RESEARCH ARTICLE

Hepatoprotective Effects of B-1,3-(D)-Glucan on Bortezomib-Induced Liver Damage in Rats

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Abstract

The aim of this study was to evaluate the effects of β -1,3-(D)-glucan as an antioxidant and tissue protective agent and study the biochemical, histopathologic, and immunohistochemical effects of first therapeutic proteasome inhibitor bortezomib on the liver for treating relapsed multiple myeloma. The experiment included 36 adult male rats, which were divided into four treatment groups: control (healthy); bortezomib-treated; β-1,3-D-glucan-treated; and bortezomib + β-1,3-(D)-glucan-treated. Each group was subdivided into two subgroups based on time of sacrifice (48 or 72 h). After the experiments, superoxide dismutase (SOD) activity and lipid peroxidation (LPO) amounts were determined, and immunohistochemical and histopathological changes were examined in all rat liver tissues. β -1,3-(D)-Glucan treatment normalized changes of LPO and stimulated an over activity of endogenous SOD. The results of the histopathologic parameters showed that treatment with β -1,3-(D)-Glucan in the bortezomib group ameliorated the development of non-specific reactive hepatitis (NSRH) and Kupffer cell activation via NF-kB. Administration of β -1,3-(D)-Glucan is effective in reversing tissue damage induced by bortezomib in rat livers.

Keywords: Bortezomib, β-1,3-(D)-glucan, Oxidative stress, Liver, Histology

Sıçanlarda Bortezomib İndüklü Karaciğer Hasarında B-1,3-(D)-Glukan'ın Hepatoprotektif Etkileri

Özet

Bu çalışmanın amacı, relaps multiple miyelom tedavi etmek için kullanılan ilk terapötik proteazom inhibitörü olan bortezomibin karaciğer üzerine immunohistokimyasal, histopatolojik ve biyokimyasal etkilerini araştırmak ve bir antioksidan ve doku koruyucu ajan olarak B-1,3-(D)-glukanın etkilerini değerlendirmekdi. Deney; kontrol (sağlıklı), bortezomib ile tedavi, B-1,3-(D)-glukan ile tedavi ve bortezomib + B-1,3-(D)-glukan ile tedavi olmak üzere dört tedavi grubuna bölünen 36 yetişkin erkek sıçan içerdi. Her bir grup sakrifikasyon zamanına (48 veya 72 saat) göre iki alt gruba ayrıldı. Deneylerin bitiminden sonra, süperoksit dismutaz (SOD) aktivitesi ve lipid peroksidasyon (LPO) miktarları ölçüldü ve tüm sıçan karaciğer dokularında immünohistokimyasal ve histopatolojik değişiklikler incelendi. B-1,3-(D)-glukan ile tedavi LPO değişikliğini normalize etti ve endojen SOD aktivitesi aşırı uyardı. Histopatolojik parametrelerin sonuçları, bortezomib grubunda B-1,3-(D)-glukan ile tedavi NF-kB yoluyla Kupffer hücre aktivasyonunu ve non-spesifik reaktif hepatit (NSRH) gelişimini regüle ettiğini gösterdi. B-1,3-(D)-glukan uygulaması, sıçan karaciğerinde bortezomibin neden olduğu geri döndürülebilir doku hasarında efektifdir.

Anahtar sözcükler: Bortezomib, β-1,3-(D)-glucan, Oksidatif stress, Karaciğer, Histoloji

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INTRODUCTION

Proteasome is a multicatalytic protein complex and the major non-lysosomal system for intracellular protein degradation in the eukaryotic cells. Proteasomes work in concert with the marking protein ubiquitin and generate the ubiquitin-proteasome pathway. This pathway is responsible for the controlled degradation of a wide range of proteins, including cellular regulators that control processes such as the cell cycle, apoptosis, inflammation, cell migration, angiogenesis, and transcription [1]. Many studies have shown that the proteasome system can act as a cellular defense mechanism because it prevents the accumulation of aggregation misfolded/oxidized proteins generated by post-translational modification errors or oxidative stress [2].

Bortezomib, a dipeptidyl boronic acid, is the first reversible 26S proteasome inhibitor used for the treatment of multiple myeloma (MM) in humans [3]. The initial target for bortezomib use in MM was the blocking of pathways of nuclear factor-kappa B (NF-kB) by limiting proteasomal degradation of inhibitor of kappa B alpha (IkBα). Although the first studies argued that bortezomib could inhibit all pathways of NF-kB, later studies have shown that it activates only the canonical pathway in neoplastic cells of MM [4]. Bortezomib was also found to be effective on NF-kB pathways in cancer types other than MM [5]. The drug has been shown beneficial in animal-model studies of autoimmune diseases such as myasthenia gravis, psoriasis, arthritis, and autoimmune encephalomyelitis [6].

