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Effect of lycopene on lipid peroxidation and glutathione-dependent enzymes induced by T-2 toxin in vivo

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Abstract

Lycopene, obtained from fresh tomatoes, was incorporated into the chicks diet. The treatments were: (1) Control, (2) 1.5 mg T-2 toxin/kg body weight/day; (3) 25 mg lycopene/kg body weight/day, (4) 1.5 mg T-2 toxin plus 25 mg lycopene/kg body weight/day. Male broiler chicks, 7–28 days of age, were provided with feed and water ad libitum. Every 7 days, malondialdehyde (MDA) and glutathione (GSH) levels, and enzymatic activities of glutathione-*S*-transferase (GST), γ-glutamyltransferase (GGT) and glutathione peroxidase (GP) were evaluated in liver homogenates. Compared to the controls after 7 days of treatment, T-2 toxin increased hepatic MDA concentration (128%). A significant consumption of endogenous antioxidant GSH (45%) was induced as well as a marked increase in hepatic enzymatic activities of GST, GGT, and GP (312, 187, and 324%, respectively). Addition of T-2 plus lycopene, at an approximate ratio of 1:17 in the diet, diminished some parameters measured ($P < 0.05$). Apparently lycopene participated as an antioxidant agent and also protecting the cellular level of GSH.

Keywords: Lycopene; T-2 toxin; Glutathione

1. Introduction

T-2 toxin is a mycotoxin produced by several species of the genus *Fusarium* and it is found as a natural contaminant in cereals, feed and vegetables (Jelinek et al., 1989). Toxic effects of T-2 toxin have been reported in humans and various farm and laboratory animal species. In broiler

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chickens, it causes reduced feed consumption and body weight gain (Wyatt et al., 1973), severe oral lesions (Hoerr et al., 1982), altered feathering (Wyatt et al., 1975), neural disturbances (Wyatt et al., 1973) and coagulopathy (Doerr et al., 1981). T-2 toxin and its metabolites induce lipid peroxidation by generating free radicals, causing disturbances in the structure of cellular membranes (Karppanen et al., 1989). Glutathione, a cellular antioxidant, plays an important role due to its direct free-radical scavenging properties and its ability to conjugate with several electrophilic intermediates which are capable of initiating lipid peroxidation (Marrs, 1996). T-2 toxin may bind to –SH groups of proteins (Ueno and Matsumoto, 1975), and some authors suggest it binds to –SH group of glutathione, reducing its cellular level. Ahmed and Ram (1986) have suggested that these biochemical changes are responsible for liver necrosis observed in T-2 animal toxicosis.

The discovery that carotenoids deactivate singlet molecular oxygen was an important advance in understanding the biological effects of carotenoids (Foote and Denny, 1968). The role of dietary antioxidants such as carotenoids, in health and disease, has attracted increasing attention at the present time, and numerous trials are currently in progress to ascertain the benefits of these compounds in the diet (Nierenberg et al., 1997). Lycopene is a red pigment present mainly in tomato (0.09 mg/g) (Mangles et al., 1993); and it presents one of the highest antioxidant activities among carotenoids (Di Mascio et al., 1989). Epidemiological studies have found that dietary intake of lycopene is correlated with diminished risk for prostate cancer (Giovannucci et al., 1995), cell proliferation in various human epithelial cancer cell lines (Levy et al., 1995), and tumors incidence (Sharoni et al., 1997). In vitro studies using chicken hepatocytes indicated that the carotenoids lutein and lycopene were able to reduce the cytotoxic effect of T-2 toxin (Leal et al., 1998). Therefore, the focus of this study was to evaluate, in vivo, the effect of lycopene on lipid peroxidation and the enzymes involved in the glutathione metabolism in broiler chicks exposed to T-2 toxin.

2. Materials and methods

2.1. Animals and treatments

One-day-old male broiler chicks (Arbor Acres × Indiana River) were obtained from a commercial hatchery (Pilgrim's Pride, Querétaro, México). Chicks were placed in electrically heated cages, under continuous fluorescent lighting, with feed and water available ad libitum. Prior to the experiments, animals were housed for 7 days for acclimatization. After this period, the chicks were individually weighed and distributed according to the experimental design, which consisted of four dietary treatments: (1) Control, with 0 mg T-2 toxin and 0 mg lycopene/kg body weight/day, (2) 1.5 mg T-2 toxin/kg body weight/day, (3) 25 mg lycopene/kg body weight/day, (4) 1.5 mg T-2 toxin plus 25 mg lycopene/kg body weight/day. These doses were chosen based on preliminary studies that evaluated weight gain, feed consumption, mortality, as well as the levels of GSH and GST. A minimum T-2 toxin concentration that caused GSH and GST changes without causing mortality, and a maximum concentration of lycopene that avoided animal injury, based on histological examination (data not shown), were used. At the beginning of the study there were 90 chicks in each of the four treatment groups (three replicates of thirty chicks per dietary treatment). Feed consumption was recorded daily for each replicate. After 7, 14 and 21 days, 30 animals from each treatment group (ten chicks by replicate), were individually weighed and sacrificed by cervical dislocation. The liver was removed for biochemical assays.

