Safety assessment of sanguiritrin, alkaloid fraction of *Macleaya cordata*, in rats

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ABSTRACT: The subchronic safety of sanguiritrin, a mixture of sanguinarine (SA) and chelerythrine (CHE) quaternary benzo[c]phenanthridine alkaloids (QBA), obtained from *Macleaya cordata* was assessed. Rats were fed a diet containing 120 ppm sanguiritrin (100 ppm QBA) for 109 days. The feed consumption and the animal weight were monitored. The content of QBA in selected tissues and plasma was determined using HPLC. It was evidenced that 2% of QBA were absorbed through the GIT while 98% were excreted in the feces. In plasma, bilirubin, urea, creatinine, glomerular filtration, AST, ALT, GMT, ALP and total antioxidant capacity were determined. In liver, GSH level, lipoperoxidation products, SOD and GPx activities and total amount of cytochrome P450 were evaluated. Damage to nuclear DNA was assessed; a ^{32}P-postlabeling assay proved that no DNA-adducts were detected in nuclear and mitochrondrial DNA in liver. No adverse effects were observed on rat organism. QBA had no influence on the gut mucosal epithelium, liver tissue and any biochemical parameters tested. Oxidative stress was not manifested during the experiment.

Keywords: benzo[c]phenanthridine alkaloids; oral administration; biochemical markers; oxidative stress; DNA damage; cytochrome P450

Medicinal plants containing quaternary isoquinolines, namely benzo[c]phenanthridine alkaloids (QBA) sanguinarine (SA) and chelerythrine (CHE) – *Sanguinaria canadensis* (rhizomes contain 4–7%, roots about 1.8% alkaloids), *Chelidonium majus* (roots contain 4.5% alkaloids) and *Macleaya cordata* (aerial part contains about 3% alkaloids) were utilized in the past in traditional medicine (North America, Europe, China) long before the isolation of pure alkaloids. QBA exert a wide spectrum of biological activities, e.g. antimicrobial, antifungal, anti-inflammatory, adrenolytic, sympatholytic, and local anesthetic, including cytotoxicity against various human normal cells and tumor cell lines (Walterova et al., 1995; Simanek et al., 2004). QBA extract from *S. canadensis* (sanguinaria) and QBA fraction from *M. cordata* (sanguiritrin) have been used in toothpastes and mouthwashes as antiplaque agents. Sanguiritrin has been applied as a veterinary preparation for mastoiditis in cows (Faddeieva and Beliaeva, 1997). The extract of isoquinoline alkaloids from the aerial part of *M. cordata* containing SA and CHE as major phytoceuticals is active component of the EU commercially successful

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preparation Sangrovit®, an additive to animal feeds. QBA in Sangrovit® improve animal performance by eliminating the need for including low levels of antibiotics in the feed, which may be associated with the risk of antibiotic resistance.

SA/CHE as well as sanguinaria exhibited a low acute oral toxicity: for sanguinarine LD_{50} = 1.7, for sanguinaria 1.4 g/kg in rats; the acute i.v. toxicity for sanguinarine LD_{50} = 29 mg/kg in rats and that for chelerythrine LD_{50} = 18.5 mg/kg in mice (Walterova et al., 1995). In subchronic studies, minor evidence for treatment-related toxicity of QBA (doses > 30 mg/kg/day; rat, monkey) has been reported (Becci et al., 1987). No toxic effects were observed in rats fed up to 150 ppm SA in the diet for two weeks and in rats treated by gavage with up to 0.6 mg SA/kg body weight for 30 days. The overwhelming majority of studies in the literature do not predict any health hazard to humans or farm animals when QBA-containing oral hygiene products or feed additives are used (Walterova et al., 1995). In our study using the swine model, a 100 ppm addition of sanguiritrin to feed resulted in an overall improvement in health and performance (Kosina et al., 2004). No adverse effects were observed on any of the hematological or biochemical parameters tested. The study showed that QBA might exert positive modulatory effect on the animal's health by functioning as an antimicrobial and anti-inflammatory remedy. On the other hand, one group has recently reported that long-term use (5 years) of toothpastes and mouthwashes containing the QBA fraction of *S. canadensis* appears to be associated with an increased prevalence of maxillary vestibular leukoplakia (Anderson et al., 2005).

From another aspect, sanguinarine and dihydrosanguinarine (DHSA) are considered to be the toxic principle of *Argemone mexicana* seed oil (Das and Khanna, 1997). Several studies suggested that singlet oxygen and hydroxyl radical are involved in argemone oil toxicity. The role of QBA in the genesis of epidemic dropsy syndrome (*A. mexicana* poisoning) has not been elucidated to date (Simanek et al., 2003). Recently two studies demonstrating the DNA damaging potential of SA in mice have been published (Das et al. 2004; Ansari et al., 2005). In both studies, after administration of a single intraperitoneal dose, the minimum genotoxically effective SA concentration of 10 mg/kg body weight was found. The authors referred to benz[c]acridine (3,4-benzacridine) as to the principal toxicant. This compound has been considered as the only metabolic product of SA biotransformation (Hakim et al., 1961; Tandon et al., 1992). Williams et al. (2000) and Vrba et al. (2004) implicated rat hepatic cytochrome CYP1A as a likely cause of the modulation of SA toxicity.

