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OXIDATIVE STRESS IN BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS EXPOSED TO MYCOTOXINS

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Abstract

One of the possible mechanism through which mycotoxins can cause cytotoxic effect is the induction of oxidative stress. This in vitro study was performed to verify the potential oxidative effect of four mycotoxins using bovine peripheral blood mononuclear cells (PBMC) culture as model. Blood samples were obtained from six healthy not pregnant and not lactating Holstein cows. The PBMC were isolated by density gradient centrifugation and incubated for 2 and 7 days with different concentration of four mycotoxins (AFB₁: 0, 5 and 20 µg/ml; T-2: 0, 2.5 and 10 ng/ml; DON: 0, 1 and 5 µg/ml; FB₁: 0, 35 and 70 µg/ml). To evaluate oxidative status concentration of reactive oxygen metabolites (ROM), intracellular thiols (SH), malondialdehyde (MDA) and gene expression of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were determined on PBMC pellet. Exposure of PBMC to AFB₁ reduced SOD mRNA and SH and increased ROM and MDA. T-2 decreased SOD and GSH-Px mRNA, and SH and increased MDA. DON reduced GSH-Px mRNA and increased MDA. FB₁ decreased SOD and GSH-Px mRNA and increased ROMs and MDA. All mycotoxins tested induced oxidative stress in a dose-time dependent manner. The model used is appropriate and sensitive to study the potential oxidant grade and cytotoxic effects of mycotoxins.

INTRODUCTION

Mycotoxins are secondary metabolites produced by moulds in foodstuffs or feeds. They can cause several diseases (mycotoxicoses) after ingestion, skin contact or inhalation in humans and animals (Betina, 1989).

Mechanisms of action through which mycotoxins induce cytotoxicity are not well clarified. One of the possible mechanisms is the induction of oxidative stress (Atroschi *et al.*, 2002). Some mycotoxins can induce production of free radicals and/or reduction of antioxidant defences (Ferrante *et al.*, 2002; Leal *et al.*, 1999; Rizzo *et al.*, 1998; Shen *et al.*, 1996).

The cytotoxic effect of mycotoxins is studied *in vivo* models or *in vitro* models using usually cells from liver or kidney. Data from literature reveal variable effects of mycotoxins on different cell systems. It has been suggested that this may be due to different sensitivity depending on the characteristics of cell metabolism (Muller et al., 2004). Considering that components of immune system are a common target of mycotoxin toxicity, the aim of this work was to evaluate the possibility to utilize lymphocytes culture as model for studying the oxidant capacity of different mycotoxins (aflatoxin B₁, T-2 toxin deoxynivalenol and fumonisin B₁).

MATERIALS AND METHODS

Animals

Six healthy not pregnant and not lactating Holstein cows of the same age and body weight were used as blood donors. Blood samples were collected via jugular venipuncture, using evacuated glass tubes coated with sodium heparin.

Isolation and treatment of peripheral blood mononuclear cells (PBMC)

Isolation of peripheral blood mononuclear cells (PBMC) was done as described by Lacetera *et al.* (2002). After isolation, PBMC were resuspended at a concentration of 1×10^6 cells/ml of RPMI-1640 enriched culture medium (ECM). Duplicate cultures were assayed, by using 24-well tissue-culture plates (Sarsted INC, Newton, USA). Each well contained 1×10^6 PBMC in 1000 μ l of ECM.

Cells were incubated in presence of different concentration of 4 mycotoxins: aflatoxin B₁ (AFB₁: 0, 5, and 20 μ g/ml), T-2 toxin (T2: 0, 2.5 and 10ng/ml), deoxinivalenol (DON: 0, 1 and 5 μ g/ml) and fumonisin B₁ (FB₁: 0, 35 and 70 μ g/ml). The effects of mycotoxin exposure were evaluated at different time of incubation (2 or 7 days). Plates were incubated in an atmosphere of 95% air and 5% CO₂ for 48 h at 39 °C. At the end of the incubation (after 2 or 7 days) plates were centrifuged at 1200 rpm for 20 minutes. Supernatant were discarded and cell pellets were stored at –80°C until analysis.

The concentration of the different mycotoxins used were chosen on the basis of their different toxicity in vitro (Charoenpornsook *et al.*, 1998, Lee *et al.*, 1999, Neldon-Ortiz and Qureshi, 1992).

Laboratory analysis

Cells were lysed using PBS solution containing Triton X-100 (0.5%) (Sigma Chemical Co., St. Louis, MO, USA) and phenylmethanesulphonyl fluoride (1mM) (Sigma Chemical Co., St. Louis, MO, USA) for 15 minutes at 4°C. On cell lysate reactive oxygen metabolites (ROMs), intracellular thiols (SH) and malondialdehyde (MDA) concentrations were determined.

Reactive oxygen metabolites (ROMs) - The concentration of ROMs was measured using a commercial kit (d-ROMs test, Diacron, Grosseto, Italy) following the manufactures' instructions. Values were as mg/dl of hydrogen peroxide.

Intracellular tiols - Intracellular thiols content (Bernabucci *et al.*, 2002; Kusmic *et al.* 2000) was determined with a commercial kit (SHp Test, Diacron, Italy) and was expressed in μ mol/L. Briefly, proteins of lymphocytes lysate were precipitated by adding 1 ml of fresh metaphosphoric acid solution (1.67 g of metaphosphoric acid, 0.2 g EDTA-disodium salt, 30 g of NaCl in 100 ml of water). The supernatant was separated

from precipitated proteins after centrifugation and filtration (Puradisk 25AS 0.2µm, Whatman plc, Maidstone Kent, UK). An aliquot of 0.5 ml of supernatant was combined with 0.5 ml of Na₂HPO₄ (300 mM). Then DTNB (5,5-dithiobis-2-nitrobenzoic acid) was added. DTNB reacts with thiols in the sample to form a colored product. Sample absorbance was read at 405 nm and the SH concentration were calculated using a standard curve.

Malondialdehyde (MDA) - MDA concentration in lymphocytes lysate was assessed by RP-HPLC after derivatization with DNPH (dinitrophenylhydrazine) at low temperature (25°C) (Fenaille *et al.*, 2001). The RP-HPLC analysis was performed on a Beckman ODS-C₁₈ column (250X4.6 mm I.D., 5 µm), and acetonitrile/ammonium acetate (50mM) (45:55 v/v) was used as mobile phase at a flow rate of 1 ml/min. The absorbance of the complex MDA-DNPH was read at 307 nm using a diode array detector (ThermoQuest, Milano, Italy). A standard curve was constructed to quantify MDA and was expressed as nmol/ml (Fenaille *et al.*, 2001).

mRNA quantification - Quantification of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) mRNA were carried out by ribonuclease protection assay (RPA).

Total RNA was isolated by homogenizing lymphocyte pellet in 1 ml of TRI-REAGENTTM solution containing phenol and guanidinium thiocyanate (Sigma-Aldrich, Milano, Italy) following the procedure described by Bernabucci *et al.* (2004).

Specific antisense ribonucleotide probes were generated using cDNAs of GSH-Px, SOD and GAPDH, which was used as internal control, that were produced from lymphocyte RNA by reverse transcription-polymerase chain reaction (RT-PCR) (Bernabucci *et al.*, 2004).

GSH-Px RNA probe – Oligonucleotide primers of GSH-Px used for RT-PCR (forward: 5'-AAC GCC AAG AAC GAG GAG AT-3'; reverse: 5'-TAA TAC GAC TCA CTA TAG GGA G GA CAG GGT TTC AAT GT-3') were designed from the bovine GSH-Px cDNA. **SOD RNA probe** – A 234 bp SOD cDNA was produced by using procedures similar to those described for GSH-Px. Oligonucleotide primers of GSH-Px used for RT-PCR (forward: 5'-TGG AGA CAA TAC ACA AGG CTG-3'; reverse: 5'-TAA TAC GAC TCA CTA TAG GGA G CT GCC CAA GTC ATC TGG TTT-3') were designed from the bovine SOD cDNA.

GAPDH RNA probe - A 177 bp GAPDH cDNA was amplified by using two synthetic oligonucleotide primers (forward: 5'-TCATCCCTGCTTCTACTGGC-3'; reverse: 5'-TAATACGACTCACTATAGGGAGC CTGCTTCACCACCT TCTTG-3') from the bovine GAPDH cDNA.

The purified riboprobes were labelled with biotin using BrighstarTM Psoralen-Biotin Kit (Ambion, Inc., Austin, Texas, USA) according to the manufacturer's instructions. The labelling was carried out by mixing the riboprobes with the Psoralen-Biotin reagent in a microtiter plate and exposing to long wavelength (365 nm) UV light. The Psoralen-Biotin reagent became covalently linked to the riboprobes in 45 min. Any excess Psoralen-Biotin reagent was removed by butanol extraction and the probe was stored at -80°C until use.

