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Original Article

Aflatoxin B1-induced oxidative stress in canine small intestinal cells

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ABSTRACT

Background: Aflatoxin B1 (AFB1) is a toxic metabolite generated by *Aspergillus* species and is commonly detected during the processing and storage of food; it is considered a group I carcinogen. The hepatotoxic effects, diseases, and mechanisms induced by AFB1 owing to chronic or acute exposure are well documented; however, there is a lack of research on its effects on the intestine, which is a crucial organ in the digestive process. Dogs are often susceptible to chronic AFB1 exposure owing to lack of variation in their diet, unlike humans, thereby rendering them prone to its effects. Therefore, we investigated the effects of AFB1 on canine small intestinal epithelial primary cells (CSIc).

Methods: We treated CSIc with various concentrations of AFB1 (0, 1.25, 2.5, 5, 10, 20, 40, and 80 μM) for 24 h and analyzed cell viability and transepithelial-transendothelial electrical resistance (TEER) value. Additionally, we analyzed the mRNA expression of tight junction-related genes (*OCLN*, *CLDN3*, *TJP1*, and *MUC2*), antioxidant-related genes (*CAT* and *GPX1*), and apoptosis-related genes (*BCL2*, *Bax*, and *TP53*).

Results: We found a significant decrease in CSIc viability and TEER values after treatment with AFB1 at concentrations of 20 μM or higher. Quantitative polymerase chain reaction analysis indicated a downregulation of *OCLN*, *CLDN3*, and *TJP1* in CSIc treated with 20 μM or higher concentrations of AFB1. Additionally, AFB1 treatment downregulated *CAT*, *GPX1*, and *BCL2*.

Conclusions: Acute exposure of CSIc to AFB1 induces toxicity, and exposure to AFB1 above a certain threshold compromises the barrier integrity of CSIc.

Keywords: aflatoxin B1, apoptosis, canine, intestinal epithelial barrier, small intestinal cell

INTRODUCTION

Grains, vegetables, and animal products and their by-products are often contaminated with fungi that may produce mycotoxins (Kumar et al., 2022). The major mycotoxins commonly found in food and feed include trichothecenes, zearalenone, ochratoxins, aflatoxins, and fumonisins. Among these mycotoxins, aflatoxins, which are well-known secondary metabolites produced by *Aspergillus* species, are considered the most toxic (Popescu et al., 2022). Aflatoxins are primarily found in tropical and subtropical regions; however, they are now detected globally owing to climate change, affecting not only developing countries but also Europe (Mahato et al., 2019).

Over 20 types of aflatoxins have been identified, which have all been derived from *Aspergillus* species, and they present a threat to both animals and humans (Negash, 2018). The most well-known types are B1, B2, G1, G2, M1, and M2, with B1 being the most potent carcinogenic mycotoxin (Popescu et al., 2022). Based on the characteristics of fungi, warm and humid conditions enhance aflatoxin production. B1, B2, G1, and G2 predominantly occur in plant-based foodstuffs such as grains, whereas M1 and M2 are primarily detected in animal-derived products (Battilani et al., 2011; Negash, 2018). Commercial dog food typically includes poultry, beef, pork, and various meat by-products (Godwin, 2012). Additionally, various grains such as corn, rice, barley, and wheat are added to meet the nutritional requirements of the feed while controlling costs (Rojas et al., 2011; Cho et al., 2023). Hence, dogs may be exposed to diverse forms of aflatoxins, as evidenced by a series of documented incidents linking aflatoxin poisoning in dogs to the consumption of commercial dog food (Dereszynski et al., 2008; Arnot et al., 2012; Gomes et al., 2014). Furthermore, unlike humans, dogs are often fed the same diet. If aflatoxin-contaminated feed is provided, it is difficult to detect contamination before observable behavioral or clinical changes are exhibited in dogs, potentially leading to chronic exposure to aflatoxins and warranting caution.

The intestinal epithelial cells serve as the initial physical barrier to all ingested substances (Newman et al., 2007; Halpern and Denning, 2015). The intestinal barrier is formed by intercellular adhesions between epithelial cells. Maintaining the barrier integrity is essential to prevent bacterial infection and spread of toxins from the lumen into the systemic circulation (Luo et al., 2019). The small intestinal epithelium comprises epithelial cells and intercellular junctions, which regulate barrier permeability and nutrient uptake via specialized structures called tight junctions (Hollander, 1999). Functional impairment of the intestinal barrier occurs via epithelial cell apoptosis, reduced cell proliferation, induction of inflammation, alterations in tight junctions due to dietary or toxic substances, and bacterial infections (Koch and Nusrat, 2009). Therefore, the permeability or integrity of the intestinal barrier may represent a useful indicator for assessing barrier function and guiding subsequent interventions (Koch and Nusrat, 2009).