In this study the rats were fed 120 ppm sanguiritrin (100 ppm mixture of SA and CHE) in the diet for 109 days. The objective was to evaluate the long-term toxic effect of SA and CHE on clinical chemistry parameters in blood and parameters describing oxidative stress in blood and liver. Also we focused on the *in vivo* genotoxicity of QBA monitored by the formation of plasma 8-hydroxydeoxyguanosine, DNA adducts in liver, and of single-stranded DNA breaks in lymphocytes and hepatocytes.

MATERIAL AND METHODS

Chemicals. Sanguiritrin, QBA fraction of *M. cordata* (minimum QBA content 84%; HPLC analysis: 640.3 mg/g SA and 219.9 mg/g CHE) was purchased from CAMAS Technologies (Broomfield, USA). Sanguinarine and chelerythrine were isolated from sanguiritrin using column chromatography on alumina. SA, 98.1% purity, MP 279–282°C; CHE, 95% purity MP 200–204°C (Southon and Buckingham, 1989) was obtained. 1-Heptanesulfonic acid, glutathione reductase, NADPH, low-melting point agarose, 8-hydroxy-2´-deoxyguanosine, Kodak X-Omat AR photographic film were from Sigma (St. Louis, MO, USA). Triethylamine 99.5% was purchased from Fluka Chemika (Buchs, Switzerland). Acetonitrile and methanol, HPLC gradient grades, hematoxylin and eosin were from Merck (Darmstadt, Germany). Human CYP1A1 antibody (goat polyclonal IgG), secondary horseradish peroxidase conjugated antibody, and western Blotting Luminol Reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Protein determination by bicinchonic acid method was done using a Pierce (Rockford, Il, USA) commercial kit. Phosphoric acid and other chemicals were obtained from local distributors and were of analytical grade.

Diet preparation. For rats in the control group the standard diet for laboratory animals (KrmiMo, Tetcice, Czech Republic) was used. For the experimental group, sanguiritrin, (0.12 g) was mixed with 1 kg standard diet. A small amount of water was added, mixed well and the small granules (length ~3 cm) were prepared. The granules were dried at 37°C in a dryer and stored in paper bags kept dry.

The content of QBA in the feed (i.e. homogenity of diet) was randomly checked by HPLC analysis. Supplementation of the feed with sanguiritrin remained constant for the duration of the experiment.

Animals. All procedures with animals were approved by the Ethics Committee, Ministry of Education, Czech Republic and were in accordance with the Czech Animal Protection Act No. 167/1993 Coll. and with the Good Laboratory Practice Regulations.

Male Wistar rats (180 ± 10 g body weight) were housed under standard laboratory conditions with free access to feed and water in a room maintained at 23 ± 2°C with light/dark cycle. After 1 week animals were randomly divided into two groups (n = 8). Control group consumed *ad libitum* a standard laboratory diet, experimetal group consumed (*ad libitum* as well) a *M. cordata* – enriched standard laboratory diet (120 ppm sanguiritrin, i.e. 100 ppm QBA), for 109 days. The total amount of administered QBA depended on the individual quantity of consumed diet, which was 25 g on average. The diet consumption was checked in both groups twice per week. The health condition of animals was checked up daily and the body weight was monitored once a week. For the determination of QBA, the feces were collected on day 50 and 109, urine on day 109 only. At the end of the experiment the animals were fasted over night, access to water was *ad libitum*. A terminal i.m. anesthesia by fentanyl (4 µg/100 g body weight), medetomidin (20 µg/100 g body weight) and diazepam (0.5 mg/100 g body weight) was applied. After opening of the abdominal cavity, a macroscopic examination of the main organs (digestive tract, heart, kidney, liver, lungs and other organs) was performed. The blood was collected from the aortic bifurcation and placed in a tube containing Na_2EDTA (1 mg/ml) and NaN_3 (0.1 mg/ml). Part of the blood was used for lymphocytes isolation. Blood was centrifuged at 2 500 g for 10 min at 4°C to obtain the plasma. The plasma aliquots were stored at –80°C for determination of QBA content, biochemical analyses, and parameters of antioxidant capacity. Erythrocytes were washed with phosphate buffered saline and were stored at –80°C for the determination of parameters of oxidative stress. Liver, muscle, kidney, myocardium, intestine were removed, washed in cold phosphate buffered saline and the selected organs (liver, heart, kidney) were weighed and stored at –80°C for determination of QBA content. Liver was utilized for biochemical and genotoxic analysis and parameters of oxidative stress. The histological examination of liver and intestine was performed.

