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In vitro Arsanilic Acid Induction of Apoptosis in Rat Hepatocytes

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ABSTRACT : This paper aimed to study the toxicity of arsanilic acid on rat primary hepatocytes *in vitro* by a modification of the perfusion method. The conditions included concentrations of 0, 1.085, 10.85, 108.5, 1,085 and 10,850 mg/kg arsanilic acid in RPMI 1,640 medium at rat hepatocytes plates respectively, each group had five repeats at 37°C for 48 h. The rat primary hepatocytes survival ratio, DNA Ladder, activities of glutathione peroxidase (GSH-px), superoxide dismutase (SOD) and catalase (CAT) in hepatocytes, activity of SOD in the medium and the expression of gene bax in hepatocytes were measured at 12 h, 24 h and 48 h respectively. The results showed that arsanilic acid decreased the activities of GSH-px and SOD, and increased the activity of CAT in all dosages, and affected as positive DNA ladder. Although the SOD activities of both hepatocytes and medium in 1.085 mg/L arsanilic acid were significantly lower than the base line at 12 h, CAT activity in 10.85 mg/L arsanilic acid was significantly higher than the base line at 48 h, and all of the DNA ladders were positive, which means 1.085 mg/L arsanilic acid induced apoptosis at 24 h. The gene expression of bax was significantly upregulated in 1.085 mg/L arsanilic acid or higher for 24 h.The parameters in 1,085 mg/L and 10,850 mg/L arsanilic acid had more severe changes than the others at any time indicating that these levels of arsanilic acid were toxic hazards for hepatocyte survival. It was concluded that arsanilic acid induced a dosage- and time-dependent gene expression of bax, 1.085 mg/L arsanilic acid could be involved in rat liver cell apoptosis at 24 h. Arsanilic acid as additives in livestock feed could present potential toxic implications for farm animals. (**Key Words :** Arsanilic Acid, Apoptosis, Antioxidant Enzymes, Gene Expression)

INTRODUCTION

Arsenic is an ubiquitous element, ranking twentieth in elemental abundance in the Earth's crust (Schroeder and Balassa, 1966). Natural sources of arsenic in the environment including soils, plants and animals that have accumulated arsenic are effective growth promoters for livestock and poultries (Frost, 1953). The arsanilic acid exhibit a moderate margin of safety, chickens being especially tolerant to the organoarsenicals, showing a ratio of toxic level to maximal permissible level in the feed of at least 5:1. For these reason arsanilic acid has been in extensive use by poultry producers (Calvert and Smith, 1981). Unfortunately, arsanilic acid poisoning through diet continue to be caused by the varying amounts of a variety of organic and inorganic arsenical compounds (Osweiler et al., 1985; Cockell and Hilton, 1988; Bahri and Romdane, 1991). The arsanilic acid directly enter the environment through excrements, and contamination through biological and abiological agents (Morrison, 1969; Moore et al., 1998). The persistence of arsanilic acid in the soils, plants and feedstuffs could present potential problems. Li et al. (2002) and Yao et al. (2003) concluded that arsenicals induced oxidative damage, also inhibited immune function. Likewise, Yang et al. (2003) suggested that different dosages of arsenic exerted morphological changes in rat hippocampal neurons including degeneration, apoptosis and necrosis in various degrees. The objective of the present study was therefore, undertaken to evaluate the effect of arsanilic acid on primary cultured rat hepatocytes toxicity *in vitro*, as well as to elucidate the role of antioxidant defense system in arsanilic acid induced apoptosis and apoptosis related oxidative stress.

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MATERIALS AND METHODS

Materials

30-day-old 110-150 g Wistar rats were bought from experimental animal station of Hunan Agricultural University, arsanilic acid from Huangyan Rongyao chemical factory of Zhejiang Province, China. RPMI1640

(GIBCO BRL), Collagenase (type IV), animal cell apoptosis DNA Ladder drawing reagent kits (20T) and DNA mark were provided by Dingguo Biology Technique Development Center, MTT kits provided by BBI Corporation; Bax Immunohistochemical Reagent kits provided by (Santa Cruz Biotechnology INC., CA.).