Ribonuclease protection assay - Hybridization of total RNA with riboprobes was performed using the protocol and reagents supplied in the RPA IIITM kit (Ambion, Inc., Austin, Texas, USA) as described for the standard procedure and method reported by Bernabucci *et al.*, 2004.

For the quantitative analysis of GSH-Px and SOD mRNAs known amounts of in vitro synthesized GSH-Px and SOD sense RNA were hybridized with an excess of labeled antisense probes to construct the standard curves. Figure 1 and 2 reports the standard curves constructed for SOD and GSH-Px.

Chemiluminescent detection - The mRNA was cross-linked to the wet membrane after the transfer by heating in an oven at 80°C for 15 min. The nonisotopic detection of the probe fragments protected was performed using BrightStar™ and BioDetect™ kits (Ambion, Inc., Austin, Texas, USA) following the procedure described by the manufacturer. The membrane was washed twice in wash buffer and incubated in a blocking solution for 30 min, then with Streptavidin-Alkaline Phosphatase Conjugate for 30 min. Then blot was rinsed in wash buffer, followed by incubation in CDP-Star at room temperature for 5 min, excess liquid was shaken off and finally membrane was exposed to Kodak BioMax Light film (Eastman Kodak Company, Rochester, NY).

Densitometry - Chemiluminescent films were analyzed with the Kodak EDAS-290 densitometer and ID Image Analysis software (Eastman Kodak Company, Rochester, NY). Samples were analyzed in conjunction with the standard curve and the intensity of the probe fragments protected by specific mRNA from the target samples was compared to the standard curve to determine the absolute amounts (pg/ 10µg Total RNA) of GSH-Px and SOD mRNA

Statistical analysis

Data were analyzed using the GLM procedure of SAS® (SAS, 1999). Data relative to mRNA were log transformed. The model included fixed effects of cow, treatment (mycotoxins), mycotoxin concentrations, length of exposure (2 or 7 days), and residual error term. Differences were considered statistically significant at $P < 0.05$.

RESULTS

AFB₁ (Figures 3, 4, 5). Higher concentration of AFB₁ (20 µg/ml) lead to increase of ROMs and a reduction of intracellular thiols and both after 2 and 7 days of exposure, a reduction of gene expression of SOD that was significantly reduced also at 5 µg/ml in cells exposed for 7 days. AFB₁ increased ($P < 0.05$) MDA concentrations in a dose dependent manner.

T2 (Figures 6, 7, 8). The effect of T2 was significant at the higher concentration (10 ng/ml) and only after 7 days of exposure (chronic effect). In particular, cells showed increase of ROMs, decrease of SOD and GSH-Px gene expression and increase of MDA concentration.

DON (Figures 9, 10, 11). No changes were observed for ROMs, intracellular thiols, and SOD mRNA. A decrease of GSH-Px mRNA at the higher concentration (5 µg/ml) both after 2 and 7 days of exposure was detected. MDA concentration increased in a dose dependent manner and was higher in cells exposed for 7 days to DON compared with cells exposed for 2 days.

FB₁ (Figures 12, 13, 14). ROMs concentration was affected by mycotoxin concentration and not by time of exposure. A significant decrease of SOD mRNA was observed in a dose dependent manner only in cells exposed for 7 days to mycotoxin. GSH-Px mRNA decreased in a dose dependent manner both in cells exposed for 2 or 7 days to mycotoxin. MDA concentration increased with the increase of FB₁ concentration.

Summarising data obtained in the present study, exposure of PBMC to AFB₁, T-2 toxin, DON and FB₁ is accompanied by an increase of ROMs production, a decrease of antioxidant defences (indicating a state of oxidative stress), and by an increase of MDA (late biomarker of lipid peroxidation and cellular damage) dependent on mycotoxin concentration and time of exposure (Table 1).

Table 1. Effects of mycotoxins on oxidative status indexes.

	ROMs	SH	MDA	SOD mRNA	GSH-Px mRNA
Aflatoxin B₁	↑ C	↓ C	↑ C [§]	↓ C	n.s. [#]
T-2 toxin	↑ C, T [¥]	C, T	↑ C, T	↓ C, T	↓ C, T
Deoxinivalenol	n.s.	n.s.	↑ C, T	n.s.	↓ C, T
Fuminisin B₁	↑ C	n.s.	↑ C, T	↓ T	↓ C

no effect of concentration or time of exposure

§ effect of mycotoxin concentration

¥ effect of time of exposure

DISCUSSION

Since non specific cellular oxidative damages are often observed during toxicity (Gautier et al., 2001), a first question is to establish if the state of oxidative stress is the cause or the consequence of cellular toxicity. Several authors (Abado-Becognee *et al.*, 1997; Abel *et al.*, 1998; Ferrante *et al.*, 2002; Guerre *et al.*, 1999; Hoehler *et al.*, 1998; Leal *et al.*, 1999; Liu *et al.*, 1999; Shen *et al.*, 1996; Vilà *et al.*, 2002; Yin *et al.*, 1998) reported that some mycotoxins can cause cell membrane damage through the increase of lipid peroxidation. Results of those studies indicate the important role of ROMs production in the cytotoxicity properties of mycotoxins. Under physiological conditions ROMs are efficiently neutralized by antioxidant defences. The inactivation of free radicals is the main mechanism to contrast damages due to oxidation. Some antioxidant molecules (selenium, vitamin A, C, E, Q₁₀ coenzyme etc.) (Athrosi *et al.*, 1997; Leal *et al.*, 1999; Rizzo *et al.*, 1998) have the ability to scavenge superoxide anion (O₂^{•-}), and thus can protect membranes by oxidative damages. Other molecules such as thiol groups and in particular intracellular thiols (which provide a good estimation of total intracellular glutathione (GSH) content, since GSH represents about 95% of the intracellular thiols of red blood cells (van den Berg et al., 1992; Lakritz et al., 2002), are involved in the inactivation of lipoperoxides (LOO[•]) in hydroperoxides (LOOH). The onset and extent of GSH depletion has been proposed by some authors (Athrosi et al., 1999) as indicators of toxic metabolite formation by FB₁. MDA is recognized as an important factor in determining alteration of membrane fluidity (Chen e Yu, 1994) and increase of membrane fragility (Spickett et al., 1998) accompanying final cell death (Halliwell e Chirico, 1993).

Csonka *et al.* (2000) demonstrated that under oxidative stress conditions heart cells had lower SOD mRNA and lower SOD and GSH-Px activities. Results of the present study in general showed a decrease of gene expression of antioxidant enzymes (SOD e GSH-Px), and an increase of ROMs and MDA concentrations. The reduction of gene expression of antioxidant enzymes may be considered as the result of the well known inhibitory effect of some mycotoxins of the protein synthesis or their apoptotic effect (Eriksen and Pettersson, 2004). From this point of view, the state of oxidative stress of bovine PBMC, observed in our study, might be interpreted as the consequence instead of the cause of cellular toxicity induced by mycotoxins. According to this hypothesis it has been speculated that exposure to FB1 is responsible for a perturbation of a complex interrelationship between membrane structure and the susceptibility of cellular components to oxidative damage (Yin *et al.*, 1998). Membrane damages caused by lipid peroxidation may also contribute to the oxidative damage of DNA with the possible consequence of genotoxicity, as recently demonstrated in goat lymphocytes exposed to AFB1 (Vitali *et al.*, 2004).

Among mycotoxins tested, intracellular thiols showed a relevant reduction in a dose dependent manner only after exposure of cells to AFB₁. The difference in the effect of AFB₁ on SH content is due to the particular mechanism of detoxification of that mycotoxin. As well known, metabolism of AFB₁ includes epoxidation of the 8,9-double bond, hydroxylation of both furan and lactone rings together with oxidative demethylation, resulting in the formation of a variety of polar metabolites which are mainly conjugated as glutathione before they are excreted (Degan and Neuman, 1978, McLean *et al.* 1995). Therefore, as a consequence of exposure to AFB₁ more GSH is needed for the enzymatic conjugation reaction (Jefferies *et al.*, 2003).

CONCLUSIONS

The experimental model used to study the potential oxidant cytotoxic effect of mycotoxins resulted applicable, sensitive and not invasive. Bovine lymphocytes result influenced by mycotoxin exposure in a dose and-time-dependent manner, with damages related to oxidative stress. The present study contributes to clarify mechanisms through which mycotoxins induce oxidative stress. A possible hypothesis is that oxidative stress may be the final result of toxic action of mycotoxin, and consequent reduction of synthesis and availability of antioxidant defences, leading to cellular damages.

Lymphocyte alteration may be responsible of impairment of immune system function in animal exposed to mycotoxins, with severe consequences on susceptibility to diseases.

Figure 1. Standard concentration curve constructed using known amounts of in vitro synthesized 'sense strand RNA' hybridized with an excess of labeled antisense probe. Ribonuclease protection assay were performed on 10, 20, 50, 100 and 200 pg samples of SOD sense RNA. The reaction products were resolved on a denaturing 5% polyacrylamide gel and then quantified with the Kodak EDAS-290 densitometer and ID Image Analysis software (Eastman Kodak Company, Rochester, NY).

