Research Article

Monensin induces apoptosis in the liver tissue and primary hepatocytes of chicks

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Abstract: In this study, monensin (MON) was investigated for its apoptotic and genotoxic effects on chick liver tissue and cytotoxic and apoptotic effects on chick primary hepatocytes isolated from perfused liver tissue. Western blotting and real-time PCR (qPCR) were used to determine the apoptotic effect on primary hepatocytes and liver tissue, and the comet assay was used to determine the genotoxic effect on liver tissue. MON decreased cell viability in primary hepatocytes with increasing concentrations. When administered at concentrations of 1 and 10 μM, MON increased the levels of apoptotic p53, caspase-3, and caspase-9, and downregulated the expression of the antiapoptotic survivin, Bcl-xl, and Bcl-2 genes ($p < 0.05$). Furthermore, 100 and 125 ppm of MON increased caspase-3, caspase-9, and p53 protein levels, and downregulated Bcl-2 expression ($p < 0.05$). Bcl-xl gene expression was downregulated only in the group that received 125 ppm of MON ($p < 0.05$). Changes observed in the expression of the survivin gene were insignificant in both groups ($p > 0.05$). Moreover, 100 and 125 ppm of MON caused comet formation, which is a marker of genotoxicity, in the liver ($p < 0.05$). Our results indicate that MON induced apoptosis in both primary hepatocytes and liver tissue, and also caused genotoxicity in the liver tissue.

Key words: Monensin, apoptosis, chick, hepatocyte, liver

1. Introduction
The incorporation of anticoccidial drugs in feed for the prevention or prophylaxis of coccidiosis is a common practice in the modern poultry industry. The use of anticoccidial drugs in broiler chickens ranges from 70% to 98% in the United States, compared to 91% in Western Europe. Anticoccidial drugs are also widely used in other major poultry production regions across the world [1].

Monensin (MON) is a monovalent carboxylic polyether ionophore antibiotic produced by fungi belonging to the species *Streptomyces cinnamonensis* [2]. MON, the molecular formula of which is $C_{36}H_{62}O_{11}$, is considered a sodium ionophore as its affinity for the sodium ion (Na^+) is considerably higher compared to other ions [3,4]. MON is used as a feed additive for the control of coccidiosis in quails, chickens, turkeys, and goats; to improve growth and feed efficiency in beef cattle; and to minimize body condition losses, increase milk protein, and reduce milk fat in dairy cattle. In addition, controlled-release capsules are available for the prevention of subclinical ketosis in dairy cattle [5]. Although MON is considered a safe drug when used at therapeutic doses, it is toxic when overdosed. Owing to its narrow therapeutic index, MON is likened to a two-edged knife. Its effects on metal ions form the basis of its toxic/harmful effects on animals [6]. MON facilitates Na^+ ion influx into the cell in both hosts and parasites. An increased intracellular Na^+ ion concentration stimulates Na^+/Ca^{++} exchange, leading to an increased intracellular concentration of extracellular Ca^{++} ions [7]. Excessively increased Na^+ and Ca^{++} ions cause cytotoxicity through pH change, calcium overload, catecholamine release and lipid peroxidation in the cell [8].

Previous studies have shown that MON causes damage to tissues and organs, in particular to the heart and skeletal muscles, by changing the biochemical, hematological and lipid peroxidation parameters [9–12]. To date, no in vivo study has been conducted on the effects of MON on apoptosis and genotoxicity. Furthermore, in vitro studies are limited to several types of cancer cells, and there is no study on the effects of MON on primary hepatocytes [13–18]. In this study, we aimed to investigate the in vivo and in vitro apoptotic effects of MON, which is widely used in the poultry industry, on the chick liver.

2. Materials and methods
2.1. Reagents and chemicals
Monensin sodium, penicillin, streptomycin, fetal bovine serum, L-glutamine, Dulbecco's modified eagle medium (DMEM), ethylene diamine tetra-acetic acid (EDTA), Tris-base, milk powder, LMA, HMA, NaCl, NaOH, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), and ethidium bromide were purchased from

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Sigma (USA). Magnesium chloride was supplied from Carlo Erba Reagents (Spain). Hank's balanced salt solution (HBSS) was purchased from Santa Cruz (USA). Collagenase II, protease and phosphatase inhibitor cocktail, RIPA buffer, SuperSignal West Pico PLUS chemiluminescent substrate, and Pierce BCA Protein Assay Kit were purchased from Thermo Scientific (USA). Ethanol, dimethyl sulfoxide (DMSO) and Triton X-100 were supplied from Merck (Germany). RNAzol was supplied from the Molecular Research Centre (USA). EvoScript Universal cDNA Master and LightCycler 480 Probes Master Kit were purchased from Roche (Switzerland). Polyvinylidene fluoride (PDVF) membrane was purchased from Bio-Rad (USA). p53, anticaspase-3, and anticaspase-9 were purchased from Abcam (USA). Antirabbit and antibeta actin antibody were supplied from Cell Signaling (USA). All other chemicals and reagents were analytical grade.

