Avermectin induced liver injury in pigeon: Mechanisms of apoptosis and oxidative stress

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Abstract

Extensive use of avermectin (AVM) can result in environment pollution, and it is important to evaluate the potential impact this antibiotic has on ecological systems. Few published literatures have discussed the liver injury mechanisms induced by AVM on birds. In this study, pigeons were exposed to feed containing AVM (0, 20, 40 and 60 mg/kg diet) for 30, 60, 90 days respectively. The results showed that AVM increased the number of apoptosis and the expression level of caspase-3, 8, fas mRNA in the liver of pigeons. Ultrastructural alterations, including mitochondrial damage and chromatin aggregation, become severe with increase exposure dose. Exposure to AVM induced significant changes in antioxidant enzyme (superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px)) activities and malondialdehyde (MDA) content, augmented protein carbonyl (PCO) content and DNA-protein crosslink (DPC) coefficient, in a concentration-dependent manner in the liver of pigeons. Our results show that AVM has toxic effect in pigeon liver, and the mechanism of injury caused by AVM is closely related to apoptosis and oxidative stress.

1. Introduction

Compared to well-studied physiological and toxicological effects of AVM in insects, AVM metabolism in pigeon is still an unexplored field. Since birds belong to higher trophic level, they may play a key role in AVM circulation in the ecosystem. AVM is a widely used agent in agriculture and veterinary medicine in prevention of parasitic diseases by affecting the nervous system and paralyzing insects (Floate et al., 2005; Kövecses and Marcogliese, 2005). AVM undergo little metabolism in animals and up to 80–98% of the initial administered dose can be found in feces after metabolic process (Sun et al., 2005). Some literature reveal the target invertebrates, which were exposed to AVM with a similar mode of action-ivermectin, shows signs of ataxia and paralysis in their behavior (Ding et al., 2001). However, little is known about the toxic effect of AVM on birds.

The accumulation of AVM in the environment makes AVM a major candidate for toxicological studies. As a critical part of the digestive system and the general circulation system, liver plays an important role in metabolism and biotransformation of exogenous substances. AVM has been identified to be able to cause injury to liver cells (El-Shenawy, 2010; Maioli et al., 2012). Some other reports indicate that highly concentrated avermectins can induce apoptosis in isolated rat hepatocytes (El-Shenawy, 2010) and pigeon neuron (Wang et al., 2009). In previous studies, we carried out studies in vivo and in vitro studies. We found that AVM has cytotoxicity to brain neurons of King pigeon in vitro and the mechanism of neurotoxicity induced by AVM is closely related to apoptosis (Li et al., 2013a, 2013b). In vivo, the microscopic structures of the cerebrum, cerebellum and optic lobe altered obviously, the severity of which increased with the concentration of AVM and exposure time. Meanwhile, AVM could induce oxidative damage to the brain tissue and serum of pigeon (Li et al., 2013a, 2013b). Meanwhile, we also attentioned that the liver of the AVM-treated pigeons exhibited structural alterations, include swelling, part necrosis, and inflammatory cell infiltration. Furthermore, there were many small focal necrosis observed in liver (unpublished). The severity of which increased with AVM concentration. Based on the above experimental results we performed this study, in order to further determine the AVM toxicity mechanism to liver in pigeons.

Apoptosis can be induced by particular endogenous and exogenous factors (Mattson and Chan, 2003). It is also involves apoptotic gene network. Caspase-3 has been implicated as a critical substance in initiating apoptosis (Nicholson and Thornberry, 1997). Caspase-8 could make mitochondrial release cytochrome C by cutting Bcl-2 interacting domain (BID). When it was bound by fas
ligand, fas can activate a signal transduction pathway that eventually resulted in apoptosis (Lasham et al., 2000; Nakamura et al., 2007). Yu et al. (2008) demonstrated that chlorpyrifos induced apoptosis in mouse retina via oxidative stress.