In this study, we investigated the effects of aflatoxin B1 (AFB1) treatment on the barrier integrity of canine small intestinal epithelial primary cells (CSIc) by analyzing transepithelial electrical resistance and the mRNA expression levels of tight junction-associated markers (occludin [*OCLN*], claudin 3 [*CLDN3*], tight junction protein 1 [*TJP1*], and mucin 2 [*MUC2*]). Moreover, it has been demonstrated that AFB1 can exert direct or indirect effects on apoptosis and induce oxidative stress (Farhadi et al., 2003; Ribeiro et al., 2010; Brahmi et al., 2011). Therefore, to investigate whether AFB1 also induces apoptosis and oxidative stress in CSIc, we analyzed the mRNA expression levels of markers associated with apoptosis (*BCL2*, *Bax*, and *TP53*) and antioxidant markers (glutathione peroxidase 1 [*GPX1*] and catalase [*CAT*]).

MATERIALS AND METHODS

Cell culture

CSIc were purchased from MK-biotech (Daejeon, Korea). CSIc were cultured in Dulbecco's modified Eagle medium supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin, 10 ng/mL fibroblast growth factor (Sigma-Aldrich, St. Louis, MO, USA), and 10 ng/mL epidermal growth factor (Sigma-Aldrich, St. Louis, MO, USA) at 37℃ in a humidified 5% CO_2 atmosphere. Experiments were conducted using CSIc up to the fifth passage. AFB1 was obtained from MedChemExpress (Monmouth Junction, NJ, USA). AFB1 was dissolved in dimethyl sulfoxide to prepare a stock solution of 10 mM and stored at -20℃ in the dark prior to experiments.

Cell viability assay

Cell viability was assessed using the MTT assay kit (Abcam, Cambridge, United Kingdom) following the manufacturer's protocol. CSIc were cultured in a 96-well plate and treated with AFB1 at various concentrations (0, 1.25, 2.5, 5, 10, 20, 40, and 80 μM) for 24 h. Absorbance at 590 nm was measured using a Multiskan SkyHigh microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) in triplicate based on the manufacturer's instructions. Cell viability and cytotoxicity were calculated as follows:

$$\text{Viability (\%)} = \frac{(\text{Sample A590 nm} - \text{Blank A590 nm})}{(\text{Control A590 nm} - \text{Blank A590 nm})} \times 100$$

$$\text{Cytotoxicity (\%)} = \frac{[(\text{Control A590 nm} - \text{Blank A590 nm}) - (\text{Sample A590 nm} - \text{Blank A590 nm})]}{\text{Control A590 nm} - \text{Blank A590 nm}} \times 100$$

Transepithelial–transendothelial electrical resistance assay

CSIc were seeded onto 24-well transwell inserts containing 3-μm pore size filters (Thincert) at a density of 1×10^4 cells/well. After seeding, the cells were treated with AFB1 at various concentrations (0, 5, 10, 20, 40, and 80 μM) for 24 h. Transepithelial-transendothelial electrical resistance (TEER) was measured using a voltmeter (World Precision Instruments, Sarasota, FL, USA). The electrical resistance ranged from 180-300 Ω cm^2 and was expressed as a percentage. All TEER values were calculated after subtracting the resistance values of the culture media and the filter resistance of the transwell. Resistance was calculated as follows:

$$\text{Resistance (\%)} = \frac{\text{Sample } \Omega\ cm^2 - \text{Blank } \Omega\ cm^2}{\text{Control } \Omega\ cm^2 - \text{Blank } \Omega\ cm^2} \times 100$$

RNA extraction and quantitative reverse transcription–polymerase chain reaction (qPCR)