2.2. Diets

The composition of the diets used for two growth stages of chicks (1–20 days old and 21–28 days old, respectively) were formulated based on sorghum and soybean meal, calculated to contain 23% protein and 2900 Kcal of metabolizable energy/kg (ME/kg of diet) and 20% protein and 2900 Kcal ME/kg, respectively, as recommended by the NRC (1994).

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T-2 toxin was purchased from Sigma (St. Louis, MO), certified as 95% pure by gas chromatography/mass spectrometry. It was incorporated into the basic diet by dissolving the toxin in 85% ethanol and then mixing the appropriate quantity with 1 kg of the basic diet, which was dried at 50°C for 50 min and mixed with the rest of the basic diet, to produce a diet containing T-2. Lycopene was obtained from fresh tomatoes, following the Bierer et al. (1995) technique. The content of lycopene in the remaining residue, after hexane evaporation, was directly analyzed without any previous extraction, by high-performance liquid chromatography (HPLC) (Perkin Elmer model 400). The column was a Waters Bondapack C_{18} (10 μm, 150 × 190 mm i.d.). The mobile phase was a mixture of acetonitrile, dichloromethane and methanol (45:10:45), with a flow rate of 2 ml/min, and 25 μl volume injection. The detection was set at 470 nm. Quantitation of lycopene was based on an external standard method. The standard curve was generated with pure lycopene (L-9879, purchased from Sigma) dissolved in dichloromethane grade HPLC and added to commercial olive oil (1:9), to obtain a final lycopene concentration of 1000 ppm. The standard curve was produced by diluting the previous solution in the mixture of acetonitrile, dichloromethane and methanol (45:10:45), to obtain the necessary concentrations. The regression equation was $y = 305.5702 + 3.3511x$, where y is lycopene concentration in ppm, and x is the area under the curve. There was a good linearity over a wide range of concentrations, and a relative standard deviation of less than 5%. An aliquot of lycopene (87% pure) was dissolved in dichloromethane and mixed with commercial olive oil, then the dichloromethane was evaporated under a stream of nitrogen. The elimination of dichloromethane was confirmed by HPLC. The remaining residue was stored at − 80°C until used and incorporated into the basic diet, just before consumption; mixing the appropriate quantity to produce a diet containing lycopene.

2.4. Measurement of malondialdehyde (MDA)

Analytical procedures, to obtain the liver homogenate and the determination of MDA concentrations, were performed according to the method of Botsoglou et al. (1994). This method is rapid, sensitive and specific for measuring lipid peroxidation in animal tissue. The sample was homogenized with aqueous trichloroacetic acid in the presence of hexane and BTH, and the homogenate was centrifuged. Hexane added before blending was effective for reducing lipid peroxidation; BHT showed an outstanding antioxidant activity. Full protection against autoxidation was assured when BHT was added to the sample before the homogenization process. Following reaction with thiobarbituric acid reagent, MDA was directly quantified (at 521 nm) on the basis of the third-derivative absorption spectrum of the pink complex formed, using a Perkin Elmer Spectrophotometer Lamda 2. Further purification was not required because the derivative transformation of the conventional analytical band, at around 532 nm, eliminates spectral interference arising from other compounds (Botsoglou et al., 1994). The method gave very high recovery (94.6%) with a relative standard deviation of 2%.

2.5. Preparation of liver homogenate

A 2 g liver sample was homogenized in 4 ml of 10 mM Tris–HCl/KCl buffer (pH 7.4) using an Ultra turrax T25 (Janke & Kunkel IKA Labortechnik, Germany). The homogenate was centrifuged at 15 000 × g for 30 min. The supernatant was used for biochemical assays.

2.6. Measurement of glutathione (GSH)

Glutathione concentration in liver homogenate was determined with a fluorescence assay described by DelRaso (1992), where *O*-phthaldehyde, a nonfluorescent compound, becomes highly fluorescent when it conjugates to GSH. A Sequoia-Turner fluorometer, model 450, with fluorescence at 430 nm (emission) and excitation at 360 nm was used.

2.7. Measurement of enzymes involved in glutathione metabolism

In liver homogenate, the activities of the following enzymes were determined, with a Perkin Elmer Spectrophotometer Lamda 2. All of them have been reported as 'U/L', where 'U' are international units and 'L' the homogenate volume normalized by protein content.