Oxidative stress, defined as a state of imbalance between the concentration of reactive oxygen species (ROS) and antioxidant defense mechanisms, contributes to the development of various pathologies in organisms (Cooke et al., 2006; Valko et al., 2006). The consequence of such an imbalance is the generator of oxidative damage to biomolecules (Pampolina and Costantini, 2011). Therefore, antioxidants are important in protecting organisms against oxidative damage from pathological conditions or exogenous damage (Costantini, 2008). Carbamate derivatives such as mancozeb have also been reported to be able to cause oxidative stress, DNA damage, and activation of the mitochondrial pathway of apoptosis (Calviello et al., 2006). Toxic heavy metals led to apoptosis, in which ROS are also been reported to be able to cause oxidative stress, DNA damage, and activation of the mitochondrial pathway of apoptosis (Calviello et al., 2006).

According to OECD Guidelines for Testing of Chemicals, pigeon is a recommended species for toxicology experiments, since they are sensitive to the change of exogenous substances in the environment. Therefore, we choose pigeon as experimental animal for monitoring a recommended species for toxicology experiments, since they are sensitive to the change of exogenous substances in the environment. Therefore, we choose pigeon as experimental animal for monitoring the number of apoptosis, the expression of caspase-3, caspase-8, fas mRNA, ultrastructural characteristics, and antioxidant responses (SOD, GSH-Px, MDA), PCO content, DPC coefficient in pigeon liver that received AVM. These results provide valuable information for the possible mechanisms of apoptosis via oxidative stress exposing to AVM in the pigeon liver.

2. Materials and methods

2.1. Chemicals and materials

AVM: white crystalline 98% AVMB$_{10}$ were purchased from the China Agricultural University Technology Development Corporation. Apoptosis detection kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany). MDA assay kit (A003-1), GSH-Px assay kit (A005-1), and SOD assay kit (A001-1) were purchased from the Nanjing Jiancheng Biotechnology CO, Ltd (Nanjing, Jiangsu, China). All other reagents used in the analyses were of analytical grade and were obtained locally.

2.2. Animals and treatments

Eighty male and female American king pigeons, 2 months old (Columbia livia) and weight of 300–350 g were selected. They were randomly allocated into 4 equal groups, and were fed with either a commercial diet (Control group) or a AVM-supplemented diet (20 mg/kg diet (Low-dose group), 40 mg/kg diet (Medium-dose group), or 60 mg/kg diet (High-dose group) for 30, 60 or 90 days, respectively. Feed and tap water were supplied ad libitum. Following euthanasia with sodium pentobarbital, pigeon livers were quickly removed at the end of 30, 60, 90 days experiment. The remaining two pigeons in each group were standby for any unexpected condition. Liver tissues were collected and divided into three portions for situ apoptosis detection, ultrastructural observations and other biochemical indices detection.

All pigeon experiments were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University under the approved protocol number SRM-06.

2.3. Ultrastructural observation

Samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for 90 min, then rinsed three times in the buffer and left overnight. Later, the tissues were post-fixed for 2 h in 1% buffered OsO$_4$ dehydrated through ascending graded series of acetone, and infiltrated with Spurr’s resin. After polymerization overnight at 70 °C, semi thin sections (0.5 mm) were cut with glass knives, stained with 1% toluidine blue O in 1% sodium borate and examined with a light microscope. Ultrathin sections (80–90 nm) were cut with a diatome diamond knife, collected on 200-mesh nickel grids, stained in 5% uranyl acetate for 10 min, and then counter-stained in lead citrate for 6 min. Sections were examined and photographed using a Jeol 100S transmission electron microscope.

2.4. Assessment of apoptosis

Immediately after being excised, the liver was fixed with 4% paraformaldehyde in PBS, dehydrated through ethanol and xylene, and embedded in paraffin. Briefly, 5 μm thick sections were cut on a Microtome (NO1026, Japan) and mounted onto poly-L-lysine-coated glass slides. TUNEL staining was performed using situ terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dTTP) biotin nick-end labeling (TUNEL) technique that identifies DNA strand breaks by labeling free 3’–OH termini (In Situ Cell Death Detection Kit, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. The result was calculated by averaging the number of apoptosis per microscopic field which was expressed as average percentages of apoptosis.