Isolation and culture of rat hepatocytes

Hepatocytes were isolated from male rats by a modification of the method of Seglen (1976). All procedures on the animals were performed according to the CEE directive 86/609 on animal experimentation. Rats were anesthetized with diethylether, the pre-perfusion of the liver *in situ* was performed at a rate of 20-30 ml/min with Ca^{2+}-free Hanks balanced salt solution. The liver was then excised and the digestion was carried out by adding 0.05% (w/v) collagenase (Sigma, type IV) in Hanks balanced salt solution supplemented with $CaCl_2 \cdot H_2O$ (0.0186 g/L) at a flux rate of 40 ml/min. At this point liver was transferred to a square plate containing 100 ml of RPMI 1,640 medium supplemented with 200 mM L-glutamine, 20 ml/L essential amino acid solution and 10 ml/L non-essential amino acid solution, 1% antibiotic antimycotic stabilized solution and 100 μM L-arginine (incomplete medium). The cells were dispersed by gentle disruption with a stainless steel comb. After filtration through 200 μm Nytal mesh, parenchymal cells (hepatocytes) were separated from nonparenchymal cells (endothelial cells, Kupffer cells and stellate cells) by centrifugation at 50 g in Eppendorf Centrifuge 5,810R at 4°C for 2 minutes and then washed twice in washing buffer (Blomhoff and Berg, 1990). Then the cells were resuspended in the same medium and filtered through 63 μm Nytal mesh. The viability of the cells was more than 80%, as estimated by trypan blue dye exclusion test (Kaltenbach et al., 1958). After cell counting the cells were diluted at a concentration of 5×10^5 cells/ml with incomplete medium supplemented with 2% fetal calf serum, 0.1 U/ml insulin and 10^{-6} M dexamethasone (complete medium). The hepatocytes were then plated in 24 well-plates coated with rat tail collagen at the final cell density of 2.5×10^5 cells per well and incubated at 37°C in an humidified atmosphere of 5% CO_2 and 95% air. After 12 h incubation, the medium was changed and replaced with incomplete medium to remove dead cells. To verify the isolation method efficiency, the acid phophatase activity per mg of proteins was evaluated. According to literature data, the specific activity of acid phophatase in nonparenchimal cells is 1.7 folds the same activity in parenchimal cells (Munthe Kaas et al., 1976).

Experimental design and treatments

The hepatocytes were incubated with arsanilic acid 0, 1.085, 10.85, 108.5, 1,085 and 10,850 mg/L respectively as the final concentration in 10 ml RPMI 1,640 medium. Each group has 5 repeats. Cells were seeded at a density of 2.5×10^5 cells/ml. All cells were seeded at the same time. The media were changed every twelve, twenty-four and forty-eight hours after seeding cells, culture media containing different concentration of arsanilic acid was collected. Cells were detached from the plate by trypsinisation for collecting, counting and homogenisation. After washing twice with phosphate-buffered saline solution, the cells were treated with 1 ml Trypsin in each 10 ml flask and incubated at 37°C for approximately 5 minutes. Trypsin was inhibited by adding an equal amount of RPMI 1,640 medium to the incubation solution. After centrifugation and removal of the supernatant, the cells were suspended with 0.5 ml RPMI 1,640 medium and resuspended in 1 ml more medium for dilution, and then treated with trypan blue for counting.

Sonification of the cells: After centrifugation, the cells were sonicated for 15 seconds in bursts of 30 seconds with cooling at 0°C. The homogenate was then centrifuged at 22,000×g for 20 minutes. All enzymatic activities and protein assays were measured in the supernatants. *Glutathione peroxidase* activity was assayed using method of Mohandas et al. (1984) and expressed as μmol of GSH oxidized/min/mg protein. *Superoxide dismutase* (SOD) level in cytosolic fractions of liver was estimated using Ransod kit supplied by Randox laboratories, Ardmore, Northern Ireland, UK. Briefly, xanthine and xanthine oxidase were used to generate superoxide anion which reacts with 2-(4-indophenyl)-3(4-nitro-phenyl)-5-phenyl tetrazolium chloride (INT) to form a red formazan dye. One enzyme unit of superoxide dismutase was defined as the amount, which inhibits the INT reaction by 50%. Specific activities were defined as units/mg protein. *Catalase* (CAT) activity was assayed according to the method of Claiborne (1985) and expressed as nmol of hydrogen peroxide decomposed/min/mg protein. *Protein* contents were measured according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