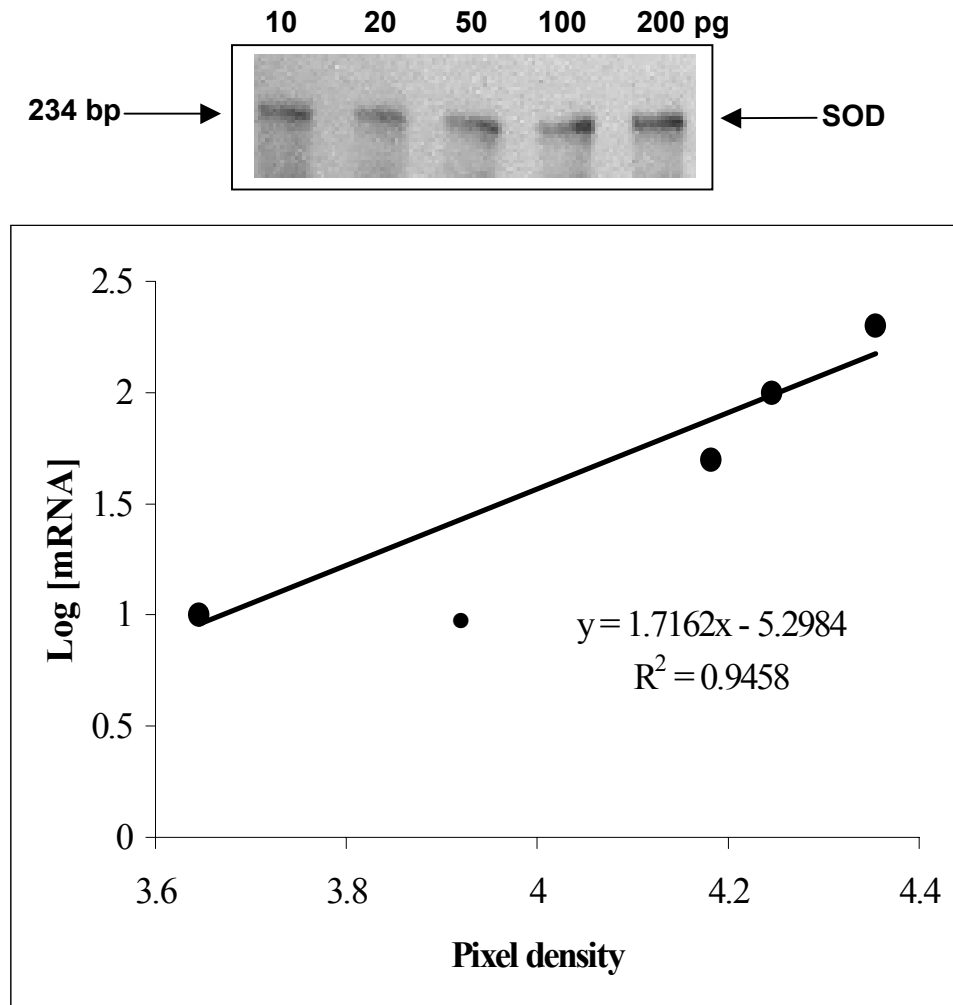


Figure 2. Standard concentration curve constructed using known amounts of in vitro synthesized 'sense strand RNA' hybridized with an excess of labeled antisense probe. Ribonuclease protection assay were performed on 10, 20, 50, 100 and 200 pg samples of GSH-Px sense RNA. The reaction products were resolved on a denaturing 5% polyacrylamide gel and then quantified with the Kodak EDAS-290 densitometer and ID Image Analysis software (Eastman Kodak Company, Rochester, NY).

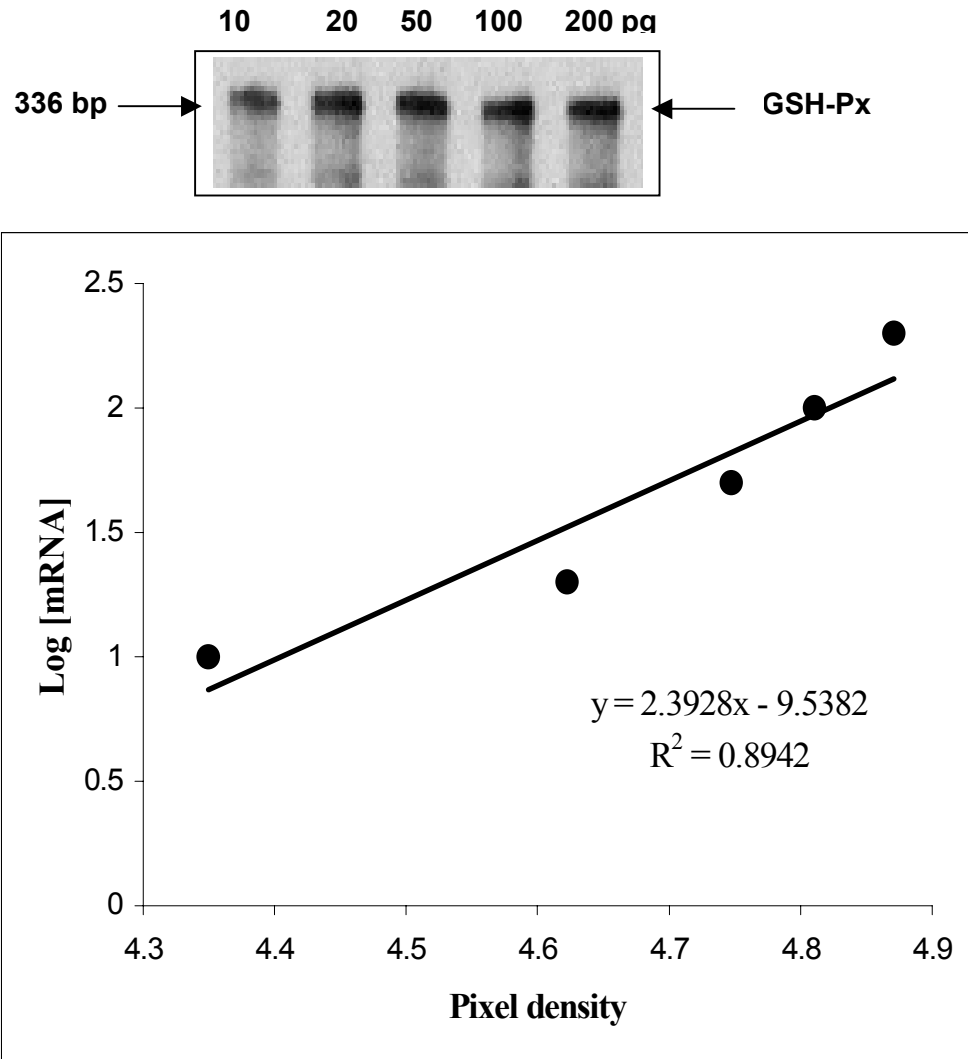


Figure 3. Reactive oxygen metabolites (ROMs) and intracellular thiols (SH) and concentrations in lymphocytes exposed for 2 or 7 days to different concentration of aflatoxin B₁ (AFB₁).

a, b = $P < 0.05$ between concentration within the day of exposure. * $P < 0.5$, between day of exposure within toxin concentration.