CSIc cultured in 24-well transwell inserts were washed with PBS and then lysed and scraped in Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was extracted using the Trizol reagent according to the manufacturer's protocol. Total RNA was quantified using a Multiskan SkyHigh microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA was synthesized using SuperScript III First-strand (Invitrogen, Waltham, MA, USA) and oligo-dT primers after treatment with DNase (Roche, Basel, Switzerland). Gene expression analysis was conducted using quantitative reverse transcription-polymerase chain reaction (Thermo Fisher Scientific, Waltham, MA, USA) with SYBR (Thermo Fisher Scientific, Waltham, MA, USA). The qPCR conditions were as follows: pre-denaturation at 95℃ for 10 min; 50 cycles at 95℃ for 20 s, 58℃ for 10 s, and 72℃ for 20 s; and elongation at 72℃ for 10 min. The primers were designed using Primer3 software (http://primer3.ut.ee/). Glyceraldehyde 3-phosphate dehydrogenase was used as a housekeeping gene, and the primer sequences for the target genes are provided in Table 1. Duplicate measurements were conducted for all samples to ensure reproducibility, and the expression levels were determined from the Ct values using the 2-delta delta Ct method (Livak and Schmittgen, 2001).

Table 1. Primer sets for quantitative reverse transcription-polymerase chain reaction analysis

Gene symbol	Description	Genbank accession number	Sequence (5′–3′)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase 1	NM_001003142	F: TGTCCCCACCCCCAATGTATC
			R: CTCCGATGCCTGCTTCACTACCTT
CLDN3	Claudin 3	NM_001003088	F: CTCATCGTCGTGTCCATCCT
			R: CGATGGTGATCTTGGCCTTG
OCLN	Occludin	NM_001003195	F: CTCAGCCGGCGTATTCTTTC
			R: GACGCGACACAGGCAAATAT
TJP1	Tight junction protein ZO-1	NM_001003140	F: GTCCCTCCTCTAATACCCGC
			R: GACTGGGGTTTCATTGCTGG
MUC2	Mucin 2, oligomeric mucus/gel-forming	XM_038425528	F: TACAACTTTGCGTCCGACTG
			R: GATGGTGTCGTCCTTGATGC
Bax	B-cell leukemia/lymphoma 2 protein associated X, Apoptosis regulator	NM_001003011	F: CGTAGAGTCTTCTTCCGAGT
			R: TGGCAAAGTAGAAGAGGGCA
BCL2	B-cell lymphoma-2	NM_001002949	F: CTTCAGGGATGGGGTGAACT
			R: CCGAACTCAAAGAAGGCCAC
TP53	Tumor protein p53	NM_001389218	F: ACTCAGATGATGCTCCCAGG
			R: CAGAGGATGATAGGGGCCAG
CAT	Catalase	NM_001002984	F: CATGCTCGACAATCAGGGTG
			R: CGAACATTGGCTGCTATGCT
GPX1	Glutathione peroxidase 1	NM_001115119	F: GAGCCCAACTTCACGCTTTT
			R: ATGAACTTGGGGTCGGTCAT

Statistical analysis

Significant differences were assessed using the Student's *t*-test and analysis of variance (ANOVA) with the R package (version 4.2.3). Tukey's test was used for post-hoc analysis of ANOVA. General linear hypothesis testing was performed to distinguish between groups. Results were considered significant at $p < 0.05$.

RESULTS

Aflatoxin B1 decreased the viability of CSIc and impaired barrier integrity

The viability of CSIc decreased when treated with 20 μM of AFB1 for 24 h (Fig. 1A). The results obtained from six repeated experiments showed that cytotoxicity increased when treated with 5 μM AFB1, but there was no difference after treatment with 10 μM AFB1 (Fig. 1B). The viabilities of 5 and 10 μM AFB1-treated cells were 85 ± 5.59% and 86 ± 7.36% relative to that of untreated cells, respectively. Cell viability decreased in a dose-dependent manner, with viabilities of 72 ± 4.70%, 70 ± 2.61%, and 59 ± 2.48% observed for 20, 40, and 80 μM AFB1, respectively. Thus, the concentration-dependent decrease in viability of CSIc by AFB1 was determined to start at 20 μM. These results were consistent with the TEER measurement results (Fig. 2). The TEER value of CSIc treated with 20 μM AFB1 was significantly reduced to 93 ± 2.53% compared to that of untreated cells. Treatment with 40 and 80 μM AFB1 resulted in TEER values of 89 ± 3.58% and 92 ± 3.09%, respectively. Additionally, mRNA expression analysis revealed a dose-dependent decrease in *CLDN3*, *OCLN*, and *TJP1* expression after 20 μM AFB1 treatment, with the lowest relative quantification value observed after 80 μM AFB1 treatment (Fig. 3). However, ANOVA analysis revealed no changes in *MUC2* expression. In summary, treatment of CSIc with AFB1 concentrations beginning at 20 μM for 24 h resulted in decreased cell viability and TEER values, accompanied by downregulation of *CLDN3*, *OCLN*, and *TJP1*, which are key markers of tight junctions in CSIc.