2.2. Animals
In this study, one-day-old Leghorn layer chicks were used. All chicks were commercially obtained at one day of age. The chicks were provided with ad libitum commercial chicken feed and water for 60 days, and were randomly assigned to 3 groups with 15 animals in each group. In addition, five animals that received drug-free and additive-free feed were divided for in vitro study. MON is used for the prevention of coccidiosis chickens reared for laying up to a maximum of 16 weeks of age. Therefore, the application period was determined as 60 days in order to demonstrate the toxicity of MON in less than 16 weeks. The minimum and maximum doses accepted as safe by the European Food Safety Authority [19] were administered to the trial groups. While the control group received drug- and additive-free feed, the other two groups were administered with 100 ppm of MON (Coxidin 200, Huvepharma) and 125 ppm of MON in feed for 60 days. At the end of the trial period, the animals were sacrificed and their liver tissue was extracted. Liver tissue samples were freshly studied on the day of extraction for the comet assay. On the other hand, liver tissue samples transferred to cryotubes were shock-frozen in a nitrogen tank at –196 ºC, and stored at –80 ºC until being used for Western blot and real-time PCR analyses. The in vivo study was carried out in accordance with the directive of the Animal Experiments Ethics Committee of Erciyes University (Approval Date and No: 16.11.2016 and 16/138).

2.3. Chick hepatocyte isolation and primary cell culture
The two-stage collagenase perfusion technique, first developed by Berry and Friend [20] and later modified by Seglen [21], was used for the isolation of chicken hepatocytes. Preoperatively, the abdominal and breast feathers of the chickens (60–65 days old) were shaved and a 10% povidone-iodine solution was used for asepsis. After

the abdominal cavity of the chicken, anesthetized by the inhalation of 2.5% sevoflurane (Abbvie, 4456), was opened, a 26G IV catheter was inserted into the portal vein. Firstly, perfusion buffer-I (at 37 °C and a 10 mL/min flow rate), containing 0.9 mM $MgCl_2$, 0.5 mM EDTA and 25 mM HEPES and HBSS (without Ca^{+2} and Mg^{+2}), and secondly, perfusion buffer-II (at 37 °C and a 25 mL/min flow rate), containing 1000 U collagenase II, was infused into the portal vein. The caudal vena cava was cut with a scalpel to allow the perfusion buffer out of the vein. Perfusion continued until the color of the liver turned pale. Primary hepatocytes were isolated in a laminar flow cabinet (SafeFast Elite), from the liver, which was extracted under sterile conditions postperfusion. A Cedex-XS (Innovatis) device was used for hepatocyte cell counts. Primary hepatocytes were cultured in DMEM supplemented with penicillin (100 IU/mL), 10% fetal bovine serum, streptomycin (100 mg/mL), and L-glutamine (2 mM) in an incubator (New Brunswick Galaxy 170R) containing a moisturised atmosphere of 5% CO_2 at 37 °C.

2.4. MTS cytotoxicity test
The MTS assay was used to determine cell viability and cytotoxicity. Primary hepatocytes were planted in a 96-well cell culture plate at an intensity of 1×10^4 cells /well and incubated at 37 °C for 24 h. Afterwards, the cells were applied with different concentrations (1, 2, 4, 5, 10, 20, and 40 μM) of MON at 37 °C for 24 h. MTS reagent was added to each well and incubated for 1 h. At the end of the incubation period, the optical density of the formazan crystals was measured on a microplate reader (BioTek Synergy H1) at a wavelength of 490 nm. The viability of the untreated control cells was accepted as 100%. The viability of the cells in the other groups was expressed as percentage (%) compared to the control cells.

2.5. Gene expression analysis
Primary hepatocytes were planted into six-well plates at an intensity of 2×10^6 cells /well and incubated at 37 °C for 24 h. Hepatocytes treated with 1 and 10 μM of MON were incubated at 37 °C for 24 h, then the cells were harvested with a scraper for RNA isolation. RNAzol was used for RNA isolation from the hepatocytes and liver tissue, EvoScript Universal cDNA Master was used for cDNA synthesis, and a LightCycler 480 Probes Master Kit was used for real-time polymerase chain reaction (qPCR) analysis. Roche's LightCycler Nano was used to determine the expression of the antiapoptotic survivin, Bcl-xl, and Bcl-2 and genes. CT values were measured and calculated using the $2^{-\Delta\Delta CT}$ formula. Values were normalized with beta-actin. The genes are shown in Table 1.

2.6. Western blot analysis
Western blot analysis was performed as described by Towbin et al. [22]. Primary hepatocytes, planted into six-well cell culture plates (2×10^6 cells per well), were

Table 1. Probe catalogue number and sequences of primers used for real-time PCR analysis.