Determination of SA/CHE in sanguiritrin, diet, feces, urine, tissues and plasma

Sample preparation. Diet and feces were extracted with acidified (1% HCl) methanol in a Soxhlet extractor for 12 h and the extracts after appropriate dilution by mobile phase (0.01M 1-heptanesulfonic acid, 0.1M triethylamine, pH 2.5 (H_3PO_4) in 25% (v/v) acetonitrile) were subjected to HPLC analysis.

Urine was diluted 1:1 by mobile phase (0.01M 1-heptanesulfonic acid/0.1M triethylamine, pH 2.5 (85% H_3PO_4) in 25% acetonitrile), vortexed for 1 min, sonicated in an ultrasonic bath for 2 min, and centrifuged at 2 500 g for 1 min at room temperature. Supernatant (50 µl) was applied onto an HPLC column.

Plasma specimens (0.5 ml) were diluted by 1 ml of 0.01M 1-heptanesulfonic acid in water, added to the conditioned (4 ml of methanol, 2 ml of water, and 1 ml of 0.01M 1-heptanesulfonic acid in water) C_{18} SPE cartridges (Speed Octadecyl C18/18%, 200 mg/3 ml, Applied Separations, USA), washed by 2 ml of 0.01M 1-heptanesulfonic acid in water, and QBA were eluted by 2 ml of 0.01M 1-heptanesulfonic acid in 95% methanol. After evaporation of the eluate and dissolution in the mobile phase (500 µl) and filtration through a 0.45 µm filter the samples (50 µl) were applied onto an HPLC column.

Tissues (1 g or maximum available amount of liver, kidney, muscle, and myocardium) were homogenized with a mechanical homogenizer in 4 ml of a 0.01M 1-heptanesulfonic acid in 95% acetonitrile. The homogenate was vortexed for 1 min, sonicated in an ultrasonic bath for 2 min, and centrifuged at 2 500 g for 1 min at room temperature. The supernatant (3 ml) was evaporated, dissolved in the mobile phase (600 µl), filtered through a 0.45 µm filter and applied (50 µl) onto an HPLC column.

HPLC analyses. HPLC analyses were carried out on a Shimadzu Class VP (Shimadzu Corporation, Tokyo, Japan), consisting of: SCL-10Avp system controller, degassing unit DGU-14A, low-pressure gradient flow control valve FCV-10Alvp, pump LC-10ADvp, auto injector SIL-10ADvp with 500 µl loop, column oven CTO-10AC, a UV detector SPD-10Avp and a fluorimetric detector RF-10Axl

using a 250/4 (5 µm) Purospher® Star RP-18e reversed phase column with 4/4 guard column containing the same sorbent (Merck, Darmstadt, Germany).

The mobile phase was 0.01M 1-heptanesulfonic acid, 0.1M triethylamine, pH 2.5 (H_3PO_4) in 25% v/v acetonitrile in a gradient with 0.01M 1-heptanesulfonic acid, 0.1 M triethylamine, pH 2.5 (H_3PO_4) in 60% v/v acetonitrile. The following gradient elution was applied (min/% B): 0/0, 1/10, 1.5/20, 11/20, 15/100, 23/100, 23.1/0, 28/Stop. The detection was carried out at 285 nm (UV) and/or 327 nm excitation and 577 nm emission wavelength (fluorimetry). To express the content of alkaloids, reference solutions in the mobile phase were used as an external standard. The concentration range used for calibration was from 0.005 to 1 µg/ml. The stock solution of sanguiritrin was prepared in methanol; standard solution was diluted from stock solution by mobile phase.

Clinical chemistry and oxidative stress parameters and determination of cytochrome P450

Clinical chemistry parameters. Levels of sodium, potassium, chlorides, bilirubin, urea, creatinine, glomerular filtration, and activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GMT), alkaline phosphatase (ALP) were analyzed. All the analyses were carried out on a ILab 600 analyser (Instrumentation Laboratory, Spain) in the Department of Laboratory Medicine, Hospital Sternberk.

Parameters of oxidative stress. Lipid peroxidation was assessed by measuring the presence of thiobarbituric acid reactive substances in the plasma or liver homogenates (Buege and Aust, 1978). The level of GSH in erythrocytes and liver homogenate was determined according to Sedlak and Lindsay (1968) using the Ellman reagent. The level of total SH-group (TSHG) was determined according to Hu (1994). An indirect spectrophotometric method used for the assessment of SOD activity in erythrocytes and liver homogenate was based on the generation of $O_2^{\cdot-}$ by a mixture of nitro blue tetrazolium, NADH and phenazine methosulfate (Ewing and Janero, 1995). The glutathione peroxidase activity was assayed by a modification of the method of Tappel (1978). The total antioxidant capacity of plasma was assessed by the chemiluminescence method using trolox as a standard (Girotti et al., 2002). The plasma and liver homogenate total antioxidant capacity was detected by cyclic voltammetry (Kohen et al., 2000). The protein concentration was determined by the Lowry method.