Determination of cell viability

Cell viability was determined by MTT test method (Mosmann, 1983) and confirmed by Trypan blue exclusion test (Kaltenbach et al., 1958). MTT (5 mg/ml) was dissolved in RPMI1,640 without phenol red. The solution is filtered through a 0.2 μm filter and stored at 2-8°C for frequent use. To determine the effects of bradykinin on cell viability, cells were treated with bradykinin (0.01 mM and 0.1 mM) for a 2 h period. After that cells were used either immediately or after an additional 24 h incubation period in incomplete medium. For the determination of cell viability, the medium has been discarded and MTT solution was

Table 1. The inhibition of arsanilic acid on hepatocytes (MTT, mg/L)

Time (h)	0	1.085	10.85	108.5	1,085	10,850
12 (OD)	0.12±0.02	0.11±0.01	0.13±0.01	0.10±0.02	0.10±0.02	0.07±0.02*
24 (OD)	0.14±0.05	0.11±0.01	0.08±0.02*	0.08±0.02*	0.05±0.01**	0.04±0.01**
48 (OD)	0.08±0.01	0.09±0.01	0.08±0.00	0.053±0.01*	0.036±0.01**	0.03±0.01**

n = 5. * Compared with control group at the same time $p<0.05$. ** Compared with control group at the same time $p<0.01$.

added and incubated for 3 h. At the end of the incubation period the MTT solution was removed and the cells and dye cristals were dissolved by adding dimethylsulfoxide (DMSO). Absorbance was measured at 570 nm in a Shimadzu UV-2100 Spectrophotometer and the results were expressed as a percentage of the absorbance of the samples.

DNA ladder in agarose gel electrophoresis

DNA fragmentation in hepatocytes was determined as reported previously (Nakamura et al., 2000). Briefly, the DNA was extracted by phenol-chloroform method. Samples were loaded into wells of 1% agarose gel in 1×TBE. DNA was visualized by ethidium bromide staining. Markers lane of DNA molecular weight standards from 100 to 1,500 bp was run.

Analysis for Bax protein expression by immuno-histochemistry

Immunostaining on formalin fixed, paraffin embedded tissue sections was done by the avidin-biotin peroxidase complex method using the ABC staining kit. (Santa Cruz Biotechnology INC., CA.) Sections of 5μm thick sections were mounted on poly-L-lysine coated slides, deparaffinized, and rehydrated through xylene and alcohol. The slides were incubated with the blocking solution (0.03% H_2O_2+ methanol) for 20min to block endogenous peroxidase. The antigenic sites were unmasked by means of three cycles of 5 min microwave irradiation in 10mM citrate buffer (pH 6.0). All the primary antibodies were obtained by Santa Cruz Biotechnology INC., CA, USA. The slides were incubated with the primary antibody against Bax used at 1:50 dilutions, for 2 h at room temperature followed by phosphate buffered saline (PBS) wash and incubation with the anti-mouse IgG for 45 min at room temperature. After PBS washing, the sections were incubated with ABC reagent for 45 min at room temperature. The reaction products were again washed in PBS then developed with diaminobenzidine tetra hydrochloride (DAB) (Sigma chemical Co., St. Louis, MO) as a chromogen. The sections were lightly counter-stained with haemotoxylin followed by dehydration, clearing, and mounting with DPX. The brown reaction product was scored by light microscopy scoring of immunohistochemistry was done by the scoring system used previously (Brambilla et al., 1996; Shabnam et al., 2004). Briefly, the percentage positivity and intensity of staining was taken into account. The appearance of cell colour in slice marked as follows: cell was no brown and yellow, negative (-); cytoplasm presented canary and nebulous, viz (+); the cytoplasm presented maize particles, viz (++); the cytoplasm presented equably puce, viz (+++). Each slice chose 10 times eyeshot (move slice at one direction, no repeat, no superpose), count electropositive cell ratio (electropositive cell percentage of whole cell in each slice). When electropositive cell ratio was less than 20%, expression of bax protein was downer; when the ratio was between 20% and 30%, the bax protein expression was more upper; when the ratio exceeded 30%, the bax protein expression was the most lofty.

Statistical analysis

The results were presented as the least squares means value±standard deviation and one-way analysis of variance was performed using the General Linear Models Procedures of the SAS software (2000). Differences among means were tested using Duncan's multiple range tests. A significant level of 0.05 was used.