2.7.1. Glutathione-S-transferase (GST)

Glutathione-S-transferase (GST) using the spectrophotometric assay of Alin et al. (1985), which uses 1-chloro-2,4-dinitrobenzene as electrophilic substrate that binds to GSH with the participation of the enzyme and forms a colored GSH-substrate complex, detected at 340 nm.

2.7.2. γ-Glutamyltransferase (GGT)

γ-Glutamyltransferase (GGT) activity was detected with the procedure described by Szasz (1969). In this spectrophotometric assay the enzyme catalyzes the transfer of γ-glutamyl group from L-γ-glutamyl-*p*-nitroaniline to glycylglycine, releasing the chromogenic product *p*-nitroaniline that absorbs strongly at 405 nm.

2.7.3. Glutathione peroxidase (GP)

Glutathione peroxidase (GP) activity was determined using the method of Lawrence and Burk (1976), which records at 340 nm the disappearance of NADPH. The action of GP is to reduce hydrogen peroxide, with coupled oxidation of NADPH.

2.8. Statistical analyses

All treatment groups were analyzed using a complete block design, with repeated measurements (Gill, 1978), where the cage formed the blocks. The effect of the cage within each treatment group was considered as the error term. All analyses of variance were conducted to obtain the treatment effect within each time level, using the general linear model procedure in the SAS software (SAS, 1994). The mean and standard deviation were calculated for all treatment groups and are presented in Tables and Figures. Means of treatment groups showing significant differences in the analysis of variance were compared using the Student–Newman–Keuls test. In all cases, a P-value of < 0.05 was used to determine significance.

3. Results

Table 1 shows the increase in feed consumption and body weight gain of the animals in different treatments. Table 2 presents the oral lesions due to the exposure to T-2 toxin and lycopene.

Table 3 shows an increased ($P < 0.05$) level of MDA in the animals exposed to T-2 toxin, in comparison with the control and lycopene treatment groups, after the first 7 days (128%); turning more severe at the end of the study (510%, 21 days). The animals exposed to lycopene plus mycotoxin showed a reduction in MDA production (24, 54 and 67% for 7, 14 and 21 days of treatment, respectively) when com-

Table 1
Effect of T-2 toxin and lycopene on feed consumption (g/chicken/day) and body weight gain (g)[a]

	Feed consumption (days)			Body weight gain (days)		
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Control	35.5 ± 7^{a}	62.6 ± 6^{a}	78.3 ± 20^{a}	256 ± 15^{a}	531 ± 48^{a}	866 ± 100^{a}
Lycopene	34.8 ± 7^{a}	57.4 ± 8^{a}	66.5 ± 22^{b}	257 ± 27^{a}	454 ± 50^{b}	847 ± 82^{a}
T-2 toxin	28.5 ± 5^{a}	50.0 ± 9^{b}	50.1 ± 18^{c}	203 ± 25^{a}	434 ± 17^{b}	634 ± 93^{c}
T-2 toxin plus lycopene	32.4 ± 6^{a}	51.6 ± 7^{b}	64.3 ± 15^{b}	244 ± 14^{a}	439 ± 50^{b}	741 ± 99^{b}

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Table 2
Oral lesions in chickens caused by ingestion of T-2 toxin[b]

Treatment	Oral lesions (%)[a]					
	Angle of the mouth			Palatine ridge and tongue		
	7 days	14 days	21 days	7 days	14 days	21 days
T-2 Toxin						
Light injury	0	0	50	0	16	50
Moderate injury	100	16	16	100	16	16
Severe injury	0	66	33	0	66	33
T-2 Toxin+lycopene						
Light injury	0	50	66	33	16	0
Moderate injury	33	0	0	33	16	68
Severe injury	0	0	0	0	0	0

[a] % Was calculated considering that control treatment did not show any injure.
[b] Chicks consumed 1.5 mg T-2/kg body weight/day and 25 mg lycopene/kg body weight/day.

Table 3
Effect of T-2 toxin and lycopene on glutathione (GSH) (nmol/mg protein) and malondialdehyde (MDA) (nmol/mg tissue) content on chicken liver[a]

Treatment	Time (days)					
	7		14		21	
	MDA	GSH	MDA	GSH	MDA	GSH
Control	216.7 ± 27.8^{c}	72.9 ± 0.9^{a}	187.5 ± 29.2^{c}	69.9 ± 0.9^{a}	284.7 ± 15.31^{c}	72.6 ± 1.0^{a}
Lycopene	215.3 ± 26.4^{c}	68.9 ± 1.0^{a}	181.9 ± 27.8^{c}	72.2 ± 1.0^{a}	284.7 ± 13.9^{c}	71.0 ± 0.9^{a}
T-2 toxin	494.4 ± 27.8^{a}	39.8 ± 0.8^{b}	677.8 ± 15.3^{a}	44.7 ± 0.9^{b}	1738.9 ± 37.5^{a}	64.1 ± 0.9^{b}
T-2 toxin plus lycopene	373.6 ± 29.2^{b}	66.9 ± 0.8^{a}	311.1 ± 25.0^{b}	66.3 ± 1.0^{a}	575.0 ± 25.0^{b}	64.6 ± 1.0^{b}

