

Lycopene prevents cisplatin-induced liver tissue damage, without affecting concentrations of TNF- α or iNOS/NO inflammatory pathway

Type

Research paper

Keywords

macrophages, inflammation, lycopene, cisplatin, liver

Abstract

Introduction

Liver inflammatory response occurring after toxic chemicals injection, such as cisplatin, causes the aggravation in liver damage. Lycopene, a carotenoid, has previously been proven to possess antioxidant, antiinflammatory and antiapoptotic properties. This study objective was to evaluate for the first time the protective effects of lycopene in cisplatin-induced liver damage based on the disturbances in serum and tissue inflammatory parameters. Also, to confirm the extent of changes in inflammation of the studied tissue, a microscopic analysis will be performed.

Material and methods

Wistar rats were divided into four experimental groups: (I) control; (II) lycopene (2 mg/kg); (III) cisplatin (10 mg/kg) and (IV) lycopene and cisplatin-treated (2 and 10 mg/kg, respectively) animals. After the experiment, we studied changes in serum liver tissue damage associated parameters (ALT, AST, and γ -GT) and liver inflammatory parameters (NO and TNF- α concentrations, myeloperoxidase and iNOS activity, as well as CD68 expression).

Results

Application of lycopene prevented a rise in evaluated serum parameters induced by cisplatin, while at the same time it did not cause any harmful effect by itself. Lycopene, alone or in combination with cisplatin, decreased the values of all studied liver inflammatory parameters.

Conclusions

We can conclude that lycopene does not prevent liver tissue inflammatory decalin seen after cisplatin application, however, it prevents tissue damage arising from this cytostatic application.

Lycopene prevents cisplatin-induced liver tissue damage, without affecting concentrations of TNF- α or iNOS/NO inflammatory pathway

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Running head: Lycopene does not affect liver inflammation

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Key words: lycopene, liver, cisplatin, inflammation, macrophages

Introduction

Cisplatin (CIS) is a platinum-containing chemotherapeutic agent, belonging to a group of alkylating agents, which can be used alone or in combination with other cytostatic drugs. Although side effects related to CP application primarily do not include hepatotoxicity, it is known that this chemotherapeutic agent administered as either high single doses or in repeated low doses significantly damages liver tissue [1]. Chemical interactions between CP and intracellular components lead to the formation of the highly electrophilic molecules which consequentially react with sulfhydryl groups, destabilize biological membranes, disrupt energy metabolism, and generate reactive oxygen species (ROS) [2].

Apart from these mechanisms of liver tissue damage, CP is known to cause the transcription of numerous pro-inflammatory genes, e.g. cyclooxygenase (COX) and inducible nitric oxide synthase (iNOS), and activates Toll-like receptor-4 (TLR-4) [3]. However, there are studies suggesting that CP causes, both in *in vitro* and *in vivo* conditions, the suppression of tissue inflammation [4,5]. In the liver, major inflammatory cells that are known to modulate tissue response to toxic chemicals are tissue macrophages *i.e.* Kupffer cells [6]. These cells have both physiological and pathophysiological role in liver tissue functioning, and their inhibition is often related to the diminution in liver damage [7].

Lycopene (**Lyp**) represents one of the most powerful natural antioxidants that can be found in tomatoes, watermelons, apricots, papayas, etc. [8]. Its antioxidant and antiradical scavenging activities are tightly related to conjugated double bonds present in **Lyp** molecule [8]. Since a great number of organ dysfunctions arrive due to impairment in cell/tissue oxidant state application of **Lyp** found its application in the traditional medicine of many nations. Also, by contributing to the prevention of apoptosis or some other signaling pathways **Lyp** can modify inflammatory response as well [8]. The effect of **Lyp** in several models of liver damage induced by different hepatotoxic agents has been previously published [9,10]. However, up to now, there are no studies that evaluate the effects of **Lyp** on CP-induced rat liver damage or to the changes in tissue inflammatory response.