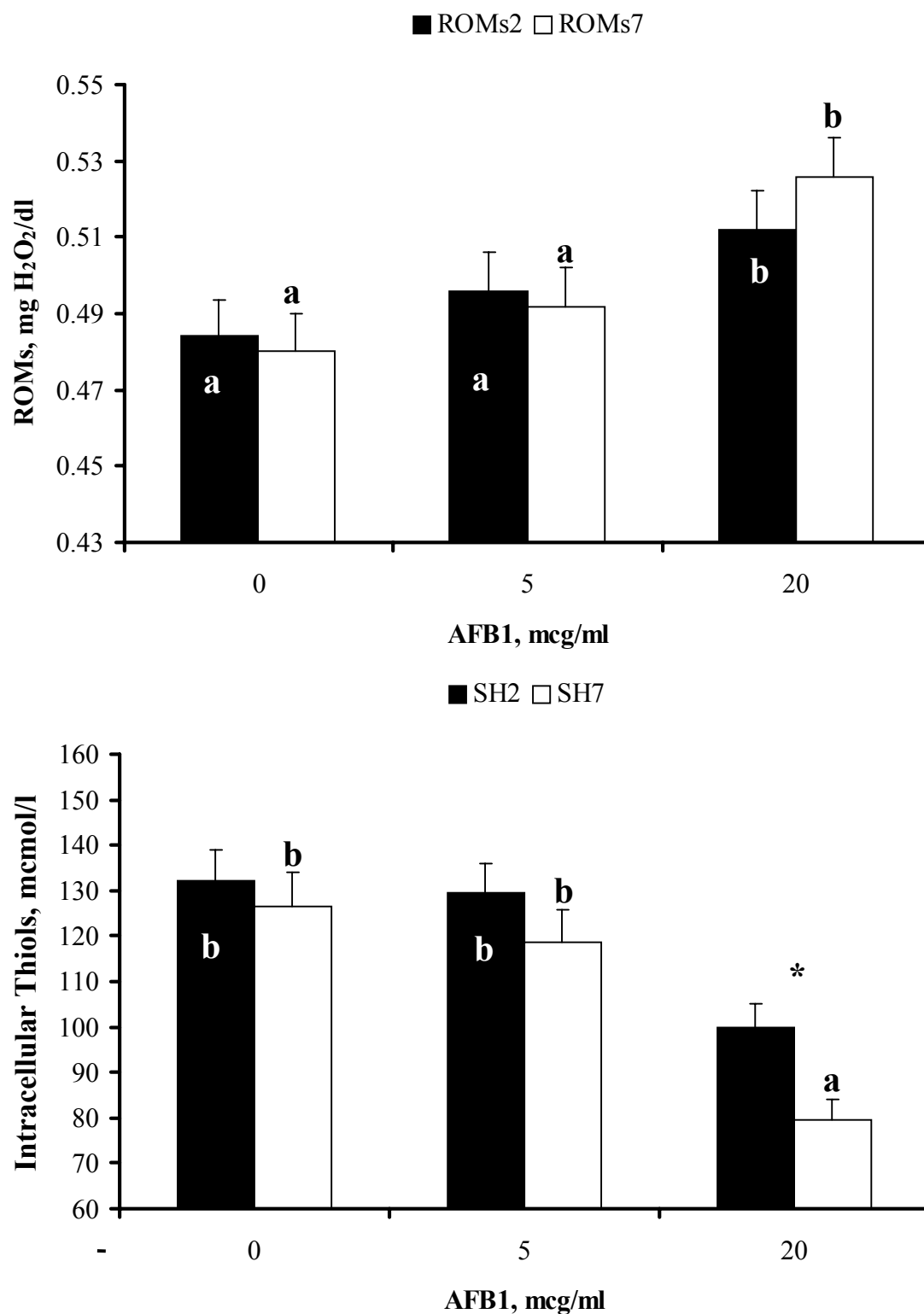


Figura 4. mRNA abundance of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) from lymphocytes exposed for 2 or 7 days to a different concentration of aflatoxin B₁ (AFB₁). mRNA is expressed as pg / 10 µg total RNA. a, b = $P < 0.05$ between concentration within the day of exposure. * $P < 0.05$, between day of exposure within toxin concentration.

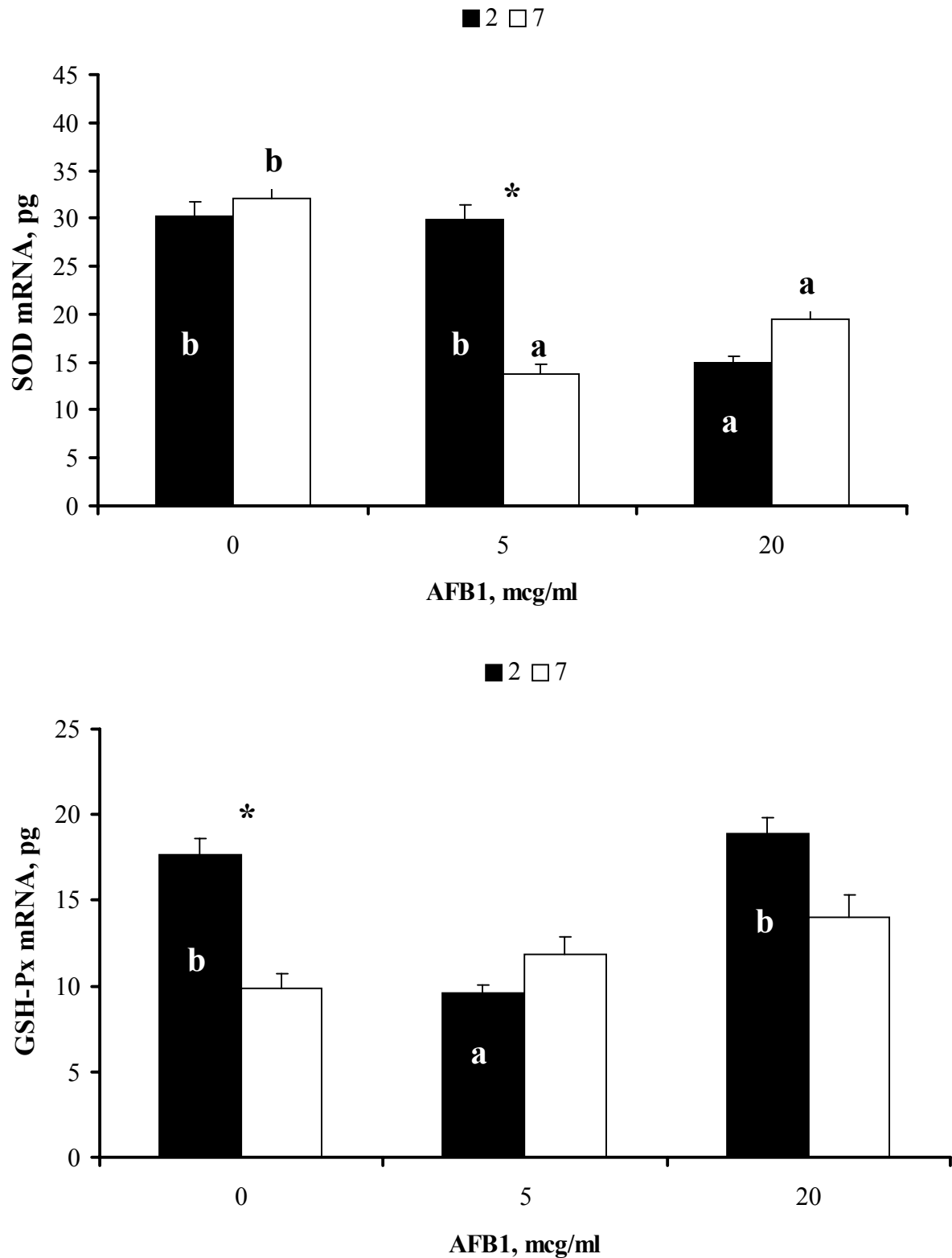


Figura 5. Malondialdehyde (MDA) concentrations in lymphocytes exposed for 2 or 7 days to different concentration of aflatoxin B₁ (AFB₁)
a, b, c = P < 0,05 tra concentrazioni entro periodo di esposizione.

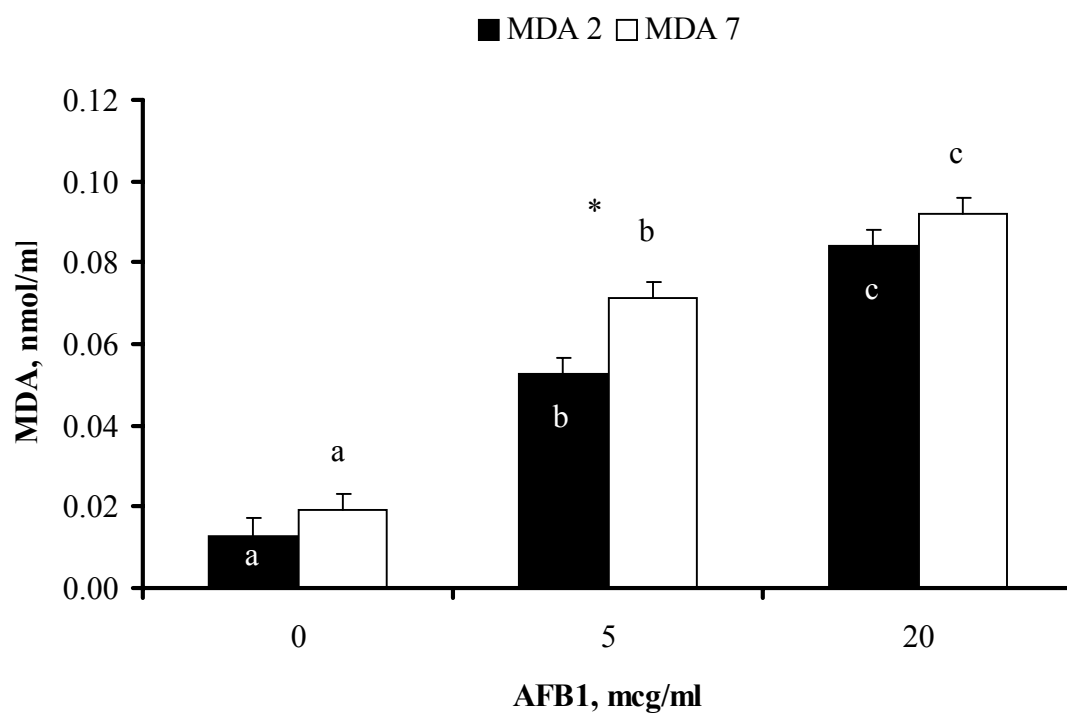


Figura 6. Reactive oxygen metabolites and intracellular thiols concentrations in lymphocytes exposed for 2 or 7 days to different concentration of T-2 toxin (T2).
a, b = $P < 0.05$ between concentrations within day of exposure; * $P < 0.05$ between day of exposure within concentration.