2.5. Determination of caspase-3, caspase-8 and fas genes expression

2.5.1. Total RNA isolation and reverse transcription

Total RNA was isolated from liver tissue samples using the TRIzol® reagent, according to the manufacturer’s instructions (Invitrogen, America). The dried RNA pellets were resuspended in 80 μl of diethyl-pyrocarbonate-treated water. The concentration and purity of the total RNA were determined spectrophotometrically at 260/280 nm. First-strand cDNA was synthesized from 2 μg of total RNA using oligo (dT) primers and Superscript II reverse transcriptase according to the manufacturer’s instructions (Takara, China). Synthesized cDNA was diluted 10 times with sterile water and stored at −80 °C.

2.5.2. Quantitative real-time PCR (qPCR)

Primer Premier Software (PREMIER Biosoft International, USA) was used to design specific primers for caspase-3, caspase-8, fas and β-actin based on the known Gallus gallus sequences under the accession numbers L08165.1, NM204725, NM204592 and XM216559. The following set of primers were used for caspase-3: forward CACCCAGCGGACAGAAT; and reverse TGCACTCATGGACCTATAGC; for caspase-8: forward CGAAGCGCTGCTCGTTA; and reverse TGGCAGCTGTTGAGATTGAT; for fas: forward GGACCGGGAAGATAGTGCAG; and reverse GCCCGGTGATGAAGACGCAA; for β-actin: forward GACCTCGTTGGAGATGT; and reverse CCTGGCATCTGGCTTCT. The amplification products were caspase-3, 135 bp; caspase-8, 104 bp; fas, 143 bp and β-actin, 197 bp. qPCR was used to detect the expression of caspase-3, caspase-8 and fas genes in liver by using SYBR Premix Ex Taq (Takara, Shiga, Japan). Reaction mixtures were incubated in the ABI PRISM 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The program was 1 cycle at 95 °C for 30 s and 40 cycles at 95 °C for 5 s and at 60 °C for 34 s. Dissociation curves were analyzed by Dissociation curve1.0 Software (Applied Biosystems) for each PCR reaction to detect and eliminate possible primer–dimer formation and nonspecific amplification. The magnitude of change in gene expression relative to controls was determined by the 2$^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (2001).

2.6. Detection of antioxidant enzyme activities

Liver tissues were homogenized in 5 ml ice-cold Tris–HCl buffer (0.01 mol/L, pH 7.4) containing 0.01% ethylenediamine tetra-acetic acid (EDTA), 0.01 mol/L saccharose, and 0.8% NaCl. The homogenization procedure was carried out for 10 min at 654 g. All procedures were performed at 4 °C. Homogenate, supernatant, and extracted samples were prepared to determine antioxidant enzyme activities (MDA, SOD, and GSH-Px assay) which according to the manufacturer’s instructions.

2.7. Determination of PCO level

PCO content was tested by 2,4-dinitrophenylhydrazine (DNPH) spectrophotometry with some modifications (Levine et al., 1990). Briefly, 100–200 mg tissue was thawed and homogenized at 10% (w/v) in HEPES buffer (pH 7.4, containing 10 mM HEPES, 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH$_2$PO$_4$, 0.6 mM MgSO$_4$, 40 mg/L PMSF, 0.5 mM L-protease inhibitor, 0.7 mg/mL pepsin inhibitor and 1.1 mM EDTA), using glass homogenizer in ice. The supernatant was used for DNPH-reaction. PCO content was calculated by the absorbance at 370 nm using the extinction coefficient of 22,000 M$^{-}\text{cm}^{-1}$ for aliphatic hydrazones, and the result was expressed as nmol carbonyl/mg protein.