[a] Chicks consumed 1.5 mg T-2/kg body weight/day and 25 mg lycopene/kg body weight/day. Values are means ± SD of 30 chicks. Values for the same time, comparing different treatments, with no common letters are significantly different ($P < 0.05$).

pared with animals exposed to T-2. Chicks receiving lycopene alone showed similar responses to the control animals ($P > 0.05$).

Chicks exposed to T-2 toxin, showed a reduction ($P < 0.05$) in the content of hepatic GSH during all the time of treatment, this response is observed in Table 3. When lycopene plus T-2 toxin were included in the diet, the level of GSH during the first 14 days was higher ($P < 0.05$) than with T-2 treatment. However, at 21 days both T-2 and chemopreventive treatment groups, had similar response, and marginally less than the untreated control groups. The level of GSH in chicks exposed to lycopene was not affected.

Fig. 1 shows increased ($P < 0.05$) GST activity in the liver of animals exposed to T-2 toxin with 7 to 21 days of treatment. The chemopreventive treatment showed an increase ($P < 0.05$) in GST activity (Fig. 1). Nevertheless, it was lower (37, 33 and 31% at 7, 14 and 21 days, respectively) than the response obtained for T-2 treatment.

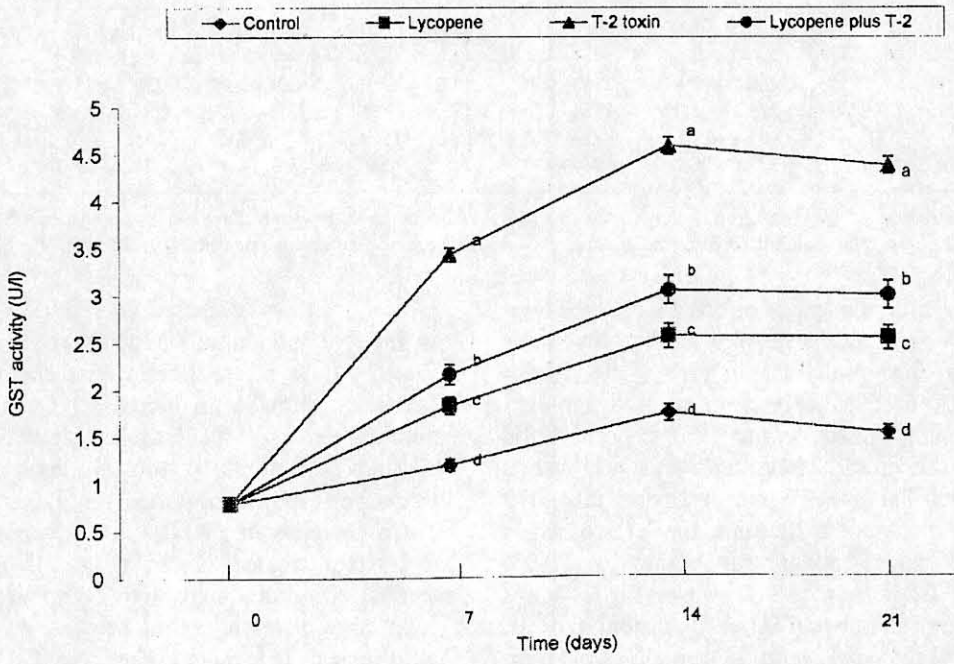
An increase in GGT activity was observed in T-2 toxin treated chicks (Fig. 2). The animals treated simultaneously with lycopene and mycotoxin showed a reduction in the enzyme activity, with respect to chicks exposed to T-2 (38, 21 and 22% for 7, 14 and 21days, respectively). However, their response was statistically different from the activity obtained with control animals ($P < 0.05$).

It was interesting to observe the activity of GP in liver of chicks exposed either to T-2 toxin treatment, or lycopene and T-2 treatment (Fig. 3). The toxin induced an increase (324%) in GP activity after 7 days of treatment. This activity decreased with time of exposure (37% after 21 days). After 14 days of treatment, the presence of lycopene in T-2 toxin treated animals gave a GP activity similar to the control animals. It is important to mention that no mortality resulted in any of the treatment groups.

4. Discussion

Body weight gains were not different in the study groups up to 14 days. At 21 days, although the feed consumption for lycopene and T-2 toxin plus lycopene treatment groups were statistically the same, the body weight gains were different giving an idea of specific toxicities. The data also suggested that lycopene was able to inhibit severe oral damage.