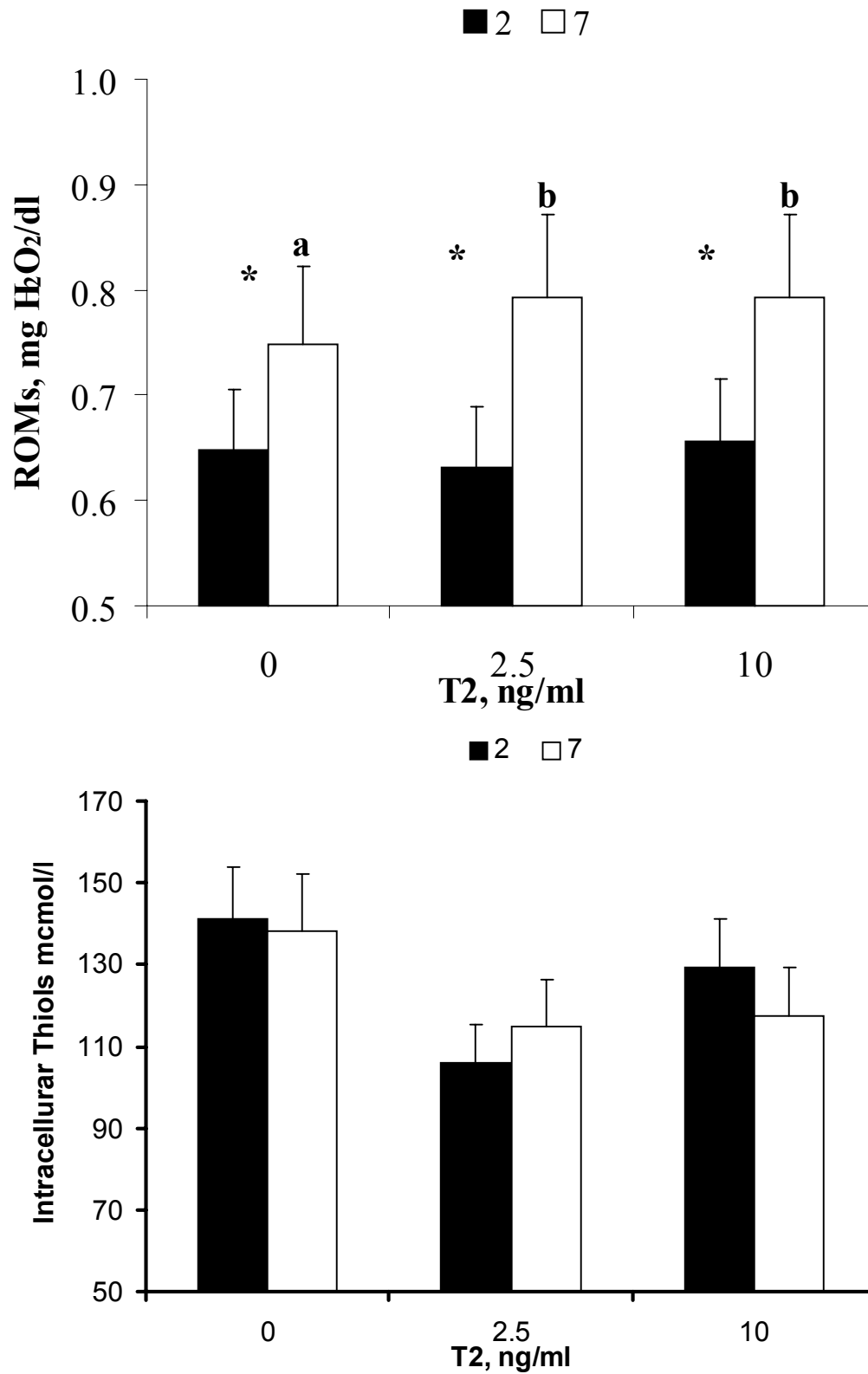


Figura 7. mRNA abundance of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) from lymphocytes exposed for 2 or 7 days to a different concentration of T-2 toxin (T2). mRNA is expressed as pg / 10 µg total RNA.
a, b = $P < 0.05$ between concentrations within day of exposure; * $P < 0.05$ between day of exposure within concentration.

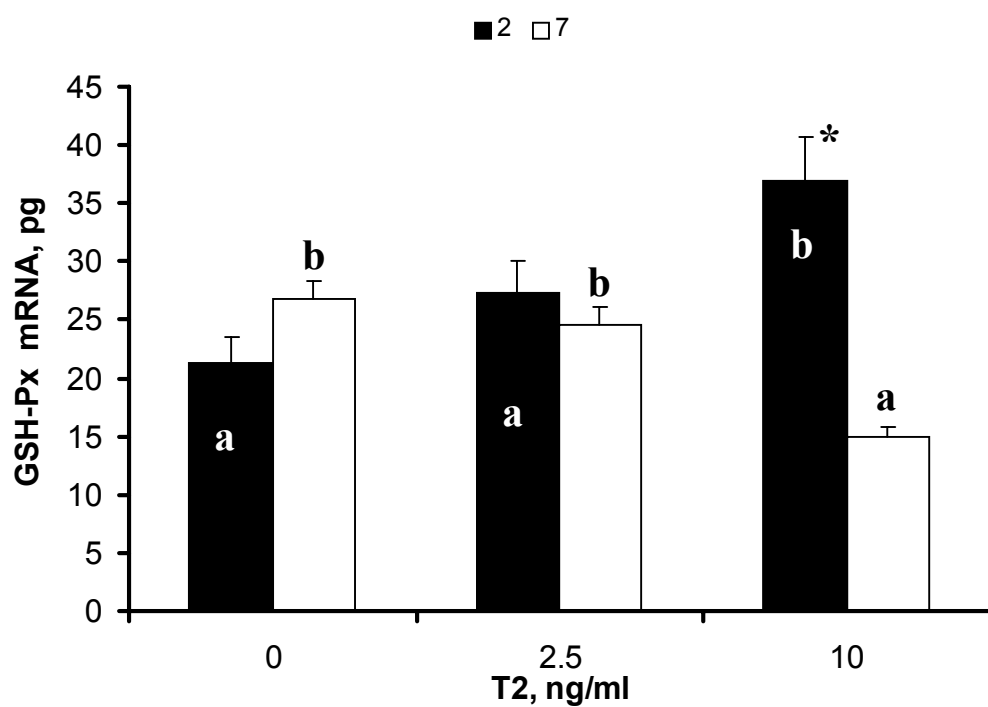
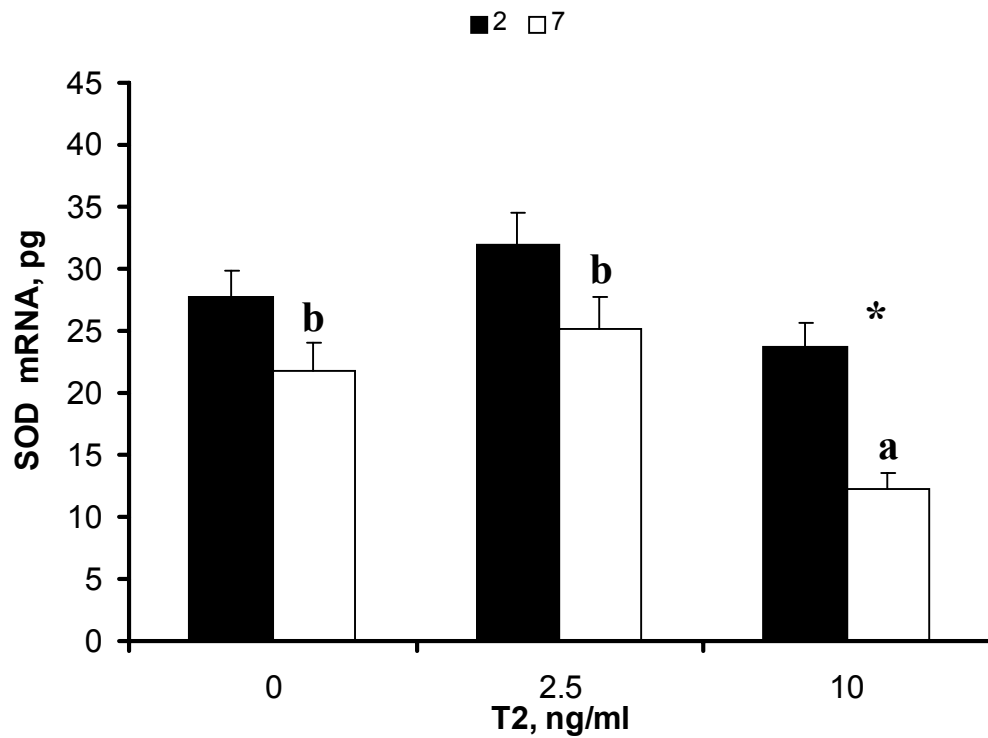


Figura 8. Malondialdehyde (MDA) concentrations in lymphocytes exposed for 2 or 7 days to different concentration of T-2 toxin (T2).

a, b = $P < 0.05$; A, B = $P < 0.01$ between concentrations within days of exposure; ** $P < 0.01$ between day of exposure within concentration.