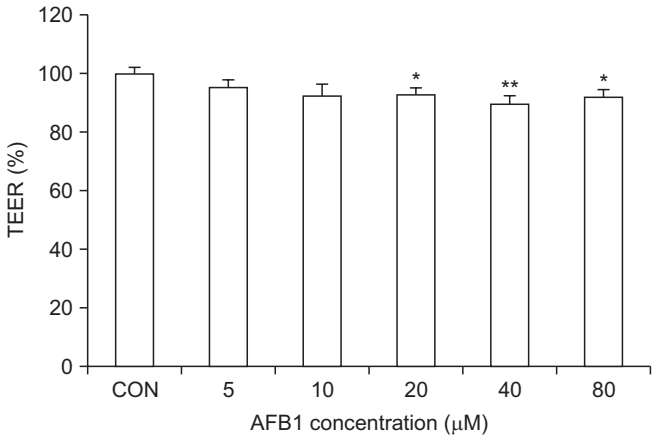

Fig. 2. Analysis of TEER in CSIc treated with AFB1. TEER in CSIc treated with AFB1 at various concentrations for 24 h. Data are presented as the mean ± SEM; Student's *t*-test, n = 6, *$p < 0.05$, **$p < 0.01$, versus CON. AFB1, aflatoxin B1; CSIc, canine small intestinal epithelial primary cells; CON, control; TEER, transepithelial-transendothelial electrical resistance.

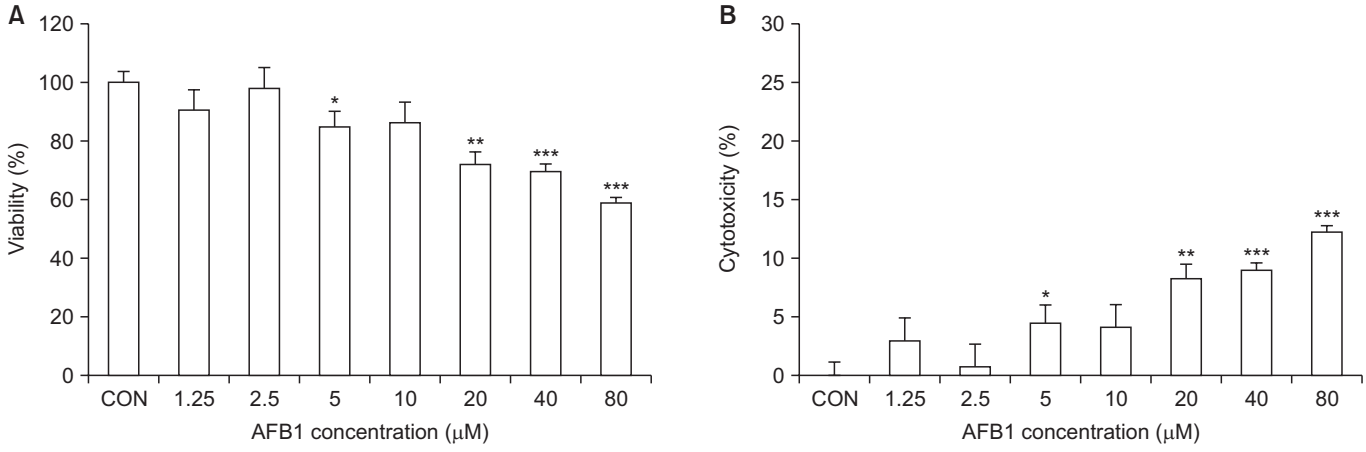

Fig. 1. Effects of different concentrations of AFB1 on CSIc viability after treatment for 24 h. (A) Cell viability and (B) cytotoxicity assay of CSIc treated with AFB1 for 24 h. Data are presented as the mean ± SEM; Student's *t*-test, n = 6, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus CON. AFB1, aflatoxin B1; CSIc, canine small intestinal epithelial primary cells; CON, control.