Lipid peroxidation is one of the responsible factors for the damage and necrosis of liver, induced by chemical compounds like mycotoxins (Suneja et al., 1989). Because biological membranes are rich in unsaturated fatty acids, the susceptibility of membranes to peroxidative attack is not rare. On the other hand, GSH status is an important factor involved in the protection against lipid peroxidation, and its concentration is maintained in part through the enzymes participating in its metabolism. In the present study, daily administration of T-2 toxin significantly increased lipid peroxidation in chick liver, as measured by the formation of MDA. Chang and Mar (1988), and Suneja et al. (1989) have also shown an increase in the level of liver lipid peroxides in rats fed with T-2 toxin. It is important to consider that GSH could be exported from the liver, which is the primary source of plasma GSH (Lauterburg et al., 1984); however, all tissues synthesize GSH to some extent. Over time, the effect of T-2 toxin treatment on GSH was diminished, and after 21



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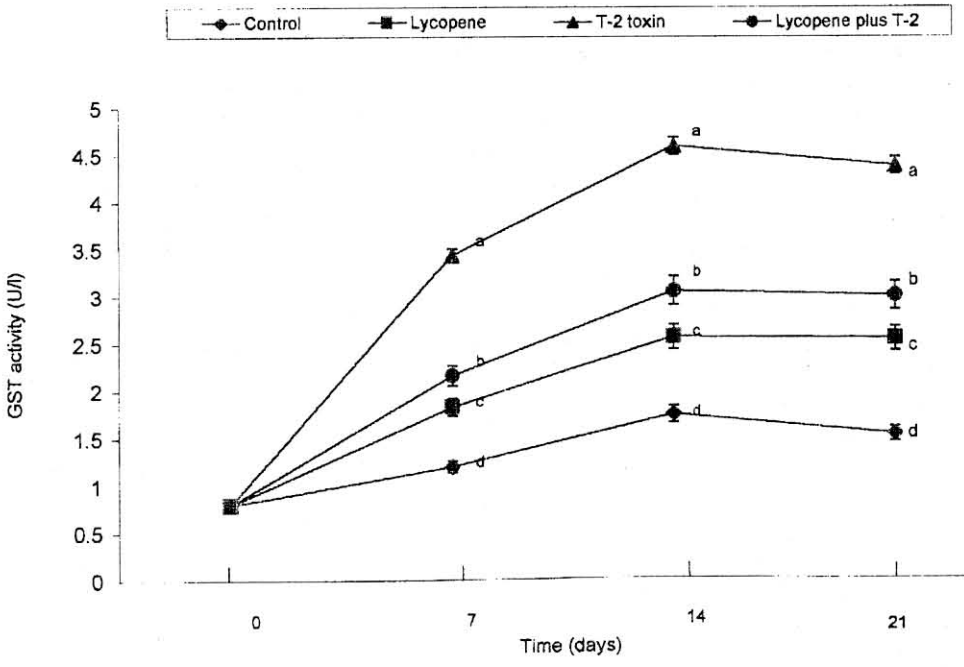

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Table 2
Oral lesions in chickens caused by ingestion of T-2 toxin[b]

Treatment	Oral lesions (%)[a]					
	Angle of the mouth			Palatine ridge and tongue		
	7 days	14 days	21 days	7 days	14 days	21 days
T-2 Toxin						
Light injury	0	0	50	0	16	50
Moderate injury	100	16	16	100	16	16
Severe injury	0	66	33	0	66	33
T-2 Toxin+lycopene						
Light injury	0	50	66	33	16	0
Moderate injury	33	0	0	33	16	68
Severe injury	0	0	0	0	0	0

[a] % Was calculated considering that control treatment did not show any injure.
[b] Chicks consumed 1.5 mg T-2/kg body weight/day and 25 mg lycopene/kg body weight/day.

Table 3
Effect of T-2 toxin and lycopene on glutathione (GSH) (nmol/mg protein) and malondialdehyde (MDA) (nmol/mg tissue) content on chicken liver[a]

Treatment	Time (days)					
	7		14		21	
	MDA	GSH	MDA	GSH	MDA	GSH
Control	216.7 ± 27.8^{c}	72.9 ± 0.9^{a}	187.5 ± 29.2^{c}	69.9 ± 0.9^{a}	284.7 ± 15.31^{c}	72.6 ± 1.0^{a}
Lycopene	215.3 ± 26.4^{c}	68.9 ± 1.0^{a}	181.9 ± 27.8^{c}	72.2 ± 1.0^{a}	284.7 ± 13.9^{c}	71.0 ± 0.9^{a}
T-2 toxin	494.4 ± 27.8^{a}	39.8 ± 0.8^{b}	677.8 ± 15.3^{a}	44.7 ± 0.9^{b}	1738.9 ± 37.5^{a}	64.1 ± 0.9^{b}
T-2 toxin plus lycopene	373.6 ± 29.2^{b}	66.9 ± 0.8^{a}	311.1 ± 25.0^{b}	66.3 ± 1.0^{a}	575.0 ± 25.0^{b}	64.6 ± 1.0^{b}