Gene	Right primer	Left primer	Probe catalogue no
Survivin	ggtggagacgaggtagacca	aaaatggcggcctatgct	04688040001
Beta-actin	acgagcgcagcaatatcat	gctctgactgaccgcgtta	04688996001
Bcl-xl	gagctgcgtggtggatgt	aagtgaccccatggttgtgt	04688066001
Bcl-2	actatctcgcggttgtcgtag	cttcccctcggaaaccat	04685059001

incubated for 24 h. Hepatocytes treated with 1 and 10 μM of MON were harvested with a scraper for protein isolation after 24 h of incubation. While RIPA buffer supplemented with phosphatase and protease inhibitor cocktail was used for protein isolation from the hepatocytes and liver tissue, a Pierce BCA Protein Assay Kit was used for quantitation. Proteins were mixed with the loading buffer (40 μg), boiled at 95 °C for 5 min, subjected to 5%–10% SDS-PAGE, and transferred onto a PVDF membrane (Bio-Rad, USA). The membranes were blocked with 5% milk powder in TBST buffer at RT for 1 h and were incubated overnight at +4 °C on a shaker with primary antibodies, including antibeta actin (1:500; Cell Signaling), anticaspase-3 (1:1000; Abcam), anticaspase-9 (1:1000; Abcam), and anti-p53 (1:1000; Abcam). After the membranes were incubated with secondary antirabbit antibody (1:5000; Cell Signaling) in the dark for 2 h, the bands were visualized using the Super Signal West Pico Plus chemiluminescence imaging reagent on the Bio-Rad ChemiDoc Imaging System. The intensity of each band was measured densitometrically with the ImageJ software and changes were calculated in comparison to the control and MON after normalization with β-actin.

2.7. Alkaline comet assay

The alkaline comet assay developed by Tice et al. [23] was used to determine the genotoxic effect of MON on liver tissue. Fresh liver tissue was minced with cold mincing buffer (20 mM EDTA, 10% DMSO, and HBSS, pH 7.5) to obtain a single-cell suspension. Samples were centrifuged at 1000 g for 15 min and then resuspended in cold mincing buffer. The cell suspension mixed with 0.5% LMA was embedded onto microscope slides previously precoated with a layer of 1.6% HMA. Slides were immersed in cold alkaline lysis solution for 1 h (10 mM Tris-base, 1% Triton X-100, 100 mM EDTA, 2.5 M NaCl, and 10% DMSO, pH: 10). The slides were placed inside a horizontal electrophoretic tank in cold electrophoresis buffer (200 mM EDTA, and 10 N NaOH, pH: 13) for 20 min to ensure DNA unwinding, and then electrophoresed at 25 V and 300 mA for 20 min. Next, the slides were immersed in neutralization buffer (400 mM Trizma pH 7.5) 3 times for 5 min, then dehydrated with ice-cold methanol and dried at room temperature. Subsequently, the slides were stained with 20 g/mL of ethidium bromide, visualized under a fluorescent microscope (Leica-microsystems), and quantified using the Comet image analysis software (Comet Assay IV, Perspective Instruments). Randomly selected 100 cells per sample were analyzed to determine DNA damage.

2.8. Statistical analysis

The IBM SPSS Statistics 21 package program was used for the statistical evaluation of data. Data were expressed as mean ± standard deviation. While MTS and comet assay data were analyzed by one-way ANOVA, Tamhane's and Tukey's tests were used to determine the differences between the groups. Western blot and qPCR data were evaluated by the one-sample t-test. $p < 0.05$ were regarded as statistically significant.

3. Results

3.1. In vitro results

3.1.1. Effect of MON on cell viability

Treatment with 2 μM and higher concentrations (4, 5, 10, 20, and 40 μM) of MON significantly decreased the viability of primary hepatocytes, when compared to the control group ($p < 0.05$). On the other hand, treatment with 1 μM of MON also decreased cell viability, but this change was statistically insignificant ($p > 0.05$) (Figure 1).

3.1.2. Effect of MON on apoptotic proteins

While the group treated with 1 μM of MON showed significantly increased ($p < 0.05$) caspase-3 (36 kDa), caspase-9 (19 kDa), and p53 levels (3.35-, 2.16-, and 2.98-fold, respectively) compared to the control group, the increase in the caspase-3 (18 kDa) and caspase-9 (46 kDa) levels (1.06- and 1.16-fold, respectively) was statistically insignificant ($p > 0.05$). On the other hand, in the group treated with 10 μM of MON, caspase-3 (18 kDa and 36 kDa), caspase-9 (19 kDa and 46 kDa), and p53 levels were determined to have significantly increased (2.10-, 1.68-, 3.22-, 2.41-, and 1.88-fold, respectively), in comparison to the control group ($p < 0.05$) (Figure 2).