RESULTS

Rat primary hepatocytes survival ratio

The primary hepatocytes proliferation was measured by MTT at 12 h, 24 h and 48 h respectively (Table 1). Our study shows that arsanilic acid inhibited the cell proliferation in a dose- and time-dependent manner, as assessed by MTT. At the concentration of 10.85 mg/L and higher, arsanilic acid significantly inhibited the proliferation of hepatocytes, compared with controls that were treated with arsanilic acid in a dose-dependent and time-dependent manner at concentrations between 10.85 mg/L ($p<0.05$) and 10,850 mg/L ($p<0.01$), and at time between 24 h and 48 h. No significant difference was observed when 1.085 mg/L of arsanilic acid was used ($p>0.05$).

Inspection of DNA ladder

To determine whether arsanilic acid induced apoptosis in rat hepatocytes, DNA fragmentation was measured in cells treated with arsanilic acid. Apoptosis was identified on the basis of the occurrence of DNA cleavage on agarose gel electrophoresis. Gel electrophoretic patterns of the DNA of arsanilic acid-treated or untreated cells were shown in Figure 1. No significant DNA cleavage was detectable in the control cells after 12, 24 and 48 h exposure. Cells

Table 2. Effects of arsanilic acid on the activities of antioxidant enzymes (mg/L)

Items	Time (h)	0	1.085	10.85	108.5	1,085	10,850
GSH-px in hepatocytes (Unit/mgprot)	12	22.15±2.61	21.49±1.77	20.89±1.27	18.96±2.22	18.59±1.56	18.23±1.46*
	24	22.20±2.04	21.01±1.01	19.18±1.16	17.57±2.70*	17.29±2.60*	16.58±2.04**
	48	21.46±1.02	19.91±0.90	18.50±2.46	16.82±1.81*	16.21±1.67**	15.67±2.54**
SOD in hepatocytes (U/mgprot)	12	26.58±0.66	23.89±0.56**	22.72±0.68**	21.71±0.91**	20.55±0.65**	20.51±0.88**
	24	24.55±0.45	21.93±0.63**	19.95±0.57**	19.29±0.36**	17.03±0.63**	15.95±0.42**
	48	19.91±0.82	15.39±0.58**	13.71±0.50**	12.12±0.62**	10.55±0.25**	9.56±0.58**
SOD in hepatocytes medium (U/mgprot)	12	26.19±0.61	25.60±0.40	24.91±0.34**	24.39±0.19**	24.01±0.32**	23.89±0.52**
	24	26.79±0.25	25.99±0.32*	25.30±0.41**	25.00±0.53**	24.61±0.37**	24.48±0.33**
	48	26.67±0.55	26.22±0.30	25.73±0.26**	25.27±0.18**	24.90±0.29**	24.25±0.43**
CAT in hepatocytes (mmol/L)	12	50.00±2.95	51.06±3.70	52.41±4.14	52.96±3.67	55.07±2.97	61.69±6.81
	24	73.51±2.20	76.21±1.29	76.41±2.20	80.29±1.53**	80.79±2.02**	82.56±4.68**
	48	95.19±0.91	97.23±1.40	99.60±1.78**	99.98±2.56**	104.48±0.98**	107.75±1.09**

n = 5, * Compared with normal control group at the same time $p<0.05$, ** Compared with normal control group at the same time $p<0.01$.

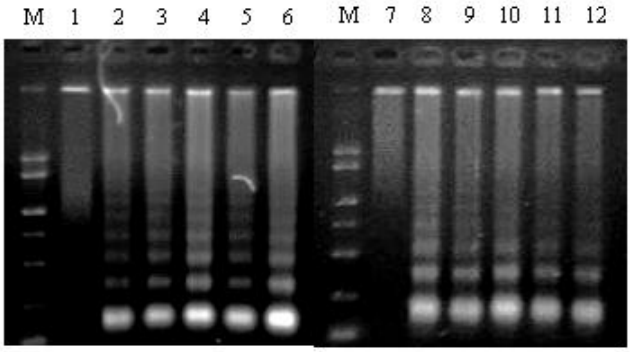

Figure 1. Dose- and time-dependent responses of DNA fragmentation of rat hepatocytes exposed to increasing arsanilic acid concentrations. DNA fragmentation was visualized as oligonucleosome-size fragments stained with ethidium bromide in agarose gels and transilluminated with UV light for photography. DNA was prepared from 4×10^6 cells incubated with 1.085, 10.85, 108.5, 1,085 and 10,850 mg/L arsanilic acid (lanes 2, 3, 4, 5 and 6 respectively) for 24 h and (lanes 8, 9, 10, 11 and 12 respectively) for 48 h and analysed as described. Lane M shows the molecular weight markers (100 bp-1,500 bp). Lane 1 and 7: control cells.

treated with 1.085, 10.85, 108.5, 1,085 and 10,850 mg/L showed a DNA ladder pattern characteristic of apoptosis after 24 h exposure, while different concentration of arsanilic acid resulted in similar condition was observed, which indicated that the 1.085 mg/L arsanilic acid was the lowest concentration of hepatocytes apoptosis for 24 h.