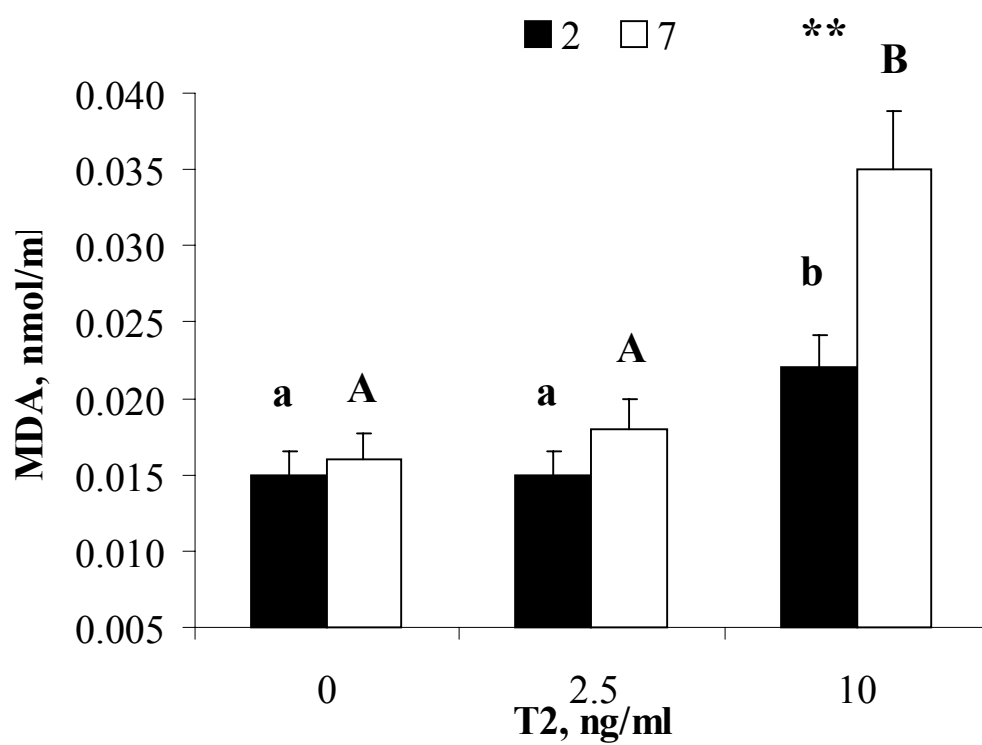


Figure 9. Reactive oxygen metabolites and intracellular thiol concentration in lymphocytes exposed for 2 or 7 days to different concentrations of deoxinivalenol (DON).

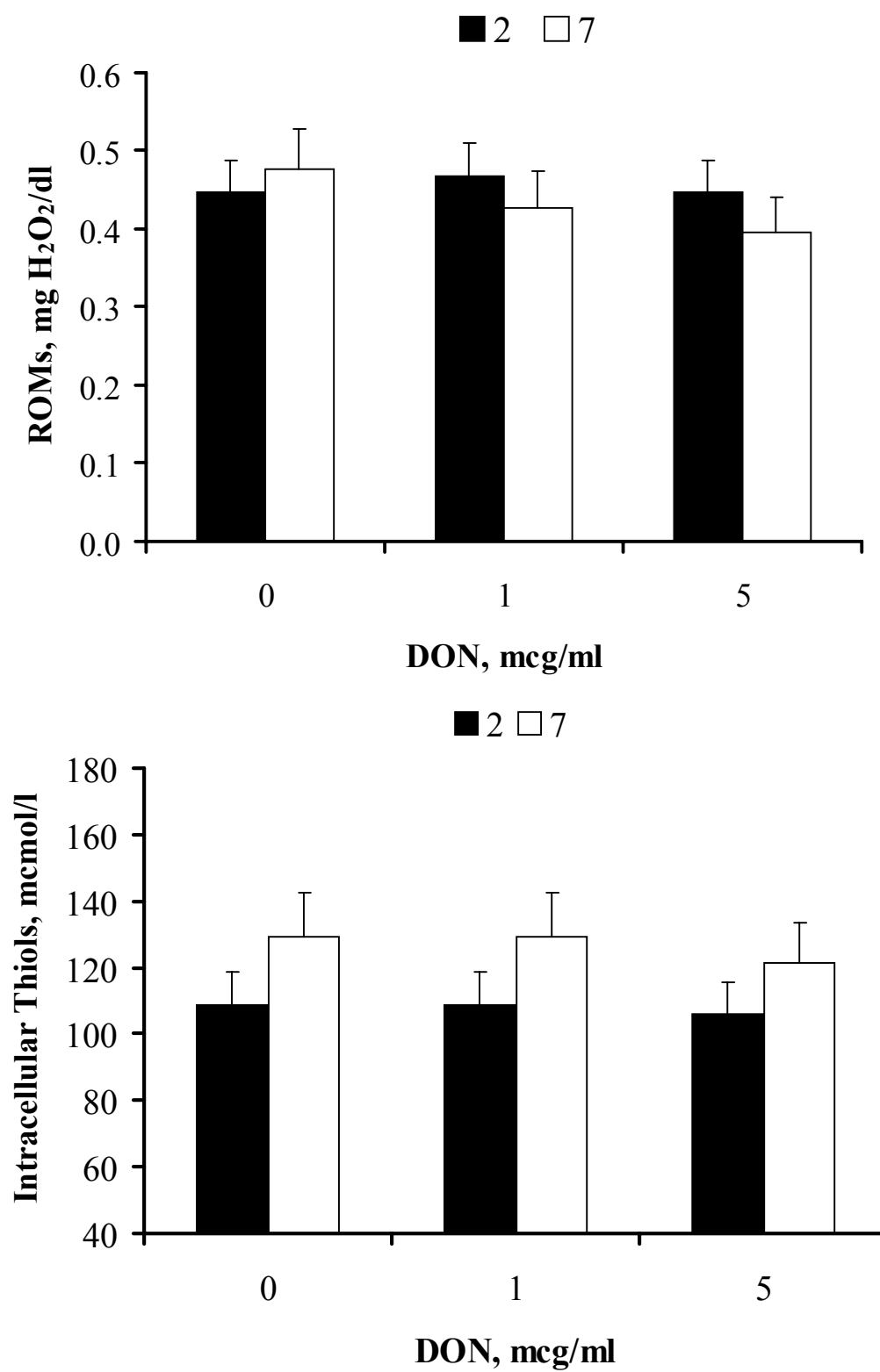


Figura 10. mRNA abundance of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) from lymphocytes exposed for 2 or 7 days to a different concentration of deoxinivalenol (DON). mRNA is expressed as pg / 10 µg total RNA. a, b = $P < 0.05$, A, B = $P < 0.01$ between concentration within the day of exposure. ** $P < 0.01$, between day of exposure within toxin.