Total content of cytochrome P450 and estimation of the CYP1A levels. The liver microsomes were isolated by differential centrifugation (Lake, 1990) and the cytochrome P450 content was measured (Omura and Sato, 1964). The microsomal protein concentration was measured by bicinchoninic acid. The level of CYP1A was detected by immunoblotting after SDS-PAGE electrophoresis using a primary antibody against human CYP1A (goat polyclonal IgG) and a secondary antibody conjugated with horseradish peroxidase using chemiluminescence detection. The cytochrome P4501A level was evaluated using a Diana 2 CCD-Camera system.

Histological examination

Liver and intestine specimens were fixed in Baker mixture, embedded in paraffin and 7 µm thick sections were cut on a rotary microtome. Sets of the histological sections were stained with hematoxylin-eosin and PAS. The histological evaluation was performed on an Olympus BX 40 light microscope.

Genotoxicity

Detection of single-stranded DNA breaks. The DNA breaks were measured in peripheral lymphocytes and liver cells using an alkaline version of Comet assay. Lymphocytes were isolated on Histopaque 1077 gradients. Liver cells (predominantly hepatocytes) were isolated after brief trypsinization of liver tissue. Cells embedded in agarose layer on microscope slides were lysed in 10mM Tris-buffered 2.5M NaCl, pH 10.0) containing 1% Triton X 100, 100mM EDTA at 4°C for 1 hour. After a 40 min period of unwinding the electrophoresis was carried out at 20 V, 4°C for 20 min (Vodicka et al., 2001). DNA damage was analyzed on an Olympus IX 70 fluorescence microscope after staining by ethidium bromide. One hundred cells per slide were analyzed and divided into four classes of DNA damage standard scale. Total DNA damage was calculated as follows: number of cells in class 1 × 1 + number of cells in class 2 × 2 + number of

cells in class 3 × 3 + number of cells in class 4 × 4. The maximal value of damage was 400.

HPLC-ECD analysis of 8-hydroxy-2′-deoxyguanosine (8-OH-dG). Blood plasma samples (0.5 ml) were deproteinated by centrifugation (60 min; 7 200 g) using a Microcon YM-10 (Millipore) apparatus.

HPLC analysis. HPLC analyses were carried out on an HPLC system containing an isocratic pump ESA-582 (ESA Inc.), a coulometric detector Coulochem III (ESA Inc.), and a column Purospher Star RP-18e, 125 × 4 mm (I.D.) with a guard column 4 × 4 mm (I.D.) containing the same sorbent (Merck, Darmstadt, Germany).

The mobile phase was 20mM sodium dihydrogenphosphate (Fluka, Buchs, Switzerland) in redistilled water, pH 5 and methanol (90:10; v/v). The flow rate was 0.8 ml/min. Samples (20 µl) were applied using a manual valve. The detection was done at the potential E_1 = 20 mV and E_2 = 450 mV vs. Pd, the guard cell 500 mV vs. Pd, sensitivity 1 µA/V. The concentration range used for calibration was from 10–100 nmol/l.

Analysis of DNA adducts. DNA was isolated from liver tissues of rats by the phenol/chloroform procedure (Stiborova et al., 1994). DNA was dissolved in sterile water and the solution stored at –80°C. One pooled sample of mitochondria was prepared from 8 animals in each group: a piece of liver (1 g) of each animal was used for the preparation of the homogenate in an isolation buffer (220mM mannitol, 68mM sucrose, 10mM Hepes-KOH, pH 7.4, 10mM KCl, 1mM EDTA, 0.1% BSA, and protease inhibitors). The homogenate was centrifuged (600 g for 10 min) to remove pellet nuclei and unbroken cells and then the supernatant was centrifuged (3 500 g for 15 min) to obtain mitochondria. The mitochondrial DNA was isolated by the phenol/chloroform procedure. DNA was dissolved in sterile water and the solution stored at –80°C. The nuclease P1 and the *n*-butanol extraction versions of the ^{32}P-postlabeling assay found previously to be suitable for detection and quantitation of sanguinarine- and chelerythrine-derived DNA adducts formed *in vitro* (Stiborova et al., 2002) were used to analyze DNA of rats exposed to sanguiritrin. The same ^{32}P-postlabeling procedure was used for DNA of control rats. The detection limit of relative adducts labelling was $0.1/10^9$ nucleotides.