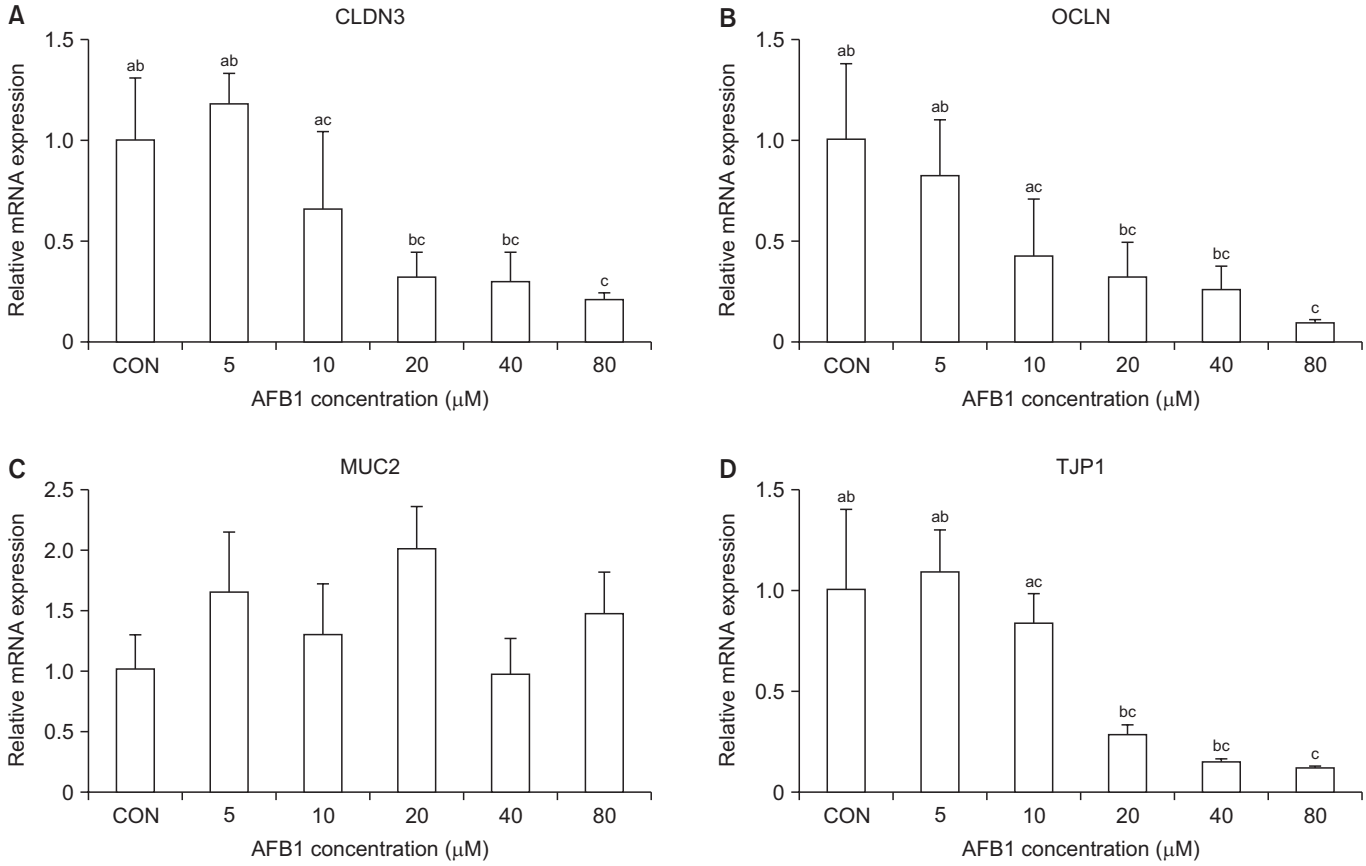

Fig. 3. Effects of different concentrations of AFB1 on the mRNA expression of tight junction-related genes including (A) *CLDN3*, (B) *OCLN*, (C) *MUC2*, and (D) *TJP1* in canine small intestinal epithelial primary cells. Data are presented as the mean ± SEM (n = 4). Different letters (ab, bc, and c) above the bars indicate significant differences between means; ab and ac indicate insignificant difference. Additionally, if the ANOVA was not statistically significant, no letter is displayed on the graph. AFB1, aflatoxin B1; CON, control; *CLDN3*, claudin 3; *OCLN*, occludin; *MUC2*, mucin 2; *TJP1*, tight junction protein 1.