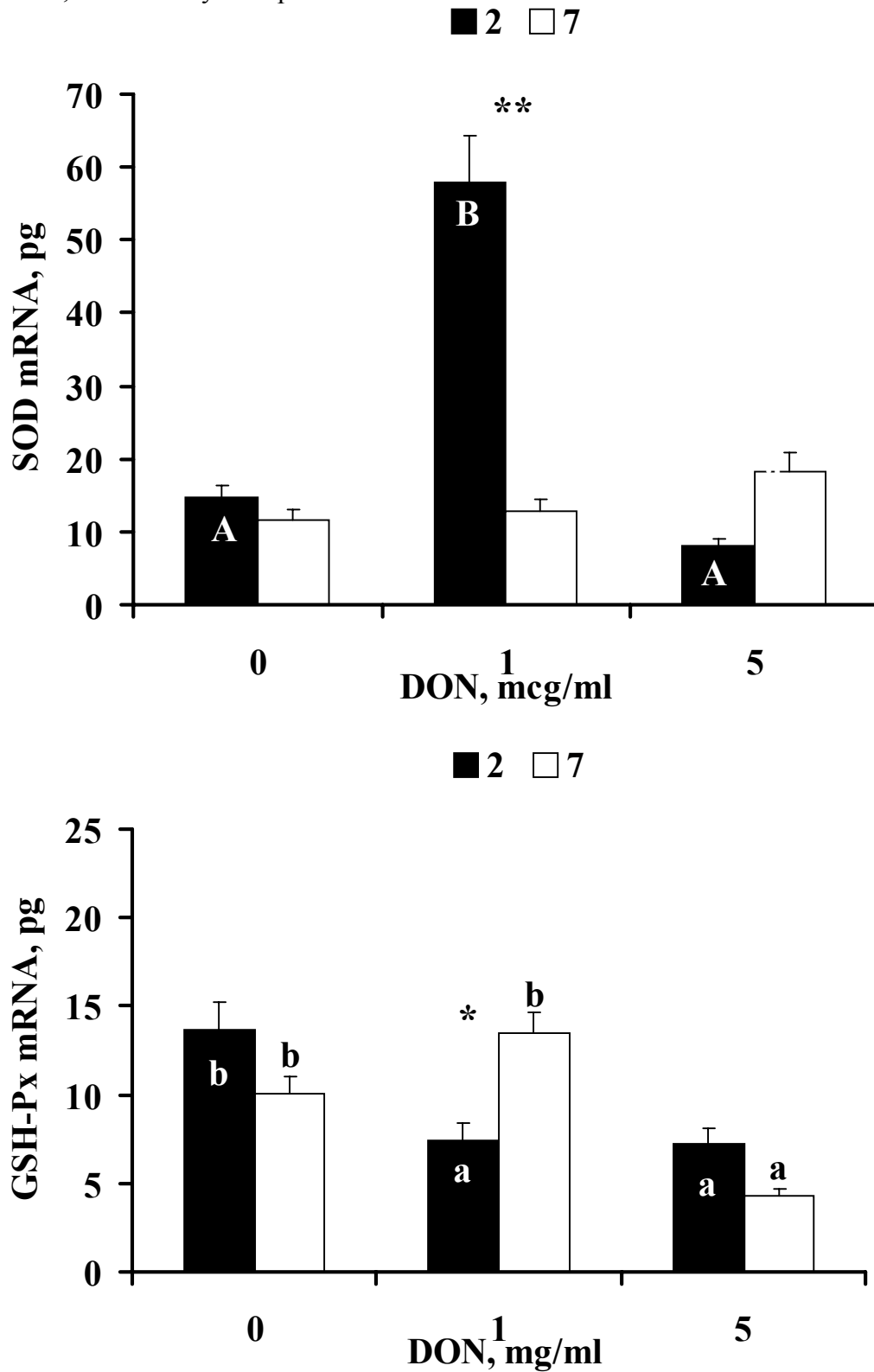


Figura 11. Malondialdehyde (MDA) concentrations in lymphocytes exposed for 2 or 7 days to different concentrations of deoxinivalenol (DON). a, b = $P < 0.05$, A, B = $P < 0.01$ between concentrations within days of exposure; * $P < 0.05$ between days of exposure within concentration.

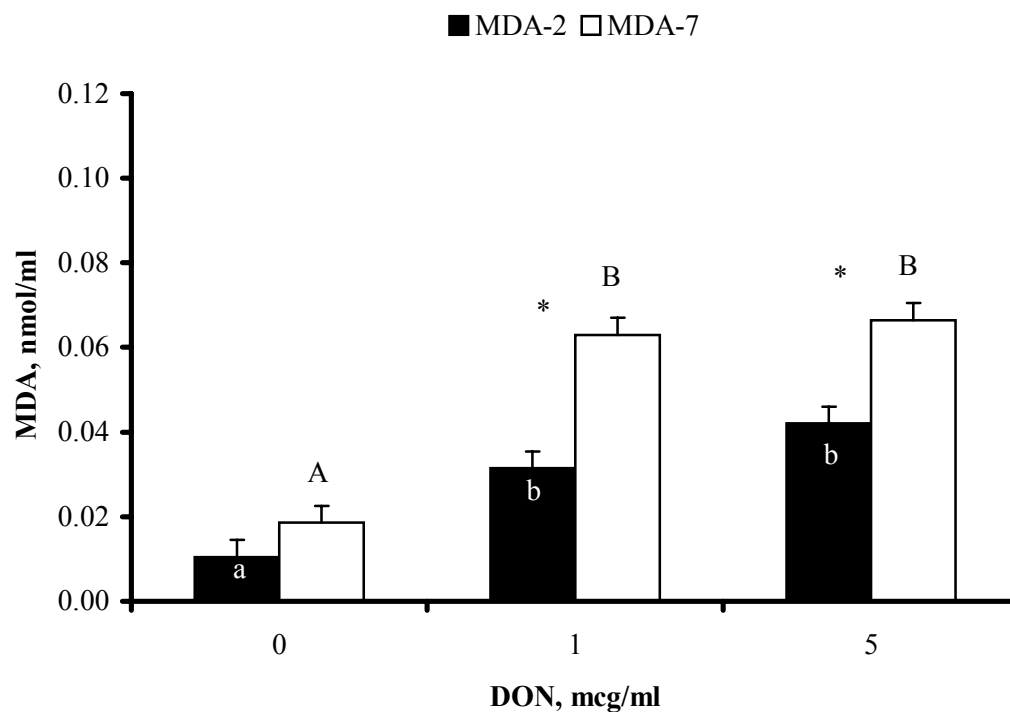


Figura 12. Reactive oxygen metabolites (ROMs) and intracellular thiols (SH) concentrations from lymphocytes exposed for 2 or 7 days to a different concentration of fumonisin B₁ (FB₁).

a, b = $P < 0.05$ between concentrations within days of exposition. * $P < 0.05$ between days of exposition within concentrations.

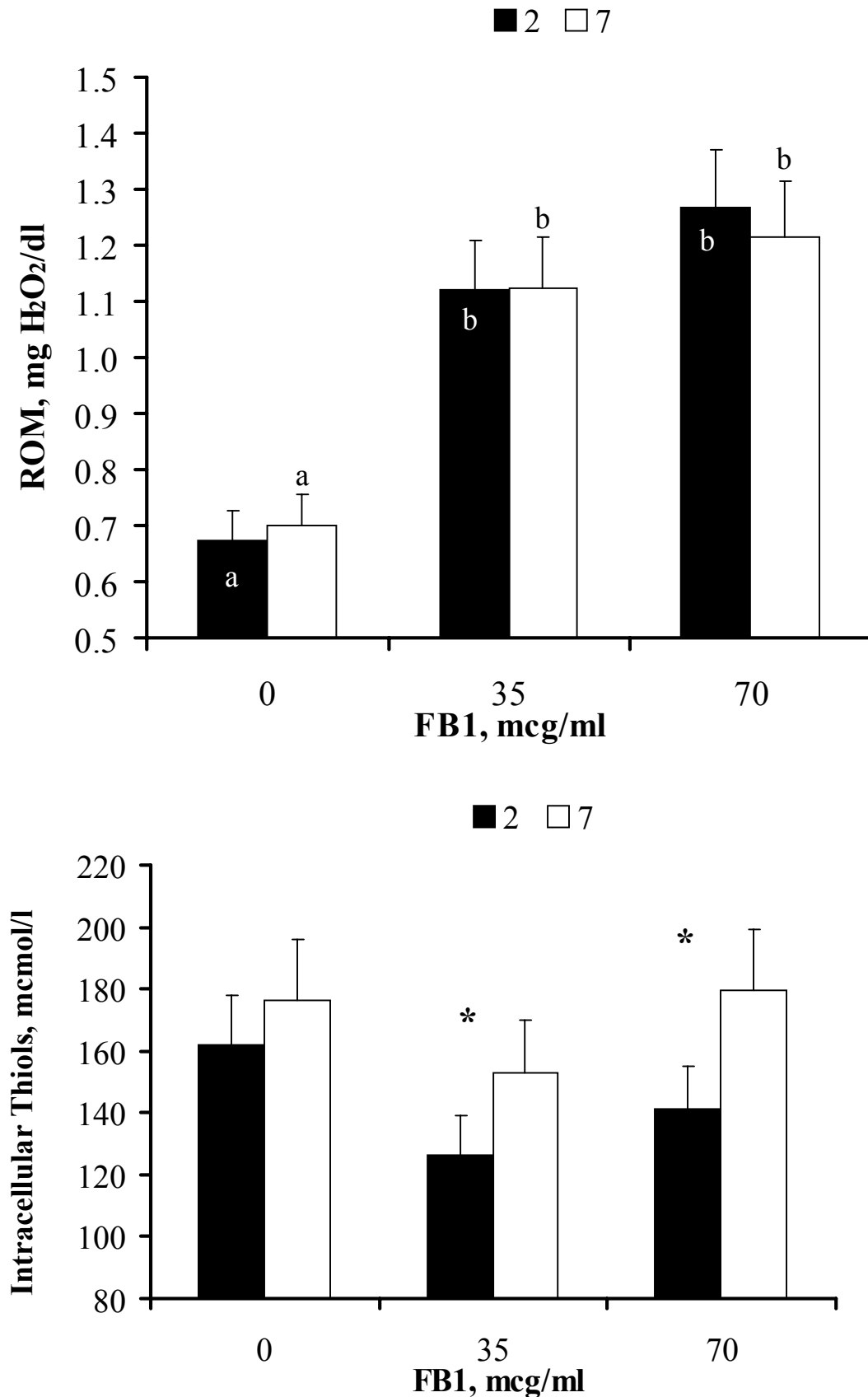


Figura 13. mRNA abundance of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) from lymphocytes exposed for 2 or 7 days to a different concentration of fumonisin B₁ (FB₁). mRNA is expressed as pg / 10 µg total RNA.
a, b = $P < 0.05$ between concentrations within days of exposition; * $P < 0.05$, ** $P < 0.01$ between days of exposition within concentration.