Statistical analysis

The values were expressed as a mean ± deviation. The *t*-test was used for calculations of significance between both groups of animals ($P < 0.05$).

RESULTS

Diet consumption and body weight

The daily QBA dose was approximately 2.36 mg per animal, which is on average 10 mg/kg body weight. The body weight, the weight of liver, right-hand kidney and heart were the same in both groups of animals (Table 1). In Figure 1, the monitored growth characteristics and feed consumption display a very similar course in both animal groups. No impairment in animal health status was observed.

Distribution of alkaloids

The content of SA and CHE in the feed, feces, plasma, liver, kidney, muscle and myocardium is

Table 1. Effect of sanguiritrin on rat body and organ weights

Weight (g)	Group of animals	
	Standard diet	Diet containing 100 ppm QBA
Body weight	526 ± 48	524 ± 41
Weight of liver	16.83 ± 1.61	17.11 ± 2.41
Weight of right-hand kidney	1.76 ± 0.35	1.80 ± 0.22
Weight of the organ/body weight (%)	1.34 ± 0.10	1.35 ± 0.18
Liver	3.20 ± 0.22	3.26 ± 0.30
Right-hand kidney	0.33 ± 0.07	0.34 ± 0.03
Heart	0.26 ± 0.03	0.26 ± 0.03

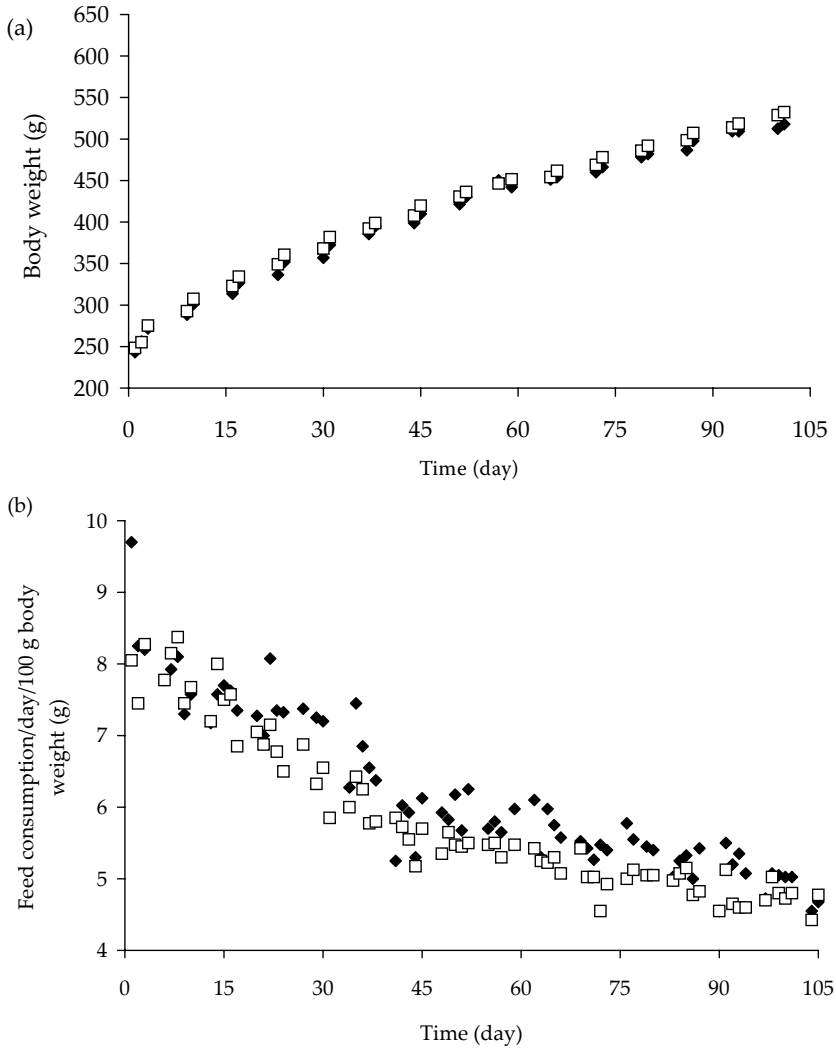

Figure 1. The effect of QBA on body weight (a) and diet consumption of rat (b)

◆ Standard diet
□ Diet containing QBA

shown in Table 2. SA was found in feces, all body tissues and plasma of the 100 ppm QBA diet group, but CHE, whose proportion in the feed was lower, was detected only in feces, liver and kidney. The retention of SA, at a level between the limit of detection and the limit of quantification, was found in the muscle and myocardium.