Having these facts in mind we aimed to evaluate the potential of **Lyp** in preventing rat liver tissue inflammatory disturbances occurring after CP administration. The hepatoprotective potential of **Lyp** would be estimated based on the changes in serum liver tissue damage associated parameters

and based on the disturbance in tissue inflammatory parameters. Also, in order to confirm the extent of changes in inflammation of the studied tissue and the effects of **Lyp** microscopic analysis will be performed.

Experimental

Drugs and chemicals

All reagents, solvents, and drugs used in this investigation were of analytical grade or better and were obtained from Sigma-Aldrich (St. Louis, MO, USA) or Richter Pharma AG (Wels, Austria). Lycopene solution was prepared daily, in corn oil, prior to the administration at a dose of 2 mg/kg.

Animals and housing

Twenty-four male Wistar rats (250-300 g) were kept under standard laboratory conditions in plexiglass cages: 22±2 °C temperature, 60% humidity, and 12/12 (light/dark) cycle. Food and water were available *ad libitum* throughout the experiment. All experimental procedures were conducted in compliance with the European Council Directive (EU Directive of 2010; 2010/63/EU) and were approved by the local Ethics committee.

Experiment design

Liver tissue damage induced by CP was performed according to a previous publication [11]. Groups of animals were treated for five days as follows:

I Group - Vehicle treated group – received a daily injection of vehicle 1 mL/kg;

II Group – **Lyp** treated group – received a daily injection of **Lyp** (2 mg/kg) for five days;

III Group - CP treated group – received CP injection (10 mg/kg) on the 3rd day of the experiment;

IV Group – CP and **Lyp** treated group – received **Lyp** for five days (2 mg/kg), and a single injection of CP (10 mg/kg) on the 3rd day.

Twenty-four hours after the last treatment all animals were sacrificed with an overdose of ketamine, a blood sample was taken by cardiac puncture, and liver tissue was removed for tissue biochemical (frozen and stored at –80 °C) and histopathological (fixated in 10% buffered formalin) analyses.

Biochemical analysis

Serum biochemical analysis

Collected blood samples were left to clot at room temperature before the serum was separated by centrifugation at 2500 rpm for 10 min. Separated serums were used for the determination of biochemical parameters that reflect the functional state of the liver. Aspartate transaminase (AST), alanine transaminase (ALT) and gamma-glutamyl transferase (γ -GT) levels were assayed by an Olympus AU680 Chemistry-Immuno Analyzer (Olympus America Inc., USA).

Tissue homogenization, protein content and inflammatory parameters determination

After defrosting tissue samples were chopped into small pieces and homogenized in ice-cold distilled water and centrifuged afterward (5000 rpm, 15 min, 4 °C). The obtained supernatant was further used for biochemical parameter estimation. Tissue protein content was determined in supernatants following Lowry's method [12].

Tissue NO concentrations were estimated based on the formed NO^{2-} , using the standard Griess reaction and the nitrite concentrations were calculated using a standard curve of sodium nitrite [13]. The activity of myeloperoxidase (MPO) was determined in tissue homogenates following the previously described method [14] which is based on the reaction between 1,2-diaminobenzene and H_2O_2 under acidic conditions.

Levels of tumor necrosis factor α (TNF- α), inducible nitric oxide synthase (iNOS) and CD68 in liver tissue homogenates were determined using Abcam (ab100785; Cambridge, United Kingdom) and CUSABIO (CSB-E08325r and CSB-E13297r; Huston, Texas, USA) ELISA kits. The range of the calibration curves for the TNF- α , iNOS and CD68 were the following 18.75-1200 pg/ml, 0.78-50 IU/mL and 0.625-40 ng/mL, respectively.

Histopathological analysis

Isolated liver tissue was fixed in formaldehyde solution (10%, w/v), further dehydrated with ethanol of differing ascending concentrations (50–100%, v/v), embedded in paraffin molds and cut into 4–5 μm -thick sections. Tissue sections were routinely stained with hematoxylin and eosin (HE), and further examined with Leica (Mycroastrms, Germany) light microscope equipped with

a Canon PowerShot S70 camera. An average number of Kupffer cells per high power field (HPF) (magnification x400) was counted on at least 10 different fields per liver sample.