3.1.3. Effect of MON on antiapoptotic genes

When compared to the control group, the expression of the survivin, Bcl-xl, and Bcl-2 genes was found to have been downregulated in the groups treated with 1 μM of
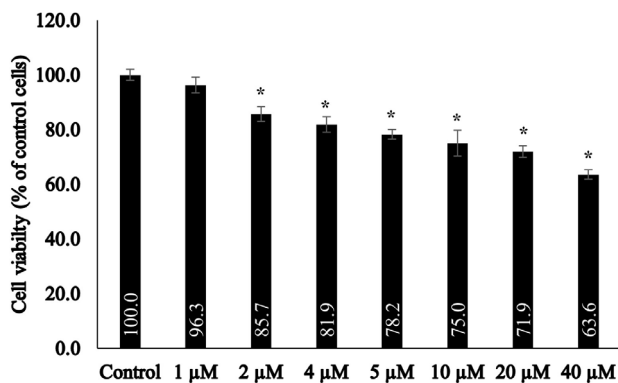
Figure 1. MTS assay to measure cell viability and cytotoxicity of hepatocytes. The values were stated as the mean ± standard deviation (n = 12). Differences according to control were considered statistically significant when *p < 0.05.

MON (0.29-, 0.36-, and 0.27-fold, respectively) and 10 µM of MON (0.30-, 0.45-, and 0.37-fold, respectively) (p < 0.05) (Figure 3).

3.2. In vivo results

3.2.1. Effect of MON on apoptotic proteins
Compared to the control group, the group treated with 100 ppm of MON displayed significantly increased caspase-3 (18 kDa and 36 kDa), caspase-9 (19 kDa and 46 kDa),

and p53 levels (1.79-, 1.45-, 2.12-, 2.22-, and 2.63-fold, respectively) (p < 0.05). In the group given 125 ppm of MON, when compared to the control group, the caspase-3 (36 kDa), caspase-9 (19 kDa), and p53 levels (2.07-, 7.58-, and 3.92-fold, respectively) were determined to have significantly increased (p < 0.05), whilst the increase in the caspase-3 (18 kDa) and caspase-9 (46 kDa) levels (1.14- and 1.25-fold respectively) was statistically insignificant (p > 0.05). (Figure 4).

### 3.2.2. Effect of MON on antiapoptotic genes
In comparison to the control group, the expression of the Bcl-2 gene was downregulated in the groups that received 100 ppm (0.50-fold) and 125 ppm (0.39-fold) of MON (p < 0.05). The expression of Bcl-xl was found to have been downregulated (0.30-fold) in only the group that received 125 ppm of MON (p < 0.05). While the expression of the survivin gene was downregulated (0.87-fold) in the group that received 100 ppm of MON and upregulated (1.75-fold) in the group that received 125 ppm of MON, when compared to the control group, these alterations were statistically insignificant (p > 0.05) (Figure 5).