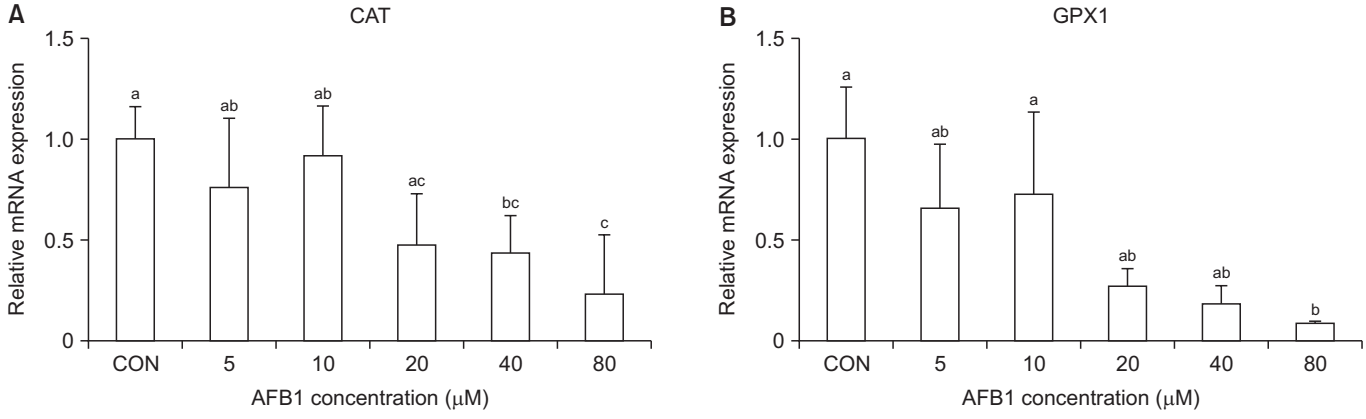

Fig. 4. Effects of different concentrations of AFB1 on the mRNA expression of antioxidant-related genes including (A) *CAT* and (B) *GPX1* in canine small intestinal epithelial primary cells. Data are presented as the mean ± SEM (n = 4). Different letters (a, b, and c) above the bars indicate significant differences between means. ab and ac indicate insignificant difference. AFB1, aflatoxin B1; CON, control; *CAT*, catalase; *GPX1*, glutathione peroxidase 1.