Statistical analysis

Data presented are mean values \pm SD and were compared using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons (GraphPad Prism, ver. 5.03; San Diego, CA, USA). Probability values (p) less than 0.05 were considered to be statistically significant.

Results

Application of CP lead to a statistically significant increase, compared to the control group, in the activity of the studied serum liver damage related enzymes (Table 1). In the group IV, the one receiving **Lyp** and CP, the serum activity of all studied enzymes was in the same range as the activity determined in untreated animals (group I), however significantly decreased compared to group III was only found for AST and γ -GT activities (Table 1).

Table 1 should be around here

Studied inflammatory parameters included NO, CD68 and TNF- α concentrations, as well as MPO and iNOS activities which were all detectable to some extent in liver tissue homogenate (Table 2). Application of **Lyp**, both alone or in combination with CP, lead to a significant decrease ($p < 0.001$) in the concentration/activity of all studied parameters compared to the control group (Table 2). Similar, almost identical, results to that of the **Lyp** treated groups for the studied biochemical parameters were obtained from livers of animals that received CP only ($p > 0.05$ vs. **Lyp** treated group).

Table 2 should be around here

The counted average number of macrophages per HPF was not found to be significantly different between the experimental groups (Fig. 1 and 2), i.e. the number of macrophages was almost identical in all four experimental groups.

Figure 1 and 2 should be around here

Discussion

Liver cell integrity and function that is known to be compromised after CP application [1] and the influence of **Lyp** was studied through the changes in liver tissue derived enzyme activities in experimental animals' serum. Here again, similar to the results of the previous publications [9,10], we found that **Lyp** protects liver tissue from the damaging agent, in this case from CP, judged by the changes in serum ALT, AST and γ -GT activities (Table 1). The mechanism that might underline this hepatoprotective activity of **Lyp** is probably related to its ability to scavenge free radicals generated by CP [8]. However, there is little information regarding the effects of **Lyp** on liver tissue inflammatory response arising after different toxicant application.

Various inflammatory stimuli are known to cause significant alterations in liver macrophage functioning by upregulating a large panel of pro-inflammatory molecules, growth factors, and reactive oxygen species [6]. Some of those molecules, such as NO, MPO, iNOS, TNF- α and CD68, which are produced by Kupffer cells were evaluated in our study in order to determine what kind of impact does **Lyp** and CP, alone or in combination, have on them. All of the evaluated biomarkers represent constitutive molecules/enzymes present in Kupffer cells, however, their activity/concentration is significantly increased in the presence of some pro-inflammatory stimulus [6].

Although CD68 doesn't have a central role in regulating innate and adaptive immune responses this lysosome/membrane-bound highly glycosylated signal peptide is still a valuable marker that enables us to track the function/activity of Kupffer cells [15]. The role of CD68 is still debatable, however, it is known that this molecule is involved in antigen processing and presentation and in some aspects in the process of apoptosis and Kupffer cell activation [5,15]. Up to now there are no studies showing the effects of CP on liver CD68 concentration, and here for the first time, we found that a single 10 mg/kg injection of CP causes a significant decrease in CD68 rat liver concentration (Table 2). On few previous occasions, results of different studies showed contradictory findings, where in one case CP application increases kidney CD68 expression [16], while in the other CP application decreased lymphoid tissue (thymus, spleen) CD68 expression [5]. Also, one of the interesting findings of this study is that **Lyp** on its own decreases CD68 expression, which corroborates the previous publication which suggests that this carotenoid possesses strong anti-inflammatory activity [9].

Various proinflammatory signals such as ROS lead to an increase in macrophage MPO activity as well as to an increase in NO production through iNOS [17]. Excesses in both MPO activity and NO concentrations are tightly related to liver tissue damage [18,19]. Here again, the results of previous studies related to CP influence on inflammatory parameters are controversial [16,20,21], however, some of them agree with the ones obtained in our study (Table 2). Regarding the effects of **Lyp** on NO/iNOS system and MPO activity the results of our study only partially agree with previous publications [22,23]. Application of **Lyp** decreased MPO and iNOS activity, as well as NO concentrations, while when it was applied together with CP it had no significant influence (compared to the CP treated group). This again underlines the studies which suggest that **Lyp** suppresses inflammatory cell function, however, this might not be a complete suppression since still a certain degree of NO, higher than in the CP group, was present.