### 3.2.3. Effect of MON on genotoxicity
Compared to the control group, there was a significant decrease in the comet head intensity, and a significant increase in the comet tail intensity in the groups that received 100 ppm and 125 ppm of MON (p < 0.05) (Figure 6).
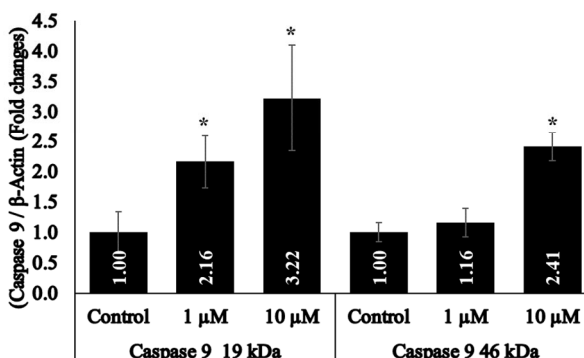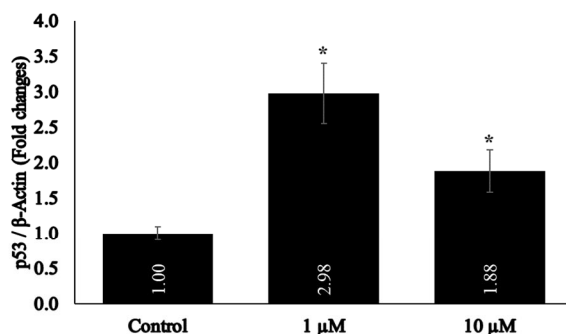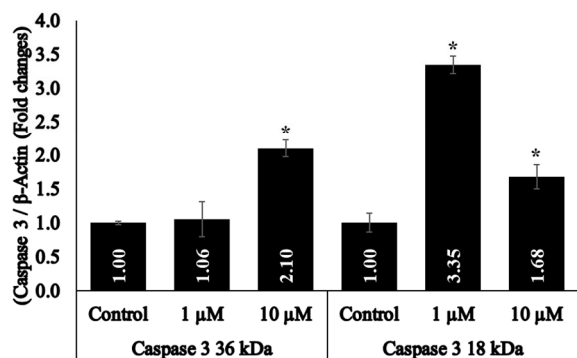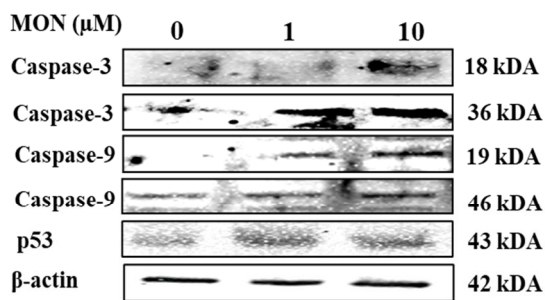
Figure 2. Protein levels of caspase-3, caspase-9, and p53 in hepatocytes were measured by Western blotting analysis. β-Actin was used as a loading control. The values were stated as the mean ± standard deviation (n = 3). Differences according to control were considered statistically significant when *p < 0.05.
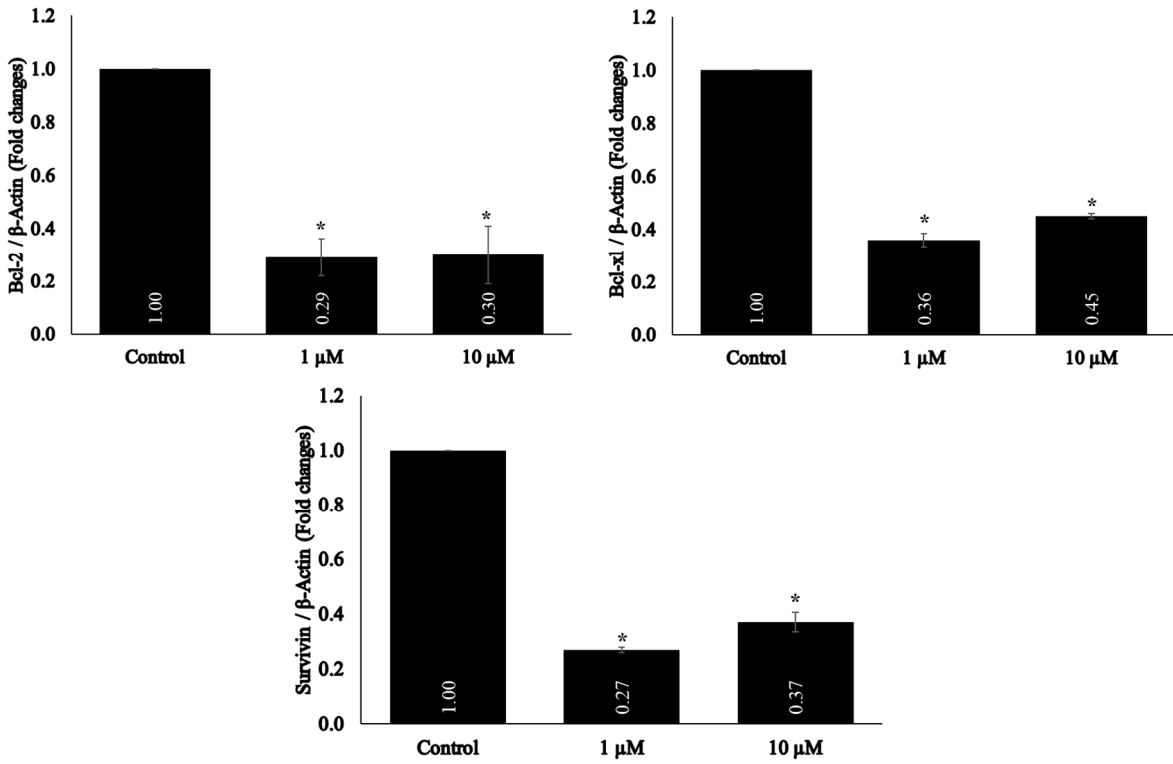
Figure 3. The gene expression levels of Bcl-2, Bcl-xl, and survivin in hepatocytes were measured by real-time PCR (qPCR) analysis. β-Actin was used as a