2.8. Measurement of DPC coefficient

Sample was washed with ice-cold phosphate-buffered saline, and was forced through a wire-mesh screen to obtain 1.5 × 10$^6$ ml$^{-1}$ single-cell suspension. The suspension was treated with sodium dodecyl sulfate (SDS, 2%) at 65 °C for 10 min, then KCl (1 M in 10 mM Tris–HCl, pH 7.4) was added followed by passing the
mixture through a 1 mL polypropylene pipette tip for six times (Sanjkar et al., 2004). The sample was cooled on ice for 5 min and centrifuged at 7267 g for 5 min at 4 °C. The supernatant containing the unbound fraction of DNA was collected. The SDS-K+ precipitate was resuspended with wash buffer (0.1 M KCl, 0.1 mM EDTA, 20 mM Tris-HCl, pH 7.4) at 65 °C for 10 min, cooled on ice for 5 min, and centrifuged as mentioned above. The precipitate was washed for three times. The final pellet was re-suspended in wash buffer with proteinase K at 50 °C for 3 h, then the digest was placed on ice for 5 min and centrifuged at 10,464 g (4 °C) for 10 min to collect supernatant. Hoechst 33258 was added to each supernatant in the dark for 30 min. Fluorescence was measured at an excitation and emission wavelength of 350 nm and 460 nm. The result was expressed as the percentage of protein-bound DNA to total DNA.

2.9. Statistical analyses

Statistical analysis of all data was performed by SPSS 13.0 software and was assessed by one-way ANOVA. The data were expressed as the mean ± SD. The different superscripts in the same time point indicate significant differences.

3. Results

3.1. Ultrastructural alterations

Cells of pigeon liver displayed structural integrity, rich cristae in mitochondriand and evenly distributed chromatin in control group (Fig. 1(a)). In AVM-treated pigeons, with the increase of exposure concentration, cells of liver appeared unclear membrane and chromatin aggregation aside. Mitochondria of liver cell swelled with lysis of cristae, and turned into vacuole in AVM-treated pigeons. The alteration degree was observed in a concentration-dependend manner (Fig. 1(b)–(d)).

3.2. Determination of apoptosis and caspase-3, caspase-8, fas mRNA level

The number of apoptotic cells was determined by the TUNEL assay. AVM caused an increasing in the number of apoptotic cells in a concentration-dependent manner in the liver compared with the corresponding control group (Fig. 2(a))–(d)). There was an increased tendency in the percentage of apoptotic cells in Fig. 2(e) which showed significant differences between any two groups in 30, 60 and 90 days (P < 0.05). The expression of caspase-3 mRNA increased at each AVM concentration compared with the corresponding control group at the same time point (Fig. 2(f)). The highest expression was observed in the high-dose group in 90 days. It showed significant differences between any two groups in 30 days (P < 0.05). Low-dose group showed significant differences compared with other three groups in 60 and 90 days (P < 0.05). Medium-dose and high-dose groups showed significant differences compared with the corresponding control groups in 60 and 90 days (P < 0.05). The caspase-8 mRNA levels was same as Caspase-3 mentioned above (Fig. 2(g)). The highest expression was observed in the high-dose group in 90 days. All treated groups showed significant differences compared with corresponding control groups except for low-dose groups in 30 and 90 days (P < 0.05). In 60 days, all treated groups showed significant differences compared with corresponding control group. The expression of fas mRNA was similar to Caspase-3 and Caspase-8 (Fig. 2(h)). In 30 days, low-dose group showed significant difference compared with high-dose group; medium-dose and high-dose groups showed significant differences compared with the corresponding control group except for low-dose groups in 30 and 90 days (P < 0.05). In 60 days, all treated groups showed significant differences compared with corresponding control group.

3.3. Determination of oxidative indexes

After AVM exposure, there was an elevated trend in the content of MDA in a dose-dependent manner compared with the corresponding control group at the same time point in liver (Fig. 3(a)). It showed significant differences between any two groups in 30 and 90 days (P < 0.05). While in 60 days, all treated groups showed significant differences compared with the corresponding control group, and there was significant difference between low-dose and high-dose groups (P < 0.05). AVM caused a downtrend in the activity of SOD in a concentration-dependent manner compared with the corresponding control group at the same time point in liver (Fig. 3(b)). Nearly 36% decreases from the corresponding control group to high-dose group in 90 days. All treated groups showed significant differences compared with the corresponding control group in 30 and 60 days (P < 0.05). While in 90 days, low-dose group showed significant differences compared with other three groups; medium-dose and high-dose groups showed significant differences compared with the corresponding control group (P < 0.05). AVM caused a same tendency as SOD in the activity of GSH-Px (Fig. 3(c)). Nearly 53% decreases from the corresponding control group to high-dose group in 90 days. All treated groups showed significant differences compared with the corresponding control group in 30 days (P < 0.05). High-dose group showed significant differences compared with other three groups in 60 days; low-dose and medium-dose groups showed significant differences compared with the corresponding control group (P < 0.05). While in 90 days, medium-dose and high-dose groups showed significant differences compared with the corresponding control group, and there was significant difference between low-dose and high-dose groups (P < 0.05).