The evaluated chemokine TNF- α is an important signaling molecule [24-26], released from Kupffer cells, which is related to liver fibrosis and activation of the hemostatic system [6]. The results of our study for the first time revealed that **Lyp** decreases TNF- α production in healthy animals, which is completely opposite from previous studies where **Lyp** increased liver TNF- α production [27]. These discrepancies could be interpreted through different doses applied to animals, as well as to the length of the experiments [27]. Regarding the effect of CP, some publications suggest that CP reduce the inflammatory response of the studied macrophages by decreasing TNF- α and interferon- γ concentrations in cell supernatants [20]. On the other hand, much higher concentrations of CP (10 μ g/ml) induce the inflammatory response, characterized by an increase in NO and proinflammatory cytokines, in macrophage culture [20].

As stated, the application of **Lyp** on its own produced the inhibition of all studied inflammation-related parameters (Table 2), but it did not reduce the number of Kupffer cells visible per HPF (Figures 1 and 2). Similar results were obtained in the groups of animals treated with CP only, or with a combination of **Lyp** and CP. The previous study found that in *in vitro* culture CP decrease macrophages viability in concentrations higher >10 μ M, while at 10 μ M CP prevented macrophage death induced by lipopolysaccharide [20]. Although we could not determine exact concentration of CP in liver tissue (or in serum) we could assume that here applied concentration of CP (10 mg/kg) did not reach 10 μ M which would possibly decrease Kupffer cell number. Also, it is interesting to

notice that the application of both **Lyp** and CP had no different impact on cell number than when the two chemicals were applied on their own.

Conclusions

In the present study, we discovered that 5-day application of lycopene to rats produces a significant decrease in liver tissue physiological inflammatory response (nitric oxide and tumor necrosis factor α concentrations, myeloperoxidase and inducible nitric oxide synthase activity, and CD68 expression) without any negative effect on liver cell function. A single dose of cisplatin also produced a significant diminution in the liver inflammatory response, studied through a panel of same parameters, which this was followed by the disturbance in hepatocytes functioning which was studied through a change in serum liver damage related enzymes activities. Co-administration of lycopene with cisplatin had no significant impact on alterations in liver inflammation levels, however, lycopene was found to prevent hepatocyte damage which arises from cisplatin application. Based on the results of this study we can conclude that lycopene's hepatoprotective activity is not only related to its ability to scavenge free radicals, but also to its ability to modulate tissue inflammatory response.

Acknowledgments

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Figure legends

Figure 1. The average number of macrophages per high power field counted in liver tissue sections obtained from different experimental groups.

Figure 2. Liver macrophages (arrowhead) found on tissue sections obtained from control (A), lycopene treated (B), cisplatin treated (C) and lycopene and cisplatin treated (D) groups (magnification x400)

Preprint

Table 1. Serum biochemical parameters obtained from different experimental groups

Parameter/Group	Group I (Vehicle treated)	Group II (Lyp treated)	Group III (CP treated)	Group IV (CP and Lyp treated)
ALT (U/L)	39.8 ± 1.8	38.8 ± 4.5	61.3 ± 11.9*	50.6 ± 5.1
AST (U/L)	89.1 ± 0.8	99.5 ± 12.4	273.3 ± 36.6*	99.5 ± 1.4 [#]
γ-GT (U/L)	0.3 ± 0.2	0.1 ± 0.1	1.5 ± 0.1*	0.2 ± 0.15 [#]

Data are presented as mean ± SD, n = 6; ANOVA, Tuckey's post hoc test * $p < 0.001$ vs. Control group (Vehicle animals); [#] $p < 0.001$ vs. CP treated group.