housekeeping gene. The values were stated as the mean ± standard deviation (n = 3). Differences according to control were considered statistically significant when *p < 0.05.
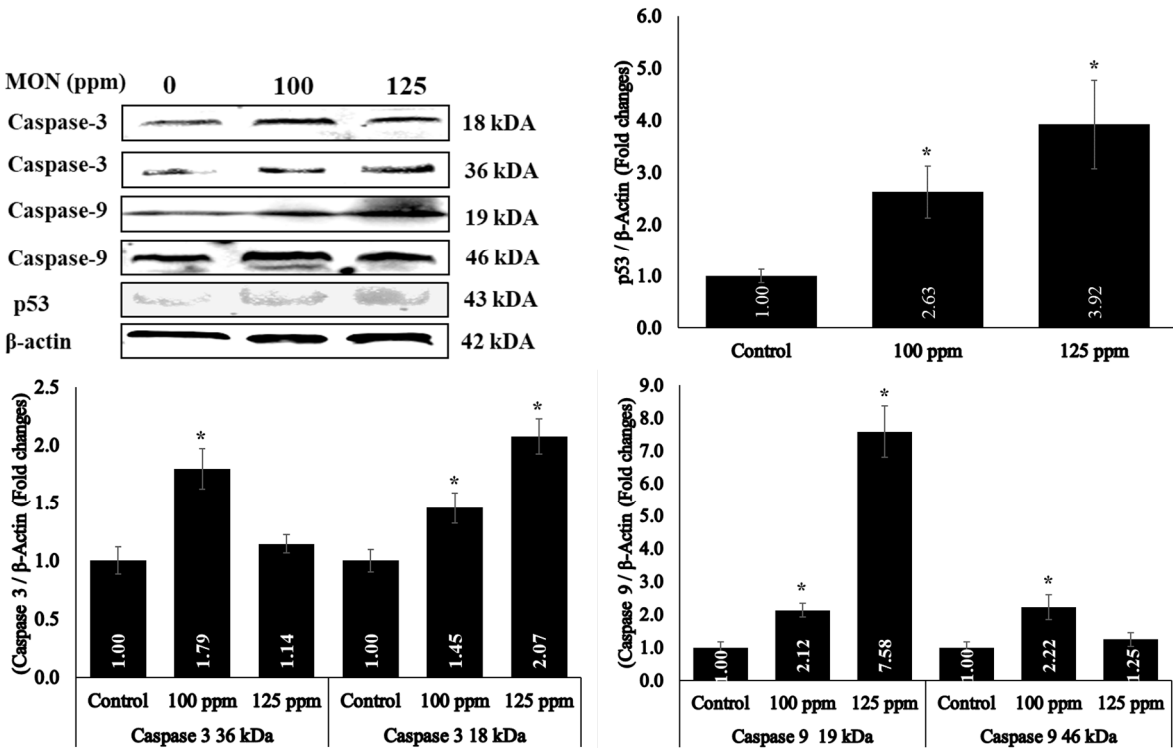
Figure 4. Protein levels of caspase-3, caspase-9, and p53 in liver tissue were measured by Western blotting analysis. β-Actin was used as a loading control. The values were stated as the mean ± standard deviation (n = 3). Differences according to control were considered statistically significant when *p < 0.05.
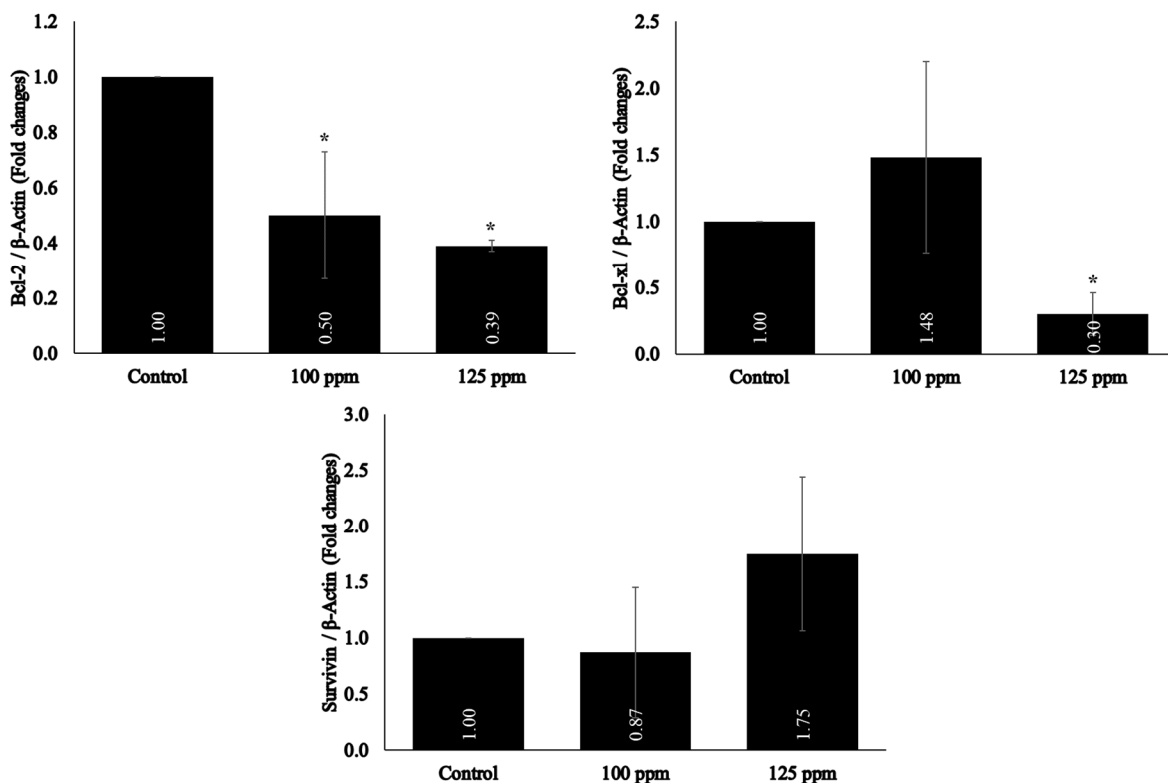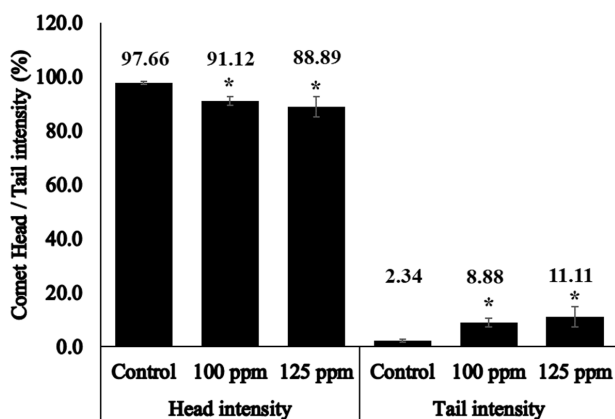
Figure 5. The gene expression levels of Bcl-2, Bcl-xl, and survivin in hepatocytes were measured by real-time PCR (qPCR) analysis. β-Actin was used as a housekeeping gene. The values were stated as the mean ± standard deviation (n = 3). Differences according to control were considered statistically significant when *p < 0.05.