[a] Chicks consumed 1.5 mg T-2/kg body weight/day and 25 mg lycopene/kg body weight/day. Values are means ± SD of 30 chicks. Values for the same time, comparing different treatments, with no common letters are significantly different ($P < 0.05$).

pared with animals exposed to T-2. Chicks receiving lycopene alone showed similar responses to the control animals ($P > 0.05$).

Chicks exposed to T-2 toxin, showed a reduction ($P < 0.05$) in the content of hepatic GSH during all the time of treatment, this response is observed in Table 3. When lycopene plus T-2 toxin were included in the diet, the level of GSH during the first 14 days was higher ($P < 0.05$) than with T-2 treatment. However, at 21 days both T-2 and chemopreventive treatment groups, had similar response, and marginally less than the untreated control groups. The level of GSH in chicks exposed to lycopene was not affected.

Fig. 1 shows increased ($P < 0.05$) GST activity in the liver of animals exposed to T-2 toxin with 7 to 21 days of treatment. The chemopreventive treatment showed an increase ($P < 0.05$) in GST activity (Fig. 1). Nevertheless, it was lower (37, 33 and 31% at 7, 14 and 21 days, respectively) than the response obtained for T-2 treatment.

An increase in GGT activity was observed in T-2 toxin treated chicks (Fig. 2). The animals treated simultaneously with lycopene and mycotoxin showed a reduction in the enzyme activity, with respect to chicks exposed to T-2 (38, 21 and 22% for 7, 14 and 21days, respectively). However, their response was statistically different from the activity obtained with control animals ($P < 0.05$).