QBA effect on of clinical chemistry and oxidative stress parameters and level of cytochrome P450

The following biochemical markers were investigated: bilirubin, urea, creatinine, ALT, AST, GMT, ALP, glomerular filtration (Table 3). They displayed no significant difference between the QBA-treated and control group of animals. We found no significant effect of QBA on the selected parameters of oxidative

Table 2. Determination of sanguinarine and chelerythrine in feces, plasma and organs[a]

Sample	SA (μg/g)	CHE (μg/g)
Diet	42.4 ± 1.6	34.9 ± 0.4
Feces	138.5 ± 0.3	86.0 ± 0.5
Plasma[b]	0.008 ± 0.002	n.d.
Liver	0.083 ± 0.013	0.024 ± 0.007
Kidney	0.011 ± 0.002	0.009 ± 0.003
Muscle[c]	0.004 ± 0.001	n.d.
Myocardium[c]	0.005 ± 0.001	n.d.

[a]rats in the experimental group were fed a dose 120 mg of sanguiritrin in 1 kg of feed; [b]μg/ml of plasma; [c]the limit of detection/quantification of QBA was 0.003/0.006 μg/g
n.d. = not determined

stress in plasma, erythrocytes or liver. No differences in the level of total liver cytochrome P450 and CYP1A between both groups of animals were found either. The results are summarized in Table 3 and Figure 2. Variations in the levels of CYP1A are caused by differences in basal expression of the enzymes.

Table 3. Effects of QBA on biochemical and oxidative stress markers in the body and the total content of liver cytochrome P450

Parameter	Unit	Group of animals	
		standard diet	diet containing 100 ppm QBA
Bilirubin[a]	μmol/l	3.40 ± 0.40	4.00 ± 1.20
Urea[a]	mmol/l	6.48 ± 0.84	6.00 ± 0.47
Creatinine[a]	μmol/l	53.0 ± 4.10	51.0 ± 6.6
ALT[a]	μkat/l	1.37 ± 0.47	1.21 ± 0.65
AST[a]	μkat/l	3.40 ± 1.35	2.58 ± 1.74
GMT[a]	μkat/l	0.03 ± 0.00	0.03 ± 0.01
ALP[a]	μkat/l	0.37 ± 0.05	0.37 ± 0.12
Glomerular filtration[a]	ml/s	4.06 ± 0.37	4.29 ± 0.54
TAC – cyclic voltammetry[a]	nA/g[d]	7.40 ± 1.56	5.70 ± 1.25
TAC – chemiluminiscence[a]	mmol/g[d]	1.12 ± 0.04	1.30 ± 0.12
SH-groups[a]	μmol/g[d]	1.78 ± 0.74	2.26 ± 0.40
TBARS[a]	nmol/g[d]	172.00 ± 9.82	165.71 ± 10.18
GSH[b]	mmol/g[d]	2.01 ± 0.28	1.95 ± 0.31
TBARS[b]	nmol/g[d]	49.29 ± 7.15	55.00 ± 7.93
GPX[b]	mmol/min/g[d]	0.40 ± 0.02	0.42 ± 0.05
SOD[b]	U/g[d]	22.94 ± 3.63	23.83 ± 0.28
TAC – cyclic voltammetry	μA/g[d]	0.39 ± 0.04	0.37 ± 0.06
GSH[c]	mmol/g[d]	26.88 ± 2.30	25.91 ± 3.19
TBARS[c]	nmol/g[d]	48.07 ± 6.49	55.00 ± 7.82
GPX[c]	mmol/min/g[d]	0.51 ± 0.07	0.68 ± 0.09
SOD[c]	U/g[d]	46.93 ± 7.53	46.53 ± 5.56
Cytochrome P450[c]	μmol/g[d]	0.54 ± 0.07	0.60 ± 0.11

[a]plasma; [b]erythrocytes; [c]liver; [d]the value was expressed per 1 gram of protein

Figure 2. Effects of QBA on liver microsomal protein level of cytochrome P450 1A1 and 1A2. Immunoblot of microsomal cytochrome P450 1A1 and 1A2; total amount of CYP1A1/1A2 in group of animal treated standard/QBA-enriched diet was: 99.30 ± 26.04/106.97 ± 42.26

Control, C – microsomes isolated from hepatoma cell line Hep G2. The animal group treated with a standard diet (1–8). The animal group treated with a diet enriched by 120 ppm of sanguiritrin (9–18)

Genotoxicity of QBA

To explain the genotoxicity of QBA, sanguinarine and chelerythrine, three different methods were used. The formation of single-stranded DNA breaks measured by Comet assay in peripheral lymphocytes and hepatocytes was done in both groups of animals. The damage to DNA was calculated as described in Material and Methods. In peripheral lymphocytes and hepatocytes of standard/QBA-enriched treated animals the DNA damage was 1.9 ± 2.8/8.6 ± 11.4 and 14.6 ± 12.0/11.7 ± 13.0. No significant differences in the formation of single-stranded DNA breaks measured by Comet assay was observed in isolated cells. The level of 8-OH-dG in the plasma was lower than 10 nmol/l, which was the detection limit of the HPLC method used. Using the ^{32}P-postlabeling procedure suitable for detection and quantitation of covalent adducts in DNA (Stiborova et al., 1994), no sanguinarine- and chelerythrine-derived DNA adducts were detected in hepatic nuclear and mitochondrial DNA of rats exposed to sanguiritrin in a diet.