Figure 6. The alkaline comet assay was used to determine the genotoxic effect of MON on liver tissue. The values were stated as the mean ± standard deviation. Differences according to control were considered statistically significant when *p < 0.05.

4. Discussion

MON is frequently used for the prevention and treatment of coccidiosis, which is an important problem in poultry breeding [5]. Although MON is considered a safe drug when administered in therapeutic doses, it is toxic in case of overdose [6, 24–26]. MON increases intracellular Ca++ concentration, leading to intracellular ROS production and Ca++ dependent apoptosis [7, 27–29]. While it has been reported that the main target organs for MON are the heart and skeletal muscles, there are studies that have proven the toxic effects of the drug on the liver. These studies have shown that MON causes a dose-dependent reduction in hepatic weight in chickens together with biochemical and histopathological changes in the liver [8–12, 30].

In the in vitro part of the study, the cytotoxic effect of 1 and 10 μM of MON on primary hepatocytes was evaluated. Although there is no previous study investigating the effects of MON on primary hepatocytes, there are several studies on the effects of MON on cell proliferation and death in different types of cancer cells. In these studies, it has been shown that increasing concentrations of MON decrease cell viability over time [13–18]. Similarly, our study has shown that MON decreases cell viability with increasing concentrations.

One and 10 μM of MON induced apoptosis by causing an increase in caspase-3, caspase-9, and p53 levels and a downregulation in Bcl-2, Bcl-xl, and survivin expressions in hepatocytes. Although the underlying mechanism of apoptosis varies, our data showed that apoptosis was

induced via the mitochondrial pathway. It is known that p53 shows antioxidant activity under low oxidative stress conditions, and causes cell death by exhibiting prooxidative activity under high oxidative stress conditions [31]. In addition, MON is reported to cause lipid peroxidation by increasing intracellular calcium levels [32]. Accordingly, in the present study, the increase in hepatocyte p53 levels was attributed to MON-induced oxidative stress. It has been reported that Bcl-2 and Bcl-xl bind to proapoptotic proteins and prevent loss in the mitochondrial membrane potential, cytochrome c release, and thus, apoptosis [33]. In our study, it is suggested that increased p53 levels cause the release of proapoptotic proteins by suppressing antiapoptotic Bcl-2, Bcl-xl and survivin, and thus increase the permeability of the outer mitochondrial membrane. Cytochrome c is released from the mitochondria into the cytosol due to the loss of the mitochondrial membrane potential and triggers apoptosome formation, which activates caspase-9. Caspase-9 activates effector procaspases, including procaspase-3, and thereby, initiates the process of apoptosis. In addition, decreased levels of survivin, which is a caspase inhibitor, can increase the levels of caspase-3 and -9 by avoiding inhibition [34, 35]. In the light of this information, in the present study, it was assumed that the downregulation of Bcl-xl, and Bcl-2 expression activated caspase-3 and caspase-9, and likewise, increased p53 levels increased caspase-3 and caspase-9 levels by downregulating the expression of survivin and preventing the inhibition of caspases. Although there is no previous study investigating the effects of MON on chicken hepatocytes, researches have been conducted on cancer cell lines. In these studies, it was determined that, in cells treated with different concentrations of MON, the level of the antiapoptotic protein Bcl-2 decreased, whilst the levels of the apoptotic proteins caspase-3 and caspase-9 increased [14–18]. In a previous study on the toxic effects of salinomycin, another ionophore antibiotic, it was reported that while the expression of apoptotic caspase-3 and caspase-9 was upregulated, that of antiapoptotic BCL-2 was downregulated in primary chicken cardiomyocytes [36]. Similar data was obtained in the present study.