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Table 2. Liver tissue inflammatory parameters obtained from different experimental groups

Parameter/Group	Group I (Vehicle treated)	Group II (Lyp treated)	Group III (CP treated)	Group IV (CP and Lyp treated)
NO (nmol/mg of proteins)	8.8 ± 0.1	2.5 ± 0.5* [#]	1.6 ± 0.3*	1.7 ± 0.5*
MPO (OD/mg of proteins)	214.3 ± 84.5	51.4 ± 4.5*	39.5 ± 3.8*	56.4 ± 7.6*
TNF- α (pg/mg of proteins)	29.3 ± 6.5	7.4 ± 2.2*	3.2 ± 1.2*	5.7 ± 3*
iNOS (IU/mg of proteins)	23.6 ± 8.1	5.4 ± 1.4*	4.9 ± 0.4*	6.9 ± 1.3*
CD68 (pg/mg of proteins)	5.7 ± 3	1.4 ± 0.4*	0.9 ± 0.05*	1.7 ± 0.3*

Data are presented as mean \pm SD, n = 6; ANOVA, Tuckey's post hoc test * p <0.001 vs. Control group (Vehicle animals); [#] p <0.001 vs. CP treated group.

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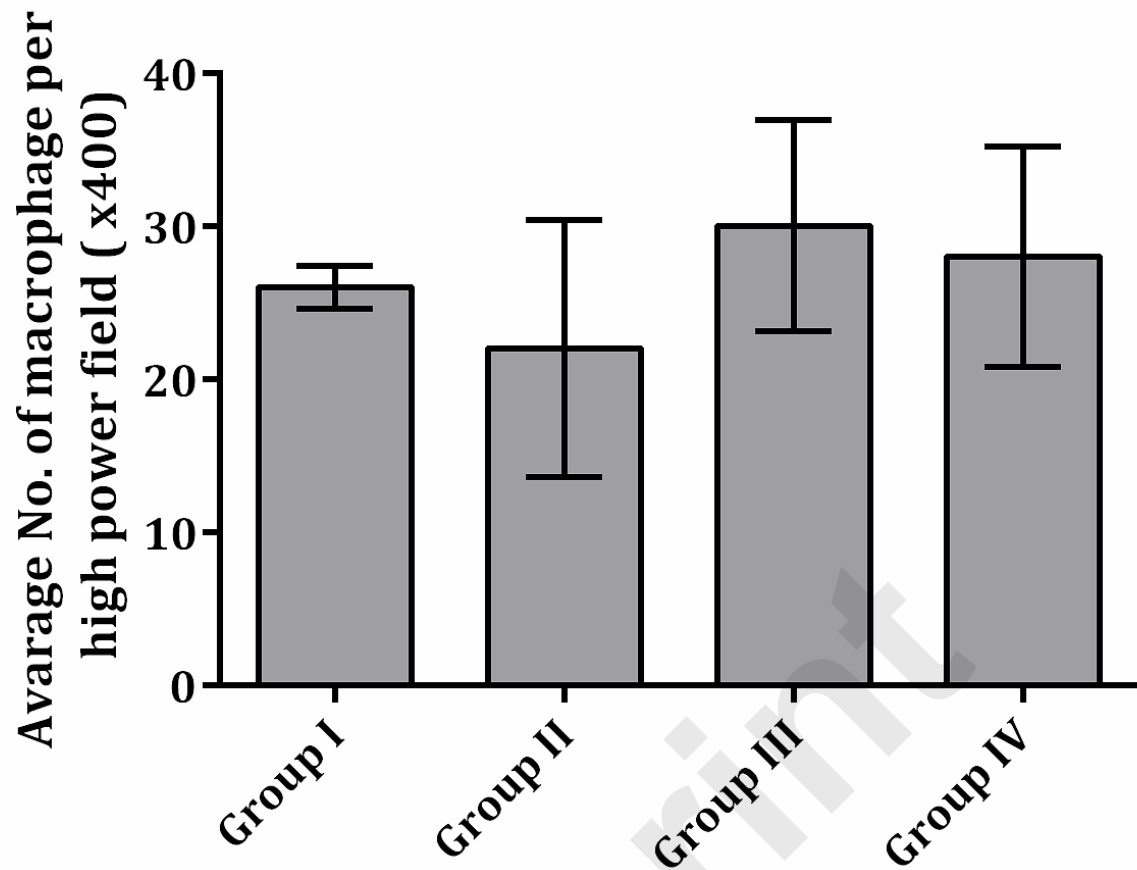


Figure 1. The average number of macrophages per high power field counted in liver tissue sections obtained from different experimental groups.