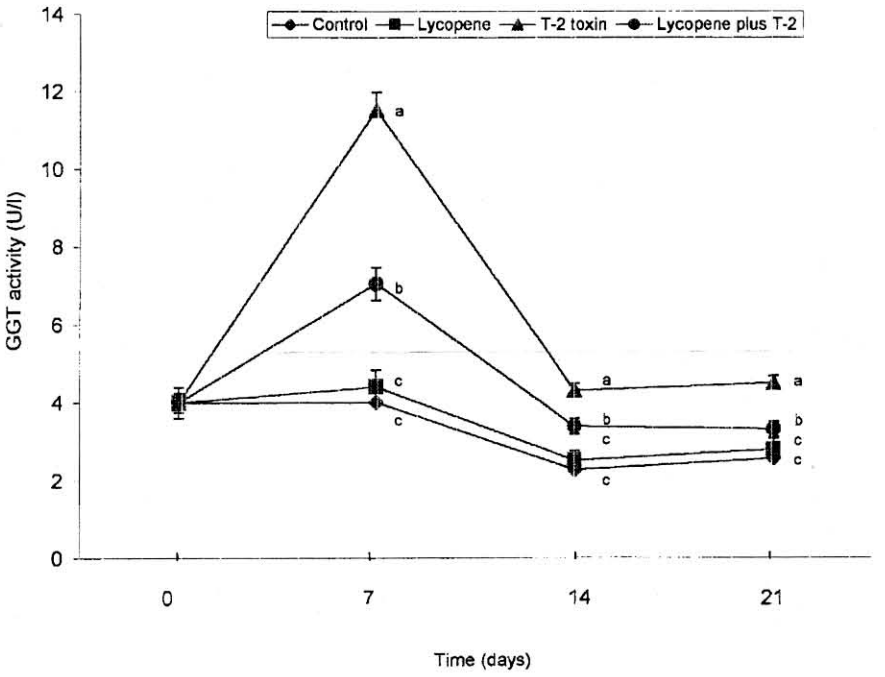


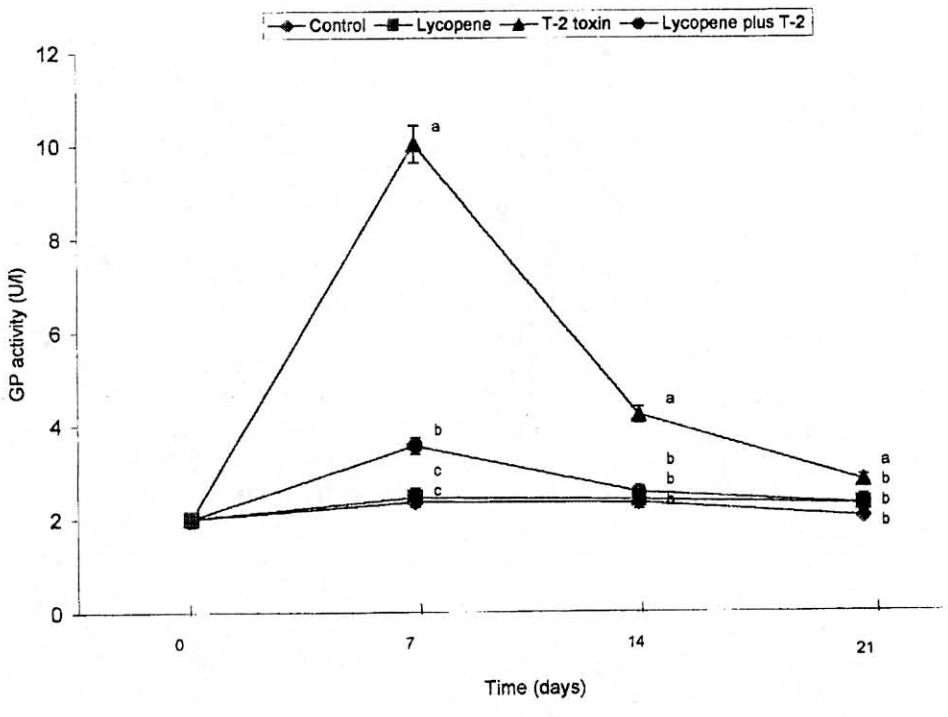
Fig. 2. Effect of T-2 toxin and lycopene on γ-glutamyltransferase (GGT) activity in chicken liver. Chicks consumed 1.5 mg T-2/kg/day and 25 mg lycopene/kg/day. Values are means ± S.D. of 30 chicks. Values for the same time with no common letters are significantly different ($P \leq 0.05$).

days the hepatic GSH levels in T-2 toxin treated animals were only marginally less than in the untreated controls. It was also interesting to note that the levels of GSH increased with respect to time only for T-2 toxin treated animals. It is known that the availability of GSH is determined by the complex interactions of utilization, transport, very active synthesis and reduction of glutathione disulfide (GSSG) and GS-SX mixed disulfides (Smith et al., 1996). However, levels of MDA showed a different trend, increasing over time, with the highest level at day 21. It has been demonstrated (Marrs, 1996) that the enzyme gamma-glutamylcysteine synthetase is feedback inhibited when cellular GSH levels are sufficient. If GSH level is decreased, as has been observed in the T-2 toxin treated animals, the rate-limiting step of GSH synthesis catalyzed by γ-glutamylcysteine synthetase probably was induced, increasing GSH biosynthesis, as was cited by Marrs (1996). The cytotoxic effect of T-2 involved alterations in some hepatic enzymes as GST, GGT and GP. After 7 days, the activity of these enzymes was markedly increased in chickens exposed to T-2. This response is indicative of cellular oxidative stress, because the presence of lipid peroxidation products apparently causes the induction of the activity of enzymes that take part in toxic compound removal. It has been observed that the presence of active oxygen species, as a primary response, induces GST and GP gene expression, and increases GSH level by feedback induction (Daniel, 1993; Zimniak et al., 1997). These authors have observed, as a secondary response, an induction on GST activity. Probably the presence of both substrates, hydrophobic and electrophylic species, obtained from T-2 action, as well as enough GSH produced by enzymatic induction, increased the activity of GST and GGT. Similar responses have been obtained with other xenobiotics (Stewart et al., 1996). As a consequence of these events, GP activity was increased as part of detoxification mechanism. Uhling and Wendel (1992) cited that a maximum rate of GP activity is

maintained when the GSH concentration remains high. This might explain the increased values of GP activity in presence of lycopene after 21 days of treatment (Fig. 3), where high levels of GSH were maintained (Table 3).

When lycopene was present in the diet of T-2 toxin treated animals, a significant reduction of detrimental effects on hepatic GSH metabolism was observed. Apparently in this experiment, lycopene participated as an antioxidant agent, protecting cellular GSH. Lycopene has been reported as the most efficient biological carotenoid singlet oxygen quencher (Di Mascio et al., 1989). Considering that lycopene is a natural compound present in human and animal diets, this experiment took advantage of its antioxidant properties. The results obtained suggest that lycopene could reduce the cytotoxicity induced by T-2, mainly by reducing lipid peroxidation. According to the doses of T-2 and lycopene tested, in the presence of 1 mg T-2, 17 mg lycopene were required to maintain at least the background concentration of the endogenous antioxidant glutathione. Probably with lower doses of lycopene, the same effect could be obtained; however, more research is needed in this regard. Because the GSH level was not affected by the presence of lycopene, the enzymes involved in its metabolism maintained their activity, although this activity was not 100% efficient when compared with the response obtained in control animals after 21 days.