Therapeutic effectiveness of anti-cancer drugs is associated with severe side effects due to their toxicity [7]. Bortezomib is one of the more widely used anti-cancer drugs for a number of cancers and is metabolized in the liver, which can develop drug toxicity due to these metabolisms [8]. A few bortezomib-based studies on the liver have shown that the 26S ubiquitin-proteasome pathway may play an etiologic role in the development of liver disorders, especially endoplasmic reticulum stress, insulin resistance, alcoholic liver disease, and lipid metabolism [9]. Earlier, an experimental study on rates revealed that the therapeutic dose of bortezomib and its deboronated metabolites M1 and M2 dealkylated to form M3 and M4 could causes liver toxicity. This study also showed a decrease in cytochrome P450 content and activity and an important increase in palmitoyl-CoA activity in *ex vivo* analyses of rat liver samples [10]. However, this present research has not found studies about oxidant and antioxidant parameters in bortezomib-induced liver toxicity. Many studies, though, have demonstrated that oxidative stress is responsiple for chemotherapeutic drug-induced liver toxicology [11]. In mitigation of chemotherapy side effects, some antioxidant drugs or agents with anti-cancer effects have been determined helpful depending on the reducing effect on oxidative stress [12]. Many studies have shown that beta-1,3-D-glucan, an antioxidant and anti-cancer agent, has protective antioxidant activity against chemotherapy-induced liver toxicity [13].

B-1,3-(D)-Glucanis a long chain polymer of D glucose from the cell wall of baker's yeast (*Saccharomyces cerevisiae*), fungi, and plants. Many experimental studies have demonstrated its pharmacological properties, especially its immunomodulator, antioxidant, and anti-tumor effects [14]. The immunomodulator effects of B-1,3-(D)-Glucan are on innate immune cells andare enlisted for immune system reinforcement in humans [15]. B-1,3-(D)-Glucan acts as an agonist on dectin-1 and complement receptor 3 (CR3) on the surface of innate immune cells [16]. Recent studies indicate that B-1,3-(D)-Glucan also promotes activation of CR3 in granulocytes and destroys iC3b-opsonized tumor cells via granulocyte bound-CR3 [17]. In addition, B-1,3-(D)-Glucan is a potent antioxidant that has been reported to prevent oxidative damage in liver and renal ischemia/reperfusion injury [18].

This present research aims to determine whether an antioxidant such as B-1,3-(D)-Glucan could provide a protective effect against bortezomib-induced liver damage, using both stereological, histopathological, and biochemical methods.

MATERIAL and METHODS

Animals

The animals were housed in facilities and the experiments conducted in accordance with international guidelines, and the studies were approved by the Institutional Animal Care and Use committee of Ataturk University. This study used 36 adult male Sprague-Dawley rats (230-250 g) from the Ataturk University Experimental Animal Laboratory (ATADEM-Approval No: 2013-03/96).

Chemicals

Bortezomib (Velcade®) was purchased as a lyophilized powder (Velcade; Janssen-Cilag, Beerse, Belgium) and dissolved in a sterile saline solution at final concentration. β-1,3-D-glucan was purchased from Sigma-Aldrich (Steinheim, Germany). All chemicals for laboratory experimentation were purchased from Sigma-Aldrich (Germany).

Experimental Design

Four groups (control; bortezomib-treated; β-1,3-D-glucan-treated; and bortezomib + β-1,3-D-glucan-treated) were formed for the research study. Bortezomib, β-1,3-D-glucan, and bortezomib + β-1,3-D-glucan groups were subdivided into two subgroups of six rats each based on time of sacrifice: 48 or 72 h after drug administration. The rats in the bortezomib and bortezomib + β-1,3-D-glucan groups were injected subcutaneously (sc) once

with 0.2 mg/kg of bortezomib on the first day of the study [19]. The rats in the bortezomib group were not given any treatment after the bortezomib injection until sacrifice. The rats in the bortezomib + β-1,3-D-glucan group were injected intraperitoneally (ip) with 75 mg/kg of β-1,3-D-glucan every day after bortezomib injection until sacrifice. The rats in the β-1,3-D-glucan group were injected ip with 75 mg/kg of β-1,3-D-glucan every day until sacrifice [13].