Effect of arsanilic acid on the activity of GSH-px, SOD and CAT in hepatocytes and SOD in culture medium

The effect of arsanilic acid exposed on three enzymes-GSH-px, SOD, CAT, in hepatocytes were depicted in Table 2. The activities of GSH-px and SOD were reduced, and CAT increased progressively at all doses. As shown in Table 2, GSH-px levels were found to be significantly decreased at 108.5 mg/L arsanilic acid on 24 h as compared to the control group ($p<0.05$), the activity of SOD were reduced

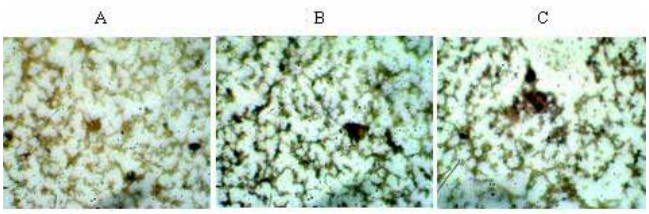

Figure 2. Hepatocyte gene expression of bax in 10.85 mg/L arsanilic acid (immunohistochemical staining). A: cultivation at 12 h (40×), B: cultivation at 24 h (40×), C: cultivation at 48 h (40×).

significantly at 1.085 mg/L arsanilic acid in hepatocytes on 12 h ($p<0.01$) and in culture medium on 24 h ($p<0.01$) as compared to the control group. However, the activities of CAT were significantly increased at 10.85 mg/L arsanilic acid on 48 h ($p<0.01$) as compared to the control.

Effect of arsanilic acid on the gene expression of bax in hepatocytes

From Table 3 and Figure 2, the results showed that the positive gene expression of bax induced by arsanilic acid were gradually increased with the amount of arsanilic acid exposure and cultured time. The gene expression of bax was significantly upregulated in 1.085 mg/L arsanilic acid and higher for 24 h as compared with the control ($p<0.01$). Hepatocyte gene expression of bax in 10.85 mg/L arsanilic acid on 24 h was more than the 12 h, and 48 h was the most expression (Figure 2). The results indicated that the 1.085 mg/L arsanilic acid induce hepatocytes apoptosis through increase the gene expression of bax.

DISCUSSION

The present study was focused to elucidate the role of antioxidant defense system in arsanilic acid toxicity and to investigate the nature of *in vitro* arsanilic acid induced hepatocytes apoptosis. The results of our study show

Table 3. Gene expression of bax in hepatocytes with different concentration of arsanilic acid

Time (h)	Concentration (mg/L)	Negative (%)	Positive (%)	+ (%)	++ (%)	+++ (%)	Expression
12	0	82.47	17.53	12.63	4.31	0.59	Low
	1.085	82.89	17.11	12.76	3.30	1.05	Low
	10.85	80.55	19.45	14.35	3.35	1.75	Low
	108.5	77.19	22.81*	14.65	5.74	2.42	Upper
	1,085	72.51	27.49*	20.19	3.97	3.33	Upper
	10,850	64.49	35.51**	20.56	8.88	6.07	High
24	0	84.52	15.48	11.29	3.55	0.645	Low
	1.085	81.42	18.58*	13.18	4.39	1.01	Upper
	10.85	71.51	28.49*	18.27	7.74	2.48	Upper
	108.5	71.00	29.00*	17.62	10.84	0.54	Upper
	1,085	68.53	31.47**	15.09	14.44	1.94	High
	10,850	66.06	33.94**	16.06	13.64	4.24	High
48	0	85.18	14.82	12.19	2.14	0.49	Low
	1.085	82.79	17.21*	12.62	3.61	0.98	Upper
	10.85	79.53	20.47*	14.19	4.51	1.77	Upper
	108.5	72.65	27.35*	18.59	7.22	1.54	Upper
	1,085	66.33	33.67**	24.01	7.81	1.85	High
	10,850	61.41	38.59**	27.25	8.02	3.32	High

n = 5, * Compared with normal control group at the same time $p<0.05$, ** Compared with normal control group at the same time $p<0.01$.