Coxidin 200 microGranulate consists of 25% monensin sodium, 15%–20% perlite and 55%–60% wheat bran [19]. Perlite, an amorphous alumina-silicate, is commercially available as HARBORLITE. Perlite is used as a filler agent in Coxidin formulation[1]. Limited data indicate that the LD50 (oral intake) of perlite in rats is greater than 10 g/kg. No toxic effects associated with perlite have been reported in animals [37]. Wheat bran is a source of insoluble fiber [38]. Wheat bran is used as a diluent agent in Coxidin formulation[2]. It was reported that wheat bran supplementation could increase nutrient digestibility in broilers [39]. Therefore, although a commercial product of MON was used for the in vivo experiments, additional ingredients could be ignored to evaluate effects on MON. In the in vivo part of the study, both doses of MON were ascertained to have triggered apoptosis by increasing apoptotic protein levels and downregulating the expression of antiapoptotic genes. It is known that MON induces oxidative stress by increasing the intracellular calcium concentration, and p53 causes cell death by exhibiting prooxidative activity under severe oxidative damage conditions [31, 32]. In addition, it is suggested that oxidative stress-induced DNA damage, which may occur upon exposure to MON, activates p53, which increases the permeability of the mitochondrial membrane, eventually facilitating the release of cytochrome c from the mitochondria into the cytosol by binding to antiapoptotic Bcl-xl, and Bcl-2. Cytochrome c triggers apoptosome formation, which activates caspase-9. Caspase-9 activates effector procaspases, including procaspase 3, and triggers the apoptosis process [34, 40]. In the present study, the increase observed in p53 levels with the administration of both MON concentrations could be a result of oxidative DNA damage induced by MON. Furthermore, it is estimated that increased p53 levels cause a downregulation in Bcl-xl, and Bcl-2 expression and an increase in caspase-3 and caspase-9 levels, thus inducing apoptosis via the mitochondrial pathway. Although there is no previous study on the apoptotic effects of MON in chickens, this subject has been investigated in mice. Park et al. [15] observed that MON increased p53 levels and decreased Bcl-2 levels in mice with experimentally induced tumors. Similar data was obtained in the present study.

Intracellular ROS, produced mostly in the mitochondria, can target structures, which are susceptible to oxidative damage, such as the DNA [41, 42]. Although DNA is a stable and well-preserved molecule, ROS can interact with it and cause various damages such as the modification of DNA bases, DNA-protein crosslinking, single and double DNA breaks, and damage to the DNA repair system [43]. DNA damage impairs respiratory chain functions by disrupting the RNA transcription of proteins involved in the electron transport chain, and also further increases ROS production, resulting in the loss of

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[2] CRL Feed Additives Authorisation (2005). Evaluation Report of the Community Reference Laboratory Feed Additives Authorisation on the Method(s) of Analysis for Coxidin® (Dossier No. FAD-2005-0003) [online]. Website https://joint-research-centre.ec.europa.eu/publications/fad-2005-0003_en/ [accessed 18.02.2022].

the mitochondrial membrane potential and impaired ATP synthesis. All these events result in apoptosis mediated by the mitochondrial pathway [41]. Based on the results of the comet assay, it is suggested that both 100 and 125 ppm of MON cause DNA damage in the liver by either directly reacting with DNA or possibly inducing free radical generation. The previous studies on the DNA-damaging effects of MON are limited. There is evidence that MON or similar drugs induce DNA damage in other species. In a study, it was reported that MON could induce DNA damage in earthworm coelomocytes (*Eisenia fetida*) by causing comet formation [44]. In addition, another study it was shown that MON induces DNA fragmentation in rat testis tissue [45]. In another study, it was observed that salinomycin, another ionophore antibiotic, caused DNA damage in chicken lymphocytes by increasing the tail length of the comet [46]. Our study is in line with the results of previous studies.

5. Conclusion
MON, which has indications for use in various animal species and is described as a double-edged blade, is considered a safe drug at therapeutic doses. However, in this study, liver damage characterized by apoptosis and genetic damage was observed even upon the administration of therapeutic doses. Therefore, it is important to use MON in combination with other anticoccidial drugs (e.g., nicarbazin) at low doses and for limited time periods to ensure the protection of animal and consumer health.

Although it is considered that the hepatotoxic effects of MON may be caused by free radicals, further more detailed studies are required for the demonstration of the highly complex and multifactorial toxic effect mechanisms of this drug.

Conflict of interest
The authors declare that there are no conflicts of interest.

Acknowledgment

Abbreviations
Bcl-2, B-cell lymphoma-2; Bcl-xl, B-cell lymphoma-extra large; DMEM, Dulbecco's modified eagle medium; DMSO, Dimethyl sulfoxide; DNA, Deoxyribonucleic acid; EDTA, Ethylene diamine tetra-acetic acid; EFSA, European Food Safety Authority; HBSS, Hank's balanced salt solution; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; HMA, High melting point agarose; LMA, Low melting point agarose; MON, Monensin; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NaCl, Sodium chloride; NaOH, Sodium hydroxide; p53, Tumor suppressor protein; PDVF, Polyvinylidene fluoride; qPCR, Quantitative polymerase chain reaction; RIPA, Radio-immunoprecipitation assay; RNA, Ribonucleic acid.

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