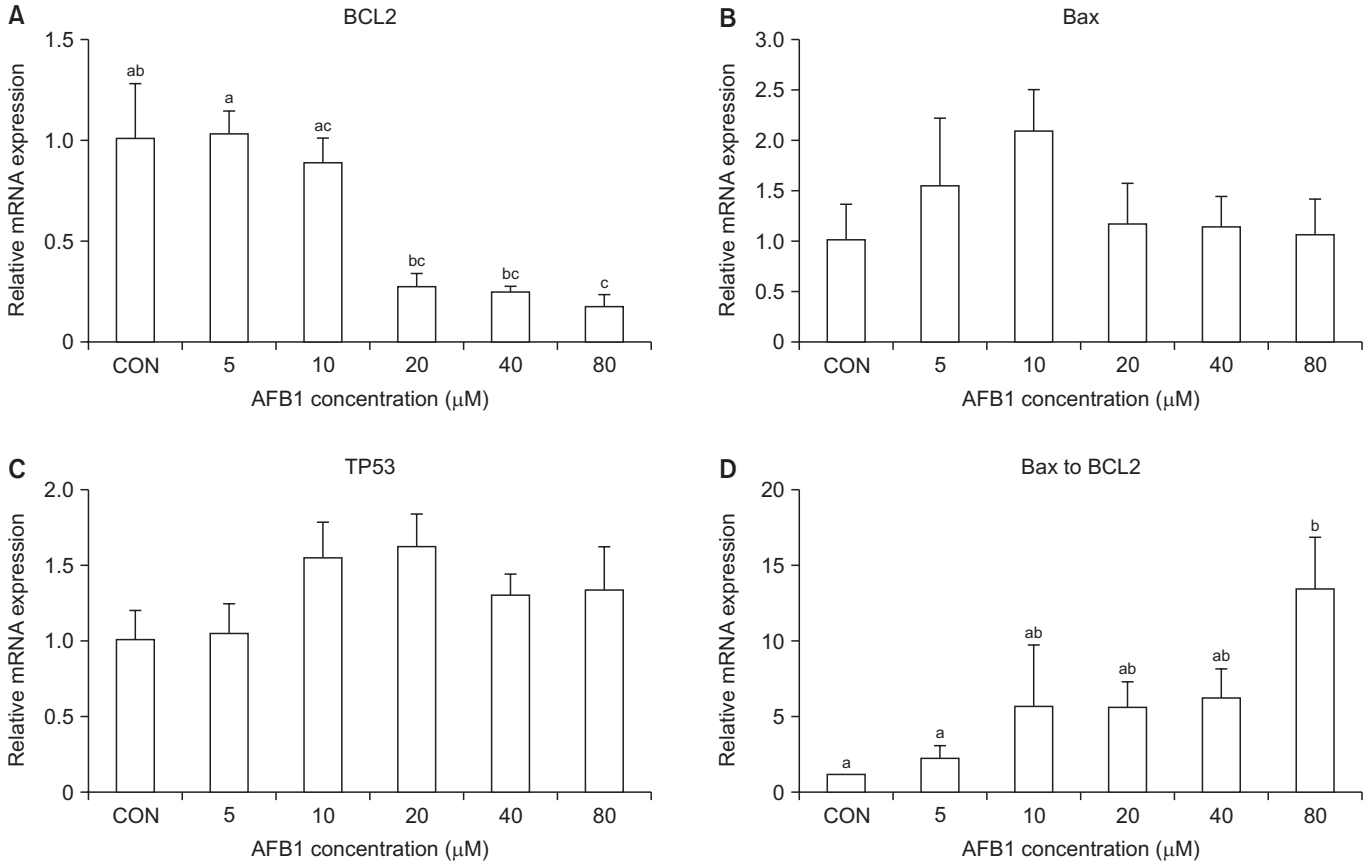

Fig. 5. Effects of different concentrations of AFB1 on the mRNA expression of apoptosis-related genes including (A) *BCL2*, (B) *Bax*, (C) *TP53*, and (D) the Bax/BCL2 ratio in canine small intestinal epithelial primary cells. Data are presented as the mean ± SEM (n = 4). Different letters (a, b, and c) above the bars indicate significant differences between means. ab indicates insignificant difference. Additionally, if the ANOVA was not statistically significant, no letter is displayed on the graph. AFB1, aflatoxin B1; CON, control; *BCL2*, B cell leukemia protein; *Bax*, BCL2-associated protein; *TP53*, tumor protein 53.

AFB1 downregulated antioxidant and apoptosis-related genes

We investigated the alterations in mRNA expression levels of antioxidant-related genes (*CAT* and *GPX1*) after exposure to AFB1 (Fig. 4). *CAT* was downregulated after treatment with 40 and 80 μM AFB1. The downregulation of *GPX1* was significant after treatment with the highest concentration of AFB1 tested (80 μM). After AFB1 treatment, the expression of *BCL2*, a key regulator of apoptosis, decreased starting at an AFB1 concentration of 20 μM, and it reached its lowest level after treatment with 80 μM AFB1 (Fig. 5A). However, AFB1 did not exert a significant effect on *Bax* and *TP53* expression (Fig. 5B and 5C). The *Bax*/*BCL2* ratio was altered after treatment with 10 μM, with a significant increase observed at the highest treatment concentration, 80 μM (Fig. 5D). Our results reveal that treatment with AFB1 in CSIc downregulated *BCL2*, a key suppressor of apoptosis, accompanied by downregulation of *CAT* and *GPX1*, which are associated with antioxidant stress.

DISCUSSION

AFB1 is considered the most potent hepatocarcinogen. Within the hepatic milieu, AFB1 is metabolized by cytochromes P450 (P450s or CYPs), a diverse family of heme-containing enzymes, leading to formation of aflatoxin B1-8,9-epoxide (AFBO), a potent liver carcinogen. Furthermore, AFBO generates DNA adducts at the reactive N7-position on guanine. Although the hepatocarcinogenic mechanism of AFB1 is known, its role in inducing malnutrition and retarding growth, which are typical negative effects of AFB1, remains unclear (Rushing and Selim, 2019). The small intestine is the first barrier against

exogenous toxins and plays an important role in digestive physiology. AFB1 can disrupt the nutrient absorption efficiency in the small intestine by regulating fibrosis and necrosis (Yunus et al., 2011; Smith et al., 2012). Furthermore, AFB1 reduces the number of intestinal villi, impairs barrier function, and reduces TEER value and barrier integrity (Gao et al., 2021; Bai et al., 2022). Additionally, AFB1 reduces cell viability in a concentration-dependent manner in various cell types (Caco-2, HEK, Hep-G2, SK-N-SH, and bovine mammary epithelial cells), including intestinal cells (Zhang et al., 2015; Zheng et al., 2018; Wu et al., 2021). Therefore, our results of reduced cell viability and TEER values in AFB1-treated CSIc support previous findings.