All six groups were sacrificed with an overdose of a general anesthetic (thiopental sodium, 50 mg/kg). The livers were then quickly removed from the rats and washed in ice-cold saline. Half of the tissues were transferred to a biochemistry laboratory and kept at −80℃ for biochemical analyses, while the other half were fixed in a 10% formalin solution for histopathological analyses.

Histopathological Analyses

Preparation of Liver Tissues for Histopathology: The livers were fixed in 10% formaldehyde, dehydrated in a graded alcohol series, embedded in paraffin wax, and sectioned using a Leica RM2125RT microtome (Leica Microsystems, Wetzlar, Germany). In this study, 4 μm thick sections from paraffin blocks were obtained using a systematic randomized sampling method (stereological method) for immunohistochemical and histopathologic examinations.

Histopathological Examination of the Liver: Sections 4-μm thick for histopathological examinations were stained with H&E and periodic acid-Schiff (PAS). All livers were examined by light microscopy for histopathological evaluation of the following parameters: H&E staining for sinusoidal expansion; inflammatory cell infiltrates; sinusoidal expansion; hyperthrophic degeneration; necrotic cell deaths, apoptotic cell deaths.

Biochemical Analyses

Preparation of Liver Tissues for Biochemical Analysis: Rat livers were kept at -80℃ for biochemical investigation. To prepare the tissue homogenates, the liver tissues were ground with liquid nitrogen in a mortar, and 0.5 g was weighed for each group and treated with 4.5 mL of an appropriate buffer. This mixture was homogenized on ice using an IKA® Ultra-Turrax homogenizer (IKA Labortechnik, Staufen, Germany) for 15 min. Homogenates were filtered and centrifuged using a refrigerator centrifuge at 4℃. The supernatants were then used to determine enzymatic activities. All assays were carried out at room temperature. To prepare the tissue homogenates, tissues were ground with liquid nitrogen in a mortar. The ground tissues (0.5 g each) were then treated with 4.5 mL of the appropriate buffer. The mixtures were homogenized on ice using an Ultra-Turrax homogenizer for 15 min.

SOD Activity: SOD activity was measured according to Sun et al.[20]. The estimation was based on the generation of O_2^- produced by xanthine and xanthine oxidase, which react with nitro blue tetrazolium (NTB) to form formazan dye. SOD activity was then measured at 560 nm by the degree of inhibition of this reaction, and was expressed as millimole per minute per milligram of tissue (mmol/min/mg tissue).

LPO Determination: The level of gastric LPO was determined by estimating MDA using the thiobarbituric acid test [21]. The rat livers were promptly excised and rinsed with cold saline. The livers were weighed and homogenized in 10 mL of 100 g/L KCl. The homogenate (0.5 mL) was added with a solution containing 0.2 mL of 80 g/L sodium laurylsulfate; 1.5 mL of 200 g/L acetic acid; 1.5 mL of 8 g/L 2-thiobarbiturate; and 0.3 mL of distilled water. The mixture was incubated at 98℃ for 1 h. Upon cooling, 5 mL of n-Butanol:pyridine (15:l) was added. The mixture was vortexed for 1 min and centrifuged for 30 min at 1875 × g. The absorbance of the supernatant was measured at 532 nm. The standard curve was obtained by using 1,1,3,3-tetramethoxypropane, and recovery was over 90%. The results were expressed as nanomol MDA per gram of tissue (nmol/g tissue).

Immunohistochemical Analysis

In addition to the histopathological analyses, activity in Kupffer cells was detected by immunohistochemical staining of NF-kB protein (p65). Immunohistochemical staining for NF-kB protein was performed by an automated method on the VENTANA BenchMark GX System (Ventana Medical Systems, Inc.) with an ultraView Universal DAB Detection Kit on 4-μ sections from a representative block in each rat. After deparaffinization to water, the antigenic determinant sites for NF-kB were unmasked in citrate buffer with steam for 60 min. The primary antibody used for NF-kB, an IgG1 class mouse monoclonal directed against the p65 (F-6) relA component of the NF-kB complex (Santa Cruz sc8008, CA), was used at a dilution of 1:80 for 32 min at 37℃. The slides were then incubated with the diluted antibody, followed by application of ultraView Universal DAB detection kit (Ventana Medical Systems, Inc.). DAB was used as a chromogen and hematoxylin as a counter stain. Similarly, processed sections from human prostate cancer were used as positive controls for p65 (RelA) immunostaining, respectively. The specificity of staining was confirmed by the inclusion of negative control slides processed in the absence of primary antibody on tissue from the same animal.