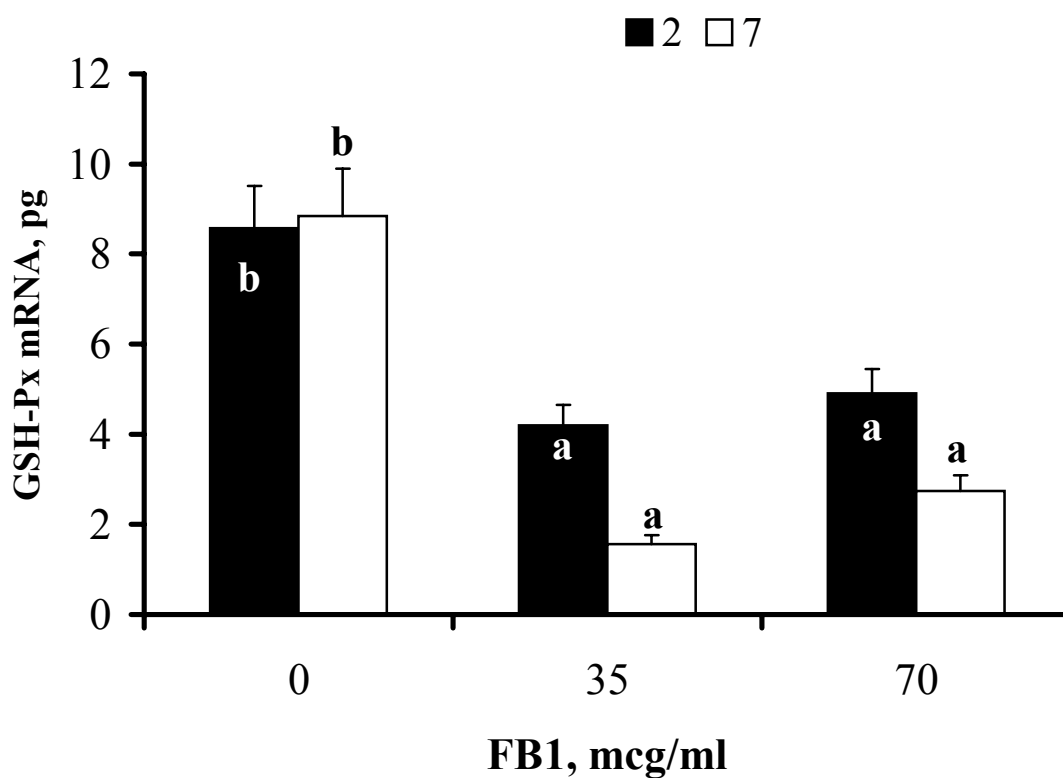
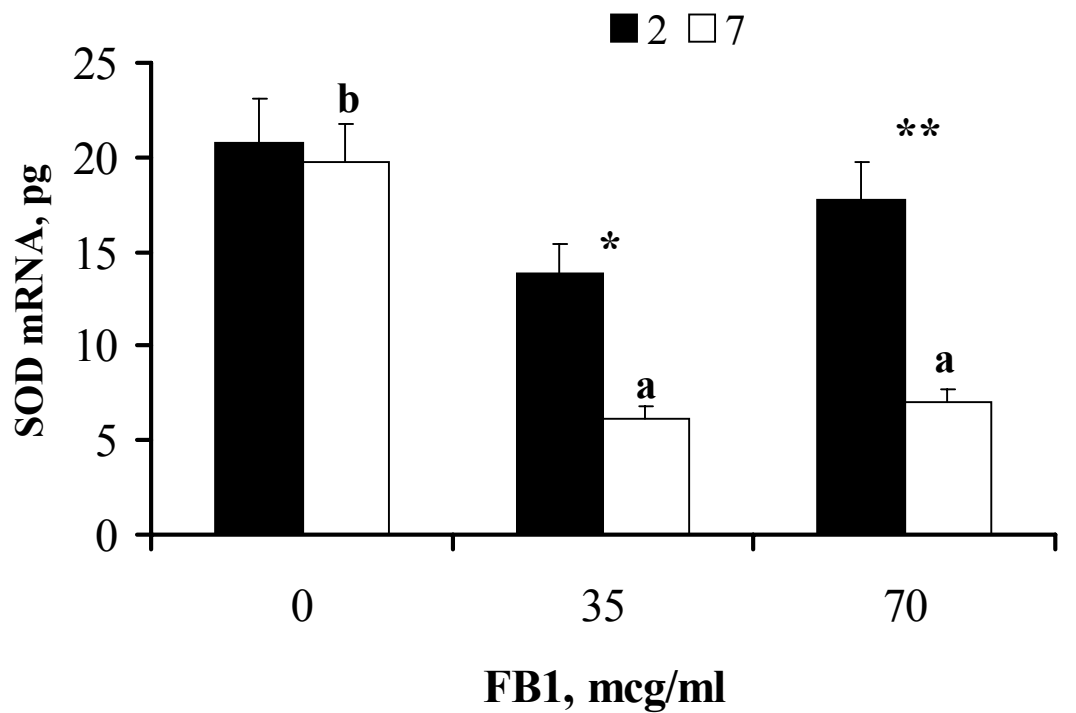
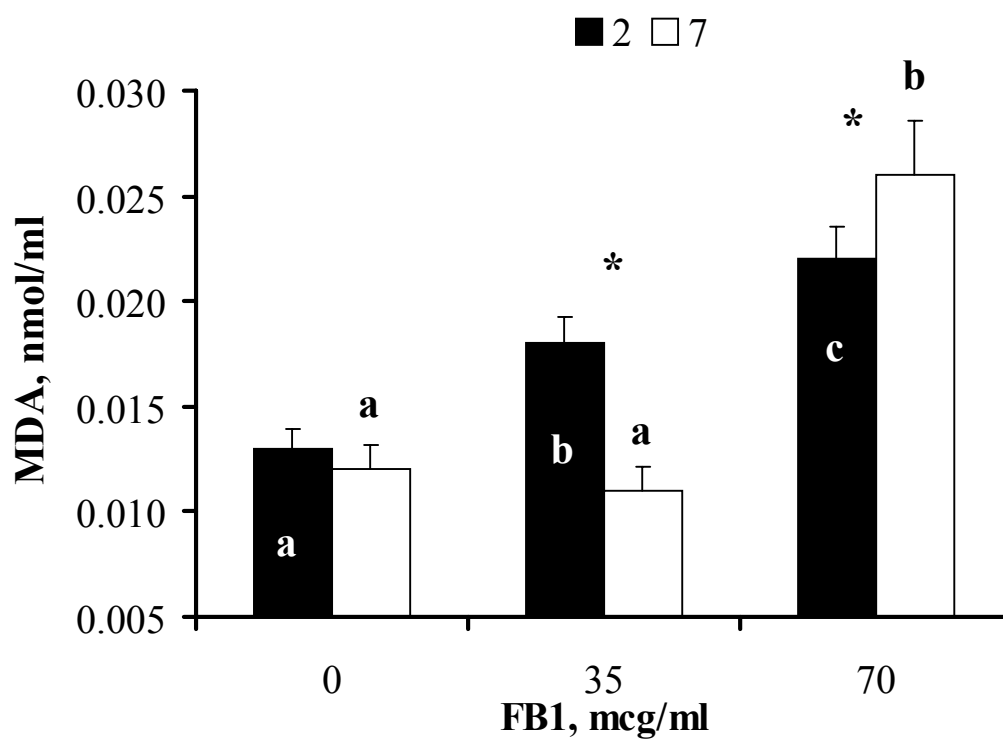


Figura 14. Malondialdehyde (MDA) concentrations in lymphocytes exposed for 2 or 7 days to different concentration of fumonisin B₁ (FB₁).

a, b, c = $P < 0.05$ between concentrations within days of exposure. * $P < 0.05$ between days of exposition within concentration.



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