Tight junctions of the intestinal epithelium play a crucial role in regulating the barrier function and transport of substances between cells. OCLN and CLDN3 are essential proteins of the tight junction that adhere to complementary molecules of adjacent cells (González-Mariscal et al., 2003). TJP1 serves as a junction adapter protein, and it interacts with transmembrane proteins such as CLDN and regulates intercellular tension (Tornavaca et al., 2015). Our results revealed a downregulation of *OCLN*, *CLDN3*, and *TJP1* in CSIc after AFB1 treatment. This is consistent with several studies demonstrating that AFB1 downregulates *OCLN*, *CLDN3*, and *TJP1* in Caco-2 and IPEC-J2 cells (Romero et al., 2016; Liu et al., 2020; Gao et al., 2021). Similar trends have been observed *in vivo* in response to AFB1 (Gao et al., 2021; Zhang et al., 2022). When CSIc were treated with AFB1 at concentrations exceeding 20 μM, a concentration-dependent decrease in the expression of intestinal tight junction-related genes was observed. These results suggest that acute exposure to AFB1 above a certain threshold disrupts the barrier integrity in CSIc. MUC2 is a glycoprotein produced by epithelial goblet cells and plays a crucial role in forming a viscoelastic gel that protects the intestinal epithelium and maintains intestinal homeostasis (Liu et al., 2020). AFB1 treatment did not affect *MUC2* expression in IPEC-J2 cells, while a significant decrease in protein expression has been reported (Zhang et al., 2022). Although the correlation between protein and mRNA expression may be low (Greenbaum et al., 2003; Koussounadis et al., 2015), further research is needed to investigate the post-transcriptional regulation of *MUC2* in intestinal cells following AFB1 treatment.

We found that AFB1 treatment downregulated *BCL2* as well as the antioxidant genes *CAT* and *GPX1*. However, the expression levels of *TP53* and *Bax* remained unchanged, suggesting that the TP53/Bax cascade is not affected by AFB1-induced DNA damage, and that *BCL2* downregulation is due to excessive ROS accumulation (Roshan et al., 2023). AFB1 generates reactive oxygen species (ROS) along with AFBO during metabolic processes mediated by cytochromes P450 (Liu and Wang, 2016; Benkerroum, 2020). Although our analysis focused on mRNA expression levels, ROS generated during AFB1 metabolism has been similarly found to generate oxidative stress leading to decreased antioxidant activity (Mughal et al., 2017). Furthermore, AFB1-induced apoptosis is alleviated by antioxidants, resulting in improved antioxidant activity and cell viability (Wang et al., 2022; Tian et al., 2023). The interrelationship between ROS and *BCL2* expression has been suggested in several studies (Hildeman et al., 2003). Excessive accumulation of ROS may be involved in the downregulation of *BCL2* and induction of apoptosis (Hildeman et al., 2003; Liu and Wang, 2016). Apoptosis is a complex mechanism involved in homeostatic regulation and defense. It can be categorized based on three main pathways: the mitochondrial pathway (endogenous), death receptor pathway (exogenous), and endoplasmic reticulum pathway. Crucial to mediating apoptosis via the mitochondrial pathway, the *BCL2* family identifies an increase in the Bax/BCL2 ratio as an indicator of apoptosis signaling activation (Huang et al., 2022). In our study, treatment with 80 μM AFB1 resulted in the activation of apoptotic signaling. This may be attributed to the excessive accumulation of ROS caused by AFB1-mediated increased ROS production and the impaired antioxidant system, compounded by AFBO.

CONCLUSION

Our results demonstrate the cytotoxicity of acute AFB1 exposure in CSIc. AFB1 downregulated genes related to the intestinal barrier integrity and those related to tight junctions. Acute exposure of AFB1 is thought to impair intestinal integrity in CSIc when the concentration exceeds a certain threshold. Moreover, the downregulation of the antioxidant-related genes *CAT* and *GPX1* after AFB1 treatment may exacerbate apoptosis induced by AFB1. Our results for CSIc are consistent with the molecu-

lar biological responses to AFB1 identified in various cell and *in vivo* experiments and support the results of previous studies.

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