In liver tissue, the numerical density of nuclear immunoreactivity for NF-kB of Kupffer cells was evaluated according to stereological analyses.In this study, unbiased counting frame and fractionator methods to estimate numerical density of NF-kB nuclear localization in the Kupffer cells were used. Each glass microscope slide was sampled using the fractionator principle of the stereology

software (Stereo Investigator® version 8.0, Micro Bright-Field, Colchester, Vermont, USA). NF-kB-positive nuclei were counted by using a 63x Leica Plan Apo objective (NA = 1.40), which allowed accurate recognition. The coefficient of error (CE) for the estimations was the last calculated value. The generally accepted highest limit of CE is 5% (22).

Data Analysis

The statistical analysis of all parameters was performed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) using the IBM® SPSS software package, version 19.00 (SPSS Inc., Chicago, IL). Statistical significance was considered P<0.05. All the results were expressed as mean±standard error of the mean (SE) for the six rats in each group.

RESULTS

Histopathological Results for Liver Toxicity

All zones (periportal, midzonal, and centrilobular) of liver acinus in the control and β-1,3-D-glucan-treated groups exhibited a typical appearance (*Fig. 1A* and *B, Table* 1). Liver tissues in the bortezomib and bortezomib + β-1,3-D-glucan-treated groups showed histopathological changes, such as cell degeneration, inflammatory cell infiltrates and foci, necrotic, and apoptotic cells (*Fig. 2A, B, C,* and *D, Table 1*). While these histopathological changes were at severe level at hour 72 (*Fig. 2B* and *Table 1*), they were at moderate level at hour 48 in the bortezomib-treated groups (*Fig. 2A* and *Table 1*). Inflammatory cell types were generally lymphocytes and macrophages, which organized both aggregates (foci) and diffuse, and were situated in the all zones of liver acinus (*Fig. 2A* and *B, Table 1*). Necrotic and degenerative cells (hypertrophic and abnormal membrane counters) were dense and focal with inflammatory responses. In addition, Councilman bodies (apoptotic cell death) and hypertrophic Kupffer cells (tissue macrophages) were observed within parenchyma in these groups (*Fig. 2A* and *B, Table 1*).

Bortezomib induced these histopathological changes, which decreased via β-1,3-D-glucan treatments at both hours 48 and 72 (*Fig. 2C* and *D, Table 1*). The liver tissues
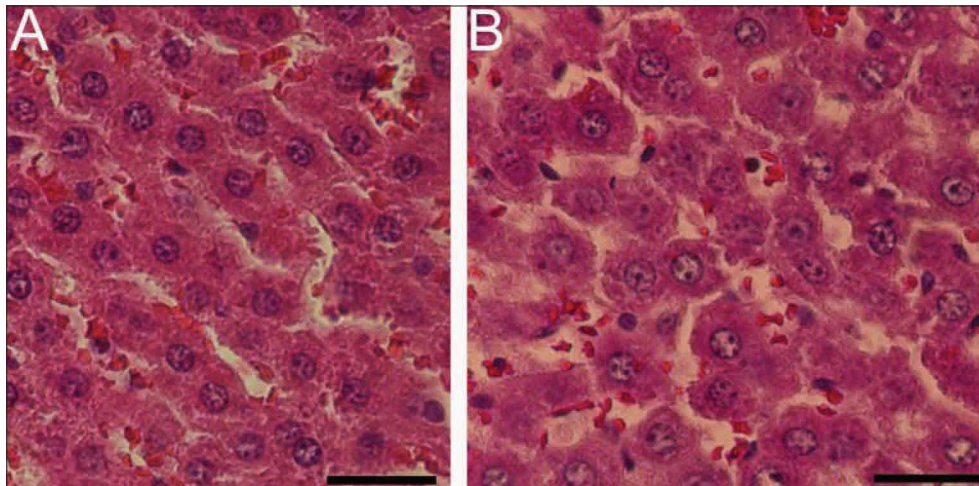

Fig 1. The normal appearance of organized plates of hepatocytes, which are separated by sinusoidal capillaries in control group **(A)** and β-1,3-D-glucan group **(B)**; Scale bars in A, B, 30 μm

Şekil 1. Kontrol **(A)** ve β-1,3-D-glukan **(B)** grubunda sinüzoidal kapillerle ayrılan hepatositlerin organize kordonların normal görünümleri. A, B 'deki ölçek barlar, 30 um

Table 1. *Histopathological score for rat liver tissues treated singly and in combination with bortezomib or β-1,3-D-glucan*