Fig. 3(d) indicated that AVM caused a significant increase of PCO level in pigeon liver in a concentration-dependent manner compared with the corresponding control group. The high-dose group significantly increased nearly 75% compared with the corresponding control group in 60 days. In 30 and 90 days, low-dose and medium-dose groups showed significant differences compared with the corresponding control groups; and high-dose groups showed significant differences compared with other three groups (P < 0.05). It showed significant differences between any two groups in 60 days (P < 0.05). AVM caused a same tendency as PCO content in DPC formation except for medium-dose group in 90 days (Fig. 3(e)), nearly 100% increases from the corresponding control group to high-dose group in 30 days. It showed significant differences between any two groups except for low-dose group when compared with the corresponding control group in 30 days (P < 0.05). In 60 days, medium-dose group showed significant difference compared with the corresponding control group; and high-dose group showed significant differences compared with other three groups (P < 0.05). While in 90 days, all groups showed same difference as 60 days except for medium-dose group which showed significant difference compared with the corresponding control group (P < 0.05).

4. Discussion

Numerous environmental toxins and pesticides have been found to cause injure the liver. The use of AVM has been raised due to its insecticidal effects. The high excretion rate of the parent compound from treated animals enables it to enter and persist in various environmental compartments which would cause damage to non-target animals. The treatment of rats with low doses of dimethoate, zineb or glyphosate alone or in combination which induced oxidative stress was able to affect brain and liver cell
survival (Astiz et al., 2009). Chlorpyrifos and dichlorvos have been proved to give rise to caspase dependent apoptosis associated to oxidative stress (Nakadai et al., 2006; Yu et al., 2008; Li et al., 2009). In previous studies, We found that the mechanism of neurotoxicity induced by AVM is closely related to apoptosis (Li et al., 2013a, 2013b). Moreover, AVM could induce the microscopic structures of the brain, oxidative damage to the brain tissue and serum of pigeon (Li et al., 2013a, 2013b). In this study, our results indicated that AVM caused ultrastructure changes with apoptotic characters and increased the expression of caspase-3, 8 and fas mRNA. In addition, AVM inhibited the activities of antioxidant enzymes and augmented the MDA and PCO content, DPC coefficient with concentration-dependent properties in pigeon liver. Thus, our findings suggest that oxidative stress plays a role in AVM-mediated apoptosis in pigeon liver.

Mitochondron plays a central role in apoptosis caused by various stimuli. In our study, AVM altered the structure of liver in pigeon, as evidenced by chromatin condensation and swelling mitochondria. And then mitochondrial bioenergetics can be disrupted, which ultimately lead to caspase-dependent apoptosis (Kleszczynski et al., 2009). In accordance with our results, it was showed that AVM causes an impairment of rat liver mitochondrial bioenergetics (Castanha Zanolli et al., 2012; Maioli et al., 2012). Meanwhile, increased number of apoptosis suggested that AVM resulted in apoptosis in pigeon liver. Apoptotic characters became more and more significant with the increase of concentration of AVM. Several molecular pathways can commit cells to apoptosis, and fas/fasL/caspases interaction is one of the important elements. Caspase-3 is the core member of caspase family, due to its pivotal roles in connecting upstream death signals and downstream apoptotic substrates (Ben-Yehudah et al., 2003). Apoptosis can also be mediated by caspase-8 activation, resulting in the formation of the death-inducing signaling complex (Boulares and Ren, 2004; Sakata et al., 2007). The fas ligand (FasL)/Fas pathway is an important pathway of apoptosis that controls cell proliferation and tissue remodeling (Gregory et al., 2011). Many studies indicated that inorganic arsenic could create oxidative damage, subsequently causing apoptosis in several kinds of cells and organs (Han et al., 2010; Singh et al., 2010). Other researchers have shown that copper oxide