These results suggest that lycopene helped to maintain the levels of cellular GSH; keeping both, the activity of some GSH enzymes involved in detoxification mechanism of T-2 toxin, and GSH normal metabolism. Functional foods, such as lycopene, could contribute to the reduction of risk of cellular damage due to contaminants in the food chain.



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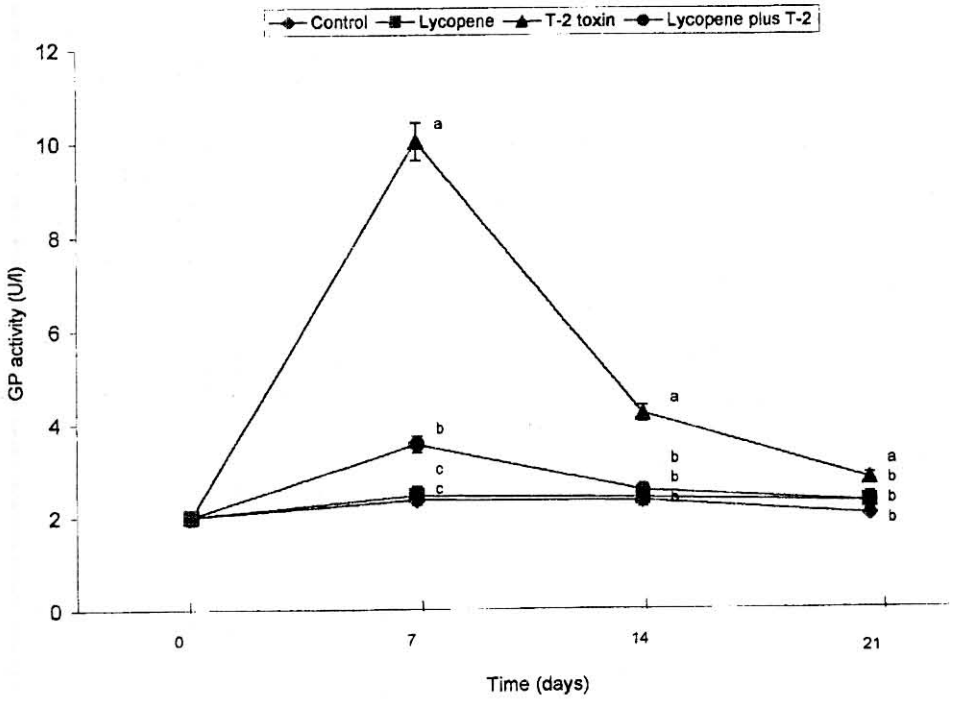

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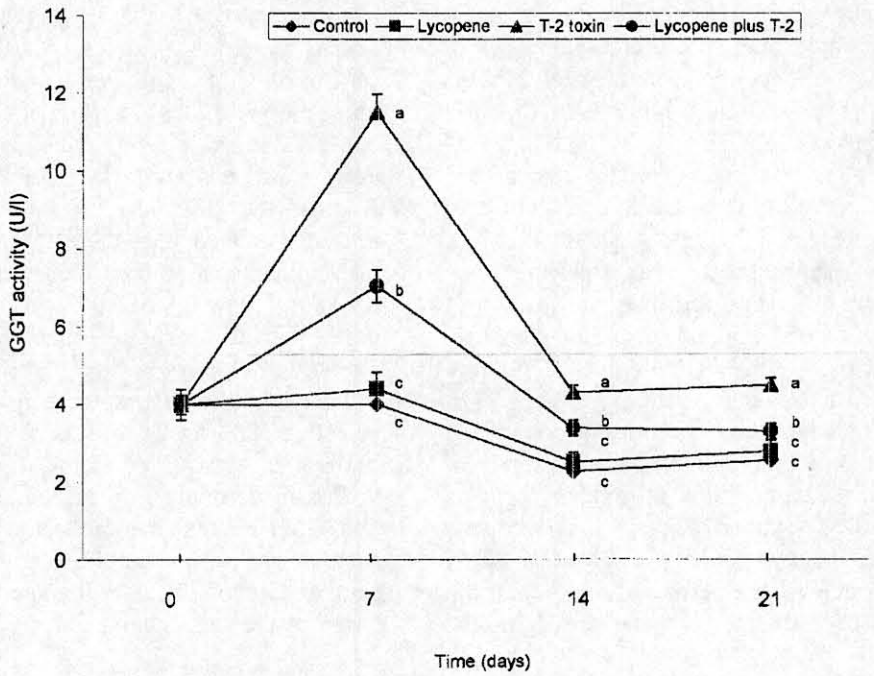

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