QBA effect on the microstructure of liver and intestine

A minor lymphocytes and polymorphonuclear infiltration in portobiliar spaces was found. The liver glycogen content was similar in both groups of animals. In the intestine there was found a higher amount of cells in stroma of villi and a mild increase of tumbler cells without inflammation. No pathological changes were found in the submucous and musculature. The histological examination of the liver and intestine did not reveal any pathological changes (Figure 3).

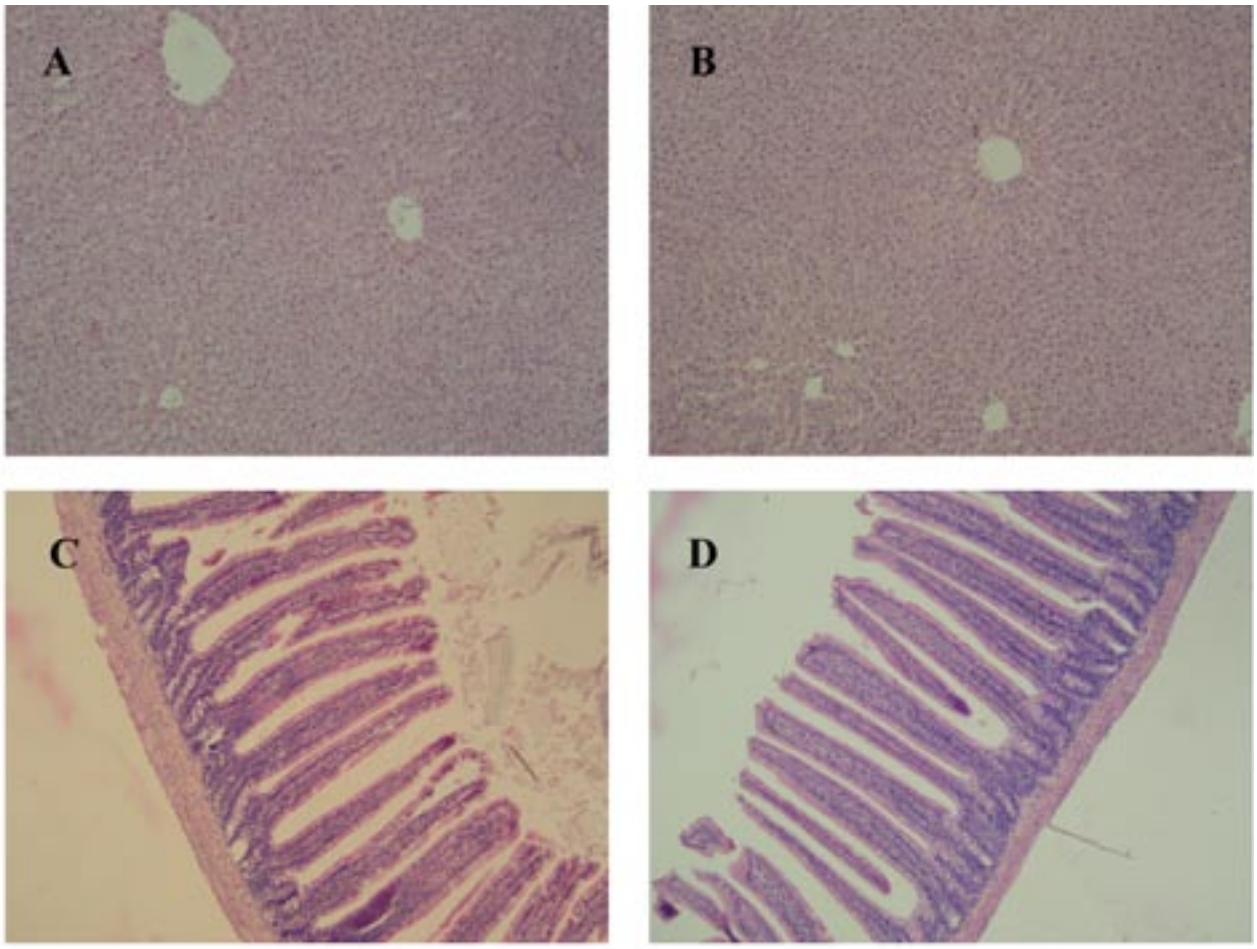

Figure 3. Selected microphotographs of the liver and intestine of the control and QBA-treated rat (60×). Liver (A) and intestine (C) of the control rat, liver (B) and intestine (D) of the QBA-treated rat

