tumours compared with the BOP-induced model time scale of 2 months. As a result of this, we used the BOP model to study the chemoprevention effect of herbal medicines on hepatocarcinogenesis. Strong nuclear staining, indicative of a high level of DNA alkylation, was observed at all time points in the livers of BOP-induced carcinogenesis, which has been shown to be related to a high tumour incidence (17). It is of note that no obvious hepatocyte necrosis was noticed in our model; we attribute this phenomenon to BOP being an ROS-inducing agent that causes free radical damage rather than ischaemic or reperfusion injury. Furthermore, 8OH-dG, a free-radical damaging indicator, was detectable in BOP-induced livers. The Sb/Bs remedy, in combination with Sel, ameliorated 8-OHdG peroxidant production in the nuclei of GST-P-positive foci, validating the methods we used.

Placental-type glutathione *S*-transferase has been reported to be unexpressed in normal hepatocytes, while it is strongly expressed in hepatoma cells and initiated cells that occur at a very early stage of chemical hepatocarcinogenesis (23, 24). The medicinal herbs with or without Sel reduced GST-P expression in BOP-induced HCC. Besides, the Sb/Bs remedy, combined with Sel increased caspase-3 and, hence, the apoptotic change of BOP-induced liver tumours. Taken together, the results suggested that Sb/Bs remedy, combined with Sel, may have chemopreventive effects on BOP-induced hepatocarcinogenesis.

Selenium in the form of selenocysteine plays an important role in many biological functions ranging

from antioxidant protection and metabolism to proper reproductive performance (25). A study of the preventive role of Sel has attributed its effect to immune modulation, but other molecular mechanisms may also be involved in achieving the treatment outcome, including a significant reduction in the ROS (10). Sel, combined with Sb/Bs remedy, significantly reduced the number of tumour foci compared with selenium alone, indicating that the combination therapy might have a synergic effect on chemoprevention and immunoprevention. Although the flavonoids in medical herbs have anticancer effects and anti-oxidant activity, chemoprevention properties have scarcely been investigated till now (26, 27).



Although Shirai *et al.* (28) reported that serum TGF-β1 in patients with HCC was higher than that in chronic hepatitis and liver cirrhosis, there is evidence indicating that serum TGF-β1 level is not well correlated with HCC. For example, Ali *et al.* (29) reported that serum TGF-β1 was significantly increased in chronic hepatitis and liver cirrhosis groups as compared with that in HCC and control groups ($P < 0.001$), while there was no significant difference between TGF-β1 in HCC and control groups ($P > 0.05$). Furthermore, TGF-β1 is shown to induce apoptosis in several human HCC cell lines (30). Chabicovsky *et al.* (31) suggested that during chemically induced liver carcinogenesis in B6C3F1 mice, basal rates of apoptosis in adenoma and carcinoma are higher than that in normal liver and can be further increased by direct injection of TGF-β1 into the tail vein. Carillo *et al.* demonstrated that IFN-α2b administration significantly decreased both the number and the volume percentage of altered hepatitis foci by an induced programmed cell death in the foci. This apoptotic effect of IFN-α2b on preneoplastic liver foci was mediated by the production of endogenous TGF-β1 from hepatocytes acting by a paracrine/autocrine way (32). Taken together, we suggested that Sb, Bs and their combination could significantly increase serum TGF-β1 level and cell apoptosis in tumour foci.

Significantly decreased body weight, daily activity and ascites were observed in the Sb-treated groups compared with the normal and vehicle groups. We attributed the above-mentioned findings to the fact

Fig. 11. Effect of the medicinal herbs, silymarin (Sil) and selenium (Sel) on serumal cytokines in an *N*-nitrosobis(2-oxopropyl)amine (BOP)-induced hepatocellular carcinoma model. The serum cytokines such as TGF-β1 (A), TNF-α (B), IL-6 (C) were measured by EIA as described in the method. TGF-β1, transforming growth factor-β1; TNF-α, tumour necrosis factor-α; IL-6, interleukin-6. $^{*}P < 0.05$ vs. vehicle group (BOP+vehicle), $\#P < 0.05$ vs. normal group (without BOP treatment).

that *S. baicalensis* could induce TNF-α secretion, a strong factor inducing cachexia in the animals (33). The chemical ingredients of Sb, baicalein, baicalin and wogonin, might be effective candidates for inducing apoptosis or inhibiting proliferation in various human HCC cell lines (34). Previously, Sb has been found to inhibit cancer cell growth *in vitro* and *in vivo* and could be an effective chemotherapeutic agent (35). In addition, baicalein decreases the 8-OH-dG content, which acts as a DNA damage marker, suggesting that the protective effect of baicalein against the cytotoxicity and genotoxicity of hepatocytes is because of its ability to quench free radicals (36). Baicalin exhibits an anti-inflammatory effect *in vivo* and *in vitro*, markedly reducing serum aminotransferase activities, protecting hepatoycte apoptosis (37). Furthermore, Saikosaponin-a and Saikosaponin-d, ingredients of Bs, have been reported to induce cell apoptosis through the caspase-3-dependent and -independent pathways in HCC cells. Besides, Saikosaponin-c exhibits anti-HBV activity and saikosaponin-d possesses potent cytotoxicity against human HCC cells (38–40).

Herbal combinations are made because of their properties of increasing apoptosis of tumour foci and decreasing the side effect of each herb alone or Sel alone. The concept of herbal combination may be applicable in clinical aspects, low-dose Sel with lessened Sb (30%) and Bs (70%) proportional dose in the Sb/Bs remedy, to elevate the survival rate and chemopreventive tumorigenesis effect of the HCC-treated group. Meanwhile, similar to chemotherapy in clinical settings, the data in this study form a good basis for a chronic study of the single compound/herb or combination therapy. It is possible that in a chronic study, the survival rate of the untreated group will be significantly lower than that in the treated groups. A long-term use of a proper ratio and dose of chemopreventive supplement combination must be sufficient to support the safe use of the therapy in a clinical scenario.

Although the model used in this study does not completely represent the human HCC development, both cancers share a common mechanism, oxidative DNA damage, which is the therapeutic target of the present study. Thus, the results of the present study may provide some clues regarding human HCC development and prevention. Besides, the agents used in the therapy groups could induce apoptosis of tumour foci and might elicit a drug effect similar to those observed in chemotherapy clinically. Accordingly, the preventive strategy has to be investigated in other HCC models before study in humans, so that the potential harmful effects of such a remedy could be expected or prevented. We used the mixture formula composed of herbal medicines that could increase the effect of treatment and decrease the side effect of a single herb as the basis of cocktail therapy. Consequently, this concept may be applicable for Sel with an Sb/Bs remedy combination and could be advantageous in the chemoprevention effect during the progression of liver carcinogenesis.

Conclusion

The Sb/Bs remedy has anti-oxidative and chemopreventive effects on carcinogenesis in a BOP-induced HCC model. The combination of anti-oxidative Sb/Bs remedy and Sel also has a synergic effect on the chemoprevention and immunoprevention activity, which might bring into perspective the clinical application of the combined use of medicinal herbs and Sel.

Acknowledgements

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