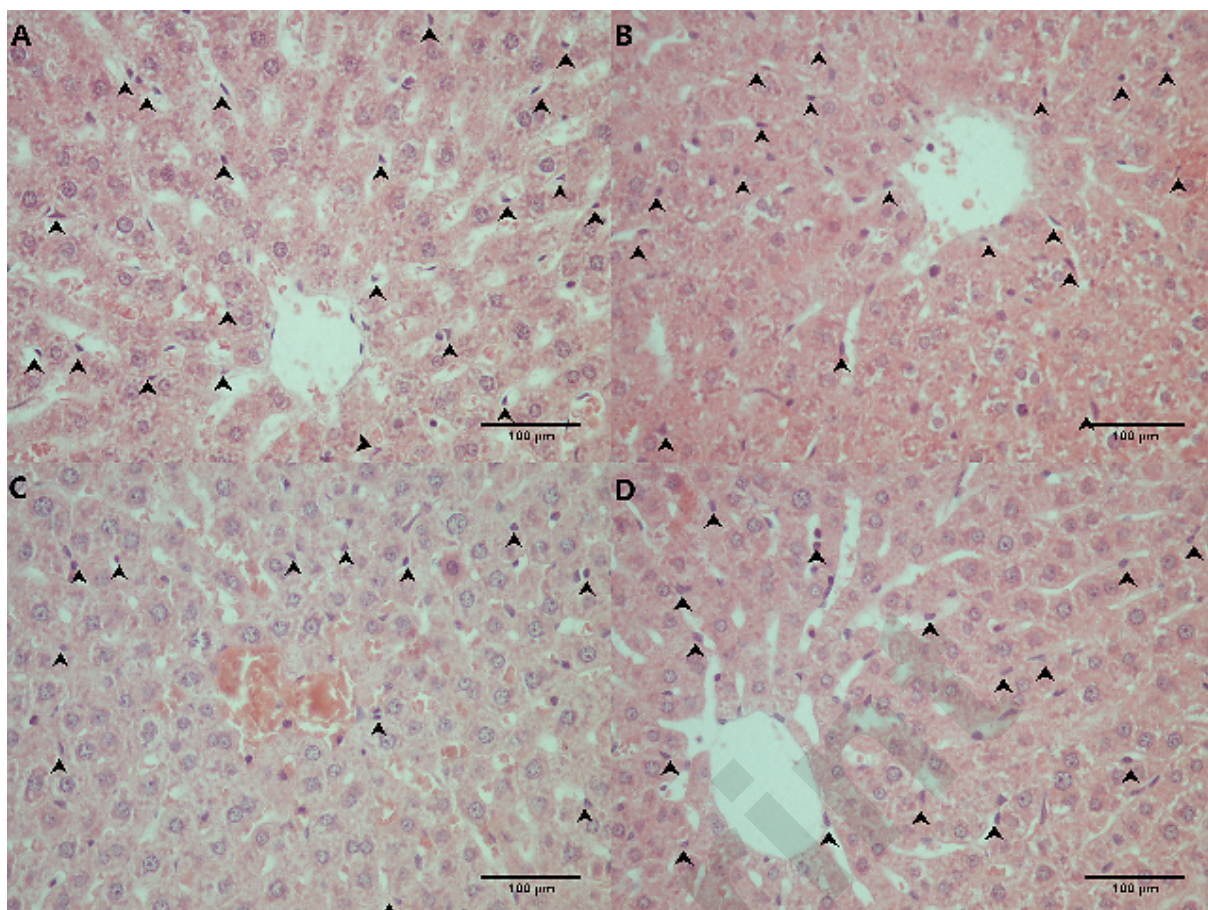


Figure 2. Liver macrophages (arrowhead) found on tissue sections obtained from control (A), lycopene treated (B), cisplatin treated (C) and lycopene and cisplatin treated (D) groups (magnification x400)