DISCUSSION

In the previous study, it was demonstrated that in pigs after a long-term feeding with a diet containing 100 ppm sanguiritrin in 1 kg feed (daily oral dose of QBA up to 5 mg/kg body weight), SA and CHE were accumulated in the GIT and their principal way of clearance was their elimination *via* feces (Kosina et al., 2004). In this study we checked whether the consumption of a daily dose of 10 mg QBA/kg body weight affects the basic metabolic parameters of rat and whether damage to DNA takes place. After a 109-day oral application of 120 ppm sanguiritrin (100 ppm QBA/kg feed) to rats, no negative effects were observed on body weight and diet consumption (Figure 1). In this work, the focus was given to the retention of the quaternary forms of SA and CHE in vital rat organs and in their possible adverse effects at a long-term oral administration. The reason for this approach was that the quaternary forms, not dihydroderivatives, are active in the organism. It has been found here that the rat body organs retained a certain part of alkaloids in the order liver > plasma ≈ kidney > muscle ≈ myocardium (Table 2). These results differ fundamentally from those of Tandon et al. (1992) in the quantity of alkaloids determined in the plasma, liver, kidney and myocardium. The quantities found here are smaller by three orders of magnitude. Such difference in QBA retention (taking into account that only a single dose was applied by Tandon et al. (1992) is hard to explain. In urine, no SA, CHE or benz[c]acridine were detected. Parameters of clinical chemistry and oxidative stress did not differ in the control and experimental group (Table 3). DNA damage in peripheral lymphocytes and hepatocytes was not demonstrated in Comet assay. Also, no formation of sanguinarine- and chelerythrine-derived DNA adducts in liver DNA or 8-OH-dG, a marker of DNA damage, in the plasma was found. The level of microsomal cytochrome CYP1A was not affected by a long-term exposure of the organism to the alkaloids (Figure 2). From the morphological evaluation, no pathological alterations of the intestinal mucous membrane and liver parenchyma (Figure 3) were observed. In short, the long-term oral administration of QBA of *M. cordata* in the dose of 10 mg/kg body weight to rats proved safe for animals, a conclusion analogous to the results of our study of a long-term QBA administration to pigs (Kosina et al., 2004).

In a recent experiment in mice, where SA was administered intraperitoneally in doses of 5, 10, and 15 mg/kg body weight, already after a 10 mg dose chromosomal aberrations and sister chromatid exchanges in bone marrow cells were found (Das et al., 2004). Damage to DNA of blood and bone marrow cells after an i.p. administration of the dose of 10 mg/kg to mice was also described in a recent work from the same laboraratory (Ansari et al., 2005). A caution is presented in these papers (based on the damage to DNA) regarding to the use of QBA-containing products in toothpaste and mouthwash and feed additives. However, it should be taken into account that the results discussed were obtained with an i.p. administration; second, the doses used were high (10 and 15 mg/kg body weight). In fact, most biologically active substances become toxic when i.p. administered in high doses, that is to say their effects in high concentration differ substantially from those after a normal dose. Also, cellular studies have shown that SA/CHE affect nonspecifically a great number of molecular targets at the level of the membranes and organelles (Simanek et al., 2004; Zdarilova et al., 2006).

Recently, in a parallel experiment it has been found that in the plasma and liver of the rats, to which a single oral dose of SA (10 mg/kg body weight) was administered, dihydrosanguinarine (DHSA) was identified as a SA metabolite and determined (Psotova et al., 2006). SA and DHSA were not detected in urine. Benz[c]acridine was found neither in urine nor in plasma and liver. In both the plasma and the liver samples, a significantly higher level of DHSA in comparison with that of SA was found. In an experiment with cell cultures of rat hepatocytes, the conversion of SA into DHSA was also proved (Zdarilova, 2006). Dihydroderivatives of SA/CHE are markedly less toxic *in vitro* compared to parent alkaloids on molecular targets in the animal cell (Vavreckova et al., 1994).

Taken together, it is evident that the results of *in vitro* experiments with QBA are not directly transferable to animals exposed to QBA after an oral administration. In cellular studies with QBA, only alkaloid concentrations higher than those existing in blood of experimental animals (after a long–term p.o. administration) are able to elicit a biological effect. Also, it is not possible to simply extrapolate the toxic effects of the QBA observed after a single i.p. dose to the whole organism exposed to oral QBA applications. Alkaloids tested were not shown to induce an oxidative stress *in vivo*. In this

respect, the toxicity of argemone oil originally ascribed to the QBA manifesting as oxidative stress, is likely to be caused by other oil components than alkaloids.

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