significant decrease in scavenging enzyme GSH-px, SOD, and along with significant elevation in CAT. It is clear from the data in this study that exposure arsanilic acid for 12 h and 24 h, both hepatocytes and culture medium SOD activities decreased in corresponding 1.085 mg/L arsanilic acids respectively, and the hepatocytes activity of GSH-px in 108.5 mg/L arsanilic acid inhibited after arsanilic acid exposed 24 h as well. These observations proved that antioxidant defense system plays an important role in the course of hepatocyte apoptosis induced by arsanilic acid. Such alteration of oxidative stress markers is suggested to be due to overuse failure of the antioxidant defense system secondary to reactive oxygen species (ROS) production as induced *in vitro* by arsanilic acid. Many studies confirmed the generation of free radicals during arsenic metabolism in cells (Yamanaka et al., 2001; Valko et al., 2006). Arsenic-mediated generation of ROS is a complex process which involves the generation of a variety of ROS. The exact mechanism responsible for the generation of all reactive species is not yet clear, but some workers have proposed the formation of intermediary arsine species (Kroemaer and Hugues, 1999; Bashir et al., 2006). Another route to the production of H_2O_2 was suggested, involving the oxidation of As (III) to As (V) which, under physiological conditions, results in the formation of H_2O_2: $H_3AsO_3+H_2O+O_2 \rightarrow H_3AsO_4+H_2O_2$ (Kamat et al., 2005). Oxidative stress has been implicated in the course of hepatocyte apoptosis that results from a variety of forms of liver injury (Czaja, 2002; Tuder et al., 2003; Han et al., 2004; Han et al., 2006).

Under normal circumstances apoptosis is a naturally occurring process by which organisms intentionally eliminate damaged cells. The cell population control system can be disrupted by toxicant exposure, including exposure to metallic compounds. The results of our biochemical assays show 10.85 mg/L arsanilic acid exposure as cultural medium additives and initiate to restrain the hepatocytes growth in a time- and dose-dependent manner and act through induction of apoptosis. Moreover, arsanilic acid was also capable in inducing apoptosis in hepatocyes (Marisa et al., 2003), this results suggested that arsanilic acid might be a target organs for hepatocytes apoptosis.

Oxidative DNA damage and DNA-protein cross-links may be the major DNA lesions induced by arsenite (Bau et al., 2002). Mice given arsenite or higher doses of arsenate show evidence of free radicals in the liver detected by a spin trap agent (Liu et al., 2000). Results such as these suggest that DNA damage induced by arsenite are mediated by ROS.

Besides oxidative stress inducing apoptosis, the expression of gene bax also accelerated hepatocyte apoptosis (Labat-Moleur and Negoescu, 2002). Bax is a member of the Bcl-2 family and may act by inhibiting the function of Bcl-2 by forming Bax/Bcl-2 complexes or by competing with other Bcl-2 targets (Brambilla et al., 1996; Jang et al., 2005). The current study showed that the expression of bax protein was significantly higher in the treated group than in the control group ($p<0.05$) in accordance with an *in vitro* study showed that bax protein expression increased after exposure to DNA-damaging agents (Shabnam et al., 2004). Overexpression of bax protein has been observed to promote apoptosis by increasing the susceptibility to anticancer drugs and radiation. Harima et al also found that a positive correlation

existed between the relative increase of bax over the course of treatment and patients' responses to treatment and activation of one of the bax pathways. In the present experiment, we discovered that arsanilic acid could increase the expression of protein bax, which indicated that the expression of gene bax was the way of arsanilic acid induced hepatocytes apoptosis. In according with our finding, Beck (1999) reported that the increased expression of protein bax was common in the course of apoptosis. Adachi et al. (2004) considered that bax translocates from the cytosol to mitochondria before mitochondrial cytochrome c release. Bax translocation was oxidative stress dependent ROS.

Apparently, it appears that the toxicity of arsanilic acid not only decrease the activity of SOD and GSH-px, but also increase the concentration of CAT in the culture medium, positive DNA ladder and the expression of gene bax. According to these results, we suggested that 10.85 mg/L arsanilic acid could be involved in the rat liver cells apoptosis. The data do demonstrate unequivocally, however, that the suitable arsanilic acid as additives in livestock feed could present potential problems.

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