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**Fig. 1.** Ultrastructure observations. The results of the liver ultrastructure observations by electron microscopy in control, low-dose, medium-dose and high-dose groups at 90 days are shown respectively in (a), (b), (c) and (d). Magnification was 15,000 x for all parts. (a) Showing nucleus contained chromatin equally distributed and normal mitochondria with cristae (red arrow) in control group; (b) showing chromatin aggregation in the cell nucleus (green arrow) in low-dose group; (c) showing mitochondria were swelling with lysis of cristae (red arrow) in medium-dose group; (d) nucleus chromatin aggregation became more severe (green arrow) and mitochondria turned into vacuole (red arrow) in high-dose group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 2. Apoptosis and related genes in liver using TUNEL assay and qPCR in liver of pigeon. The results of TUNEL assay by microscopy in control, low-dose, medium-dose and high-dose groups at 90 days are shown respectively in (a), (b), (c), and (d). While (e) shows the results of the apoptotic index. Effects of AVM on the relative mRNA expression levels of Caspase-3, Caspase-8, fas in liver of pigeon are shown in (f), (g) and (h). Bars with different letters represented statistically significant difference ($P < 0.05$) between the different doses AVM-treated groups at the same time point. Each value represents the mean ± SD ($n=6$).
nanoparticles and silica nanoparticles induced cytotoxicity, DNA damage and apoptosis in cultured human cells through oxidative stress (Ahamed et al., 2010; Akhtar et al., 2010). In the present study, we showed that caspase-3, 8 and fas were elevated on transcriptional level in pigeon liver with the increased exposure concentration of AVM. In agreement with our results, the data from experimental system in vitro revealed caspase-3 overexpression and apoptosis of gastric epithelial cells after helicobacter pylori treatment (Scotiniotis et al., 2000). In addition, diethylstilbestrol has been proved to up-regulate Fas–FasL and cause apoptosis in spermatogenic cells of adult male rats (Nair and Shaha, 2003).

Oxidants can lead to programmed cell death (Martindale and Holbrook, 2002). In the present study, AVM caused the induction of oxidative stress, including SOD and GSH-Px activities decreased, MDA content augmented, and alters biomacromolecules (especially protein and nuclei acid) which was evidenced by increased PCO and DPC in liver of pigeon after AVM exposure. Similar results have also been reported in other species. Jin et al. (2010a, b) reported that atrazine exposure caused a significant increase in MDA content in the liver tissue of common carp. Xing et al. (2012) reported that atrazine and chlorpyrifos caused significant decrease in antioxidant enzymes (SOD and GSH-Px) activities at sub-chronic exposure level in the brain and kidney of common carp. Moreover, the increased levels of PCO and DPC caused by AVM in pigeon liver mentioned above suggested that AVM disturbed the defensive capacity of antioxidant system. PCO is a sensitive indicator of protein oxidative damage. Likewise DPC is a sensitive indicator of DNA oxidative damage which cause a loss of important genetic material and cell apoptosis. Various studies indicated that exposures of environmental contaminants could cause DPC (Shaham et al., 2003). Falfushynska et al. (2012) reported increased PCO were detected after tetrazine pesticide in mollusks. Sang et al. (2009) indicated that the protein carbonyl content and DNA-protein crosslink coefficient were significantly augmented with concentration-dependent properties by exposing Wistar rats to SO₂ at 14, 28 and 56 mg/m³. Oxidative damage would further...
disturb mitochondrial structure and then increase intima permeability of mitochondria which make ion gradient disappear on both sides of the intima, leading $\Delta \Psi_m$ decline and finally resulting in apoptosis. At the same time, mitochondrial content such as cytochrome C released into the cytoplasm activating the caspase protein family and leading to the occurrence of apoptosis. They both influenced and interacted with each other in the process of apoptosis through the mitochondrial apoptosis way.

5. Conclusion

In conclusion, our study demonstrated that AVM can cause ultrastructure changes, apoptosis, and oxidative stress in pigeon liver cell. This study suggested that AVM inducing oxidative stress is an important factor for initiating apoptosis in liver, using an in vivo model of pigeon. The information presented in this study should be helpful for further understanding AVM-mediated liver injury mechanisms in pigeon.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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