Tablo 1. *Tek başına ve kombinasyon halinde bortezomib ya da β-1,3-D-Glukan ile tedavi edilen sıçan karaciğer dokuları için histopatolojik skor*

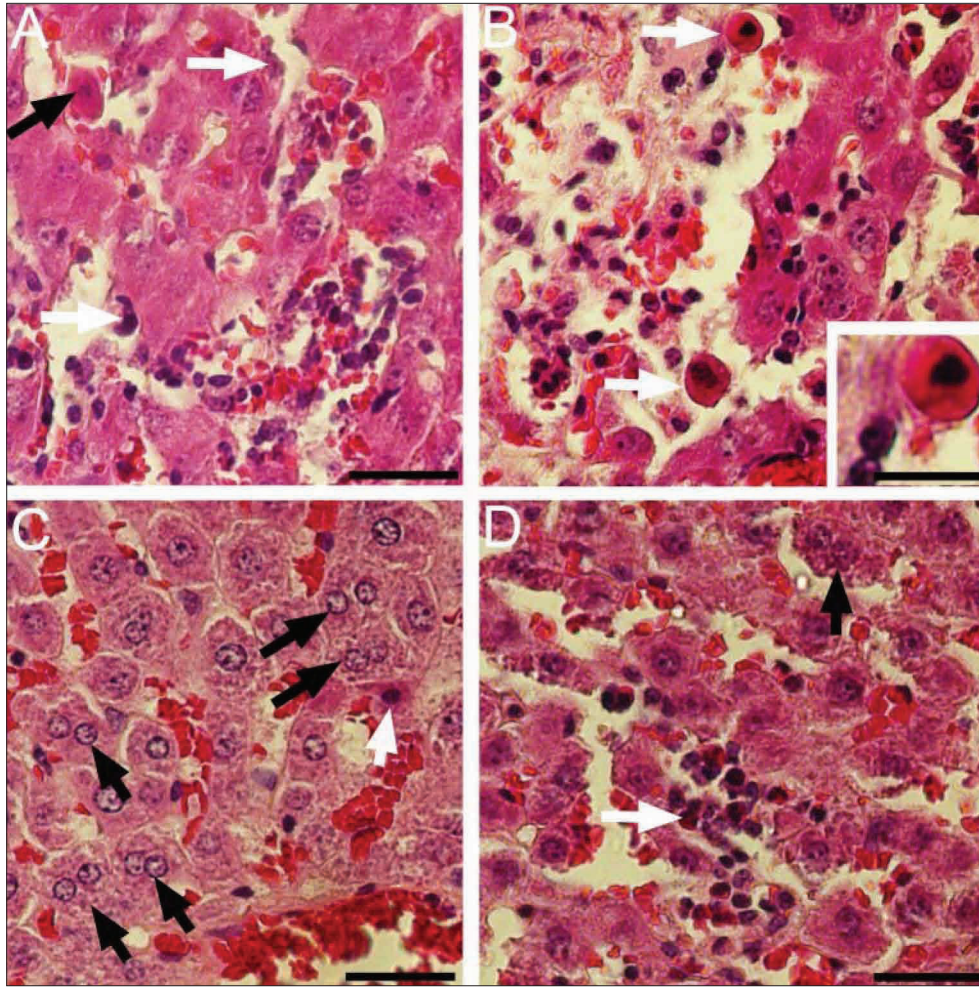| Data	Group					
	Control	β-1,3-D-glucan	Bortezomib 48th h	Bortezomib 72th h	Bortezomib+β-1,3-D-glucan 48th h	Bortezomib+β-1,3-D-glucan 72th h
Hypertrophic hepatocyte degeneration	-	-	++++	++++	++	++
Sinusoidal expansion	-	-	+++	++++	+	++
Necrotic cell deaths	-	-	+++	++++	+	++
Inflammatory cell foci or infiltrates	-	-	+++	++++	-	+
Hypertrophic Kupffer cells	-	-	+++	++++	-	+
- none; + minimal; ++ mild; +++ moderate; ++++ severe						

Fig 2. A- Irregular thickened hepatocyte plates with inflammatory cell infiltrates, Councilman bodies *(black arrow)* and hypertrophic Kupffer cells *(white arrows)* in parenchyma of livers from bortezomib-treated (hour 48) group, **B-** Severe inflammatory cell infiltrates with hepatocytes debris, Councilman bodies *(small square)* in parenchyma of livers from bortezomib-treated (hour 72) group, **C-** Necrosis in hepatocytes *(white arrow)* and moderate level hypertrophic and binucleate hepatocytes in midzonal and centrilobular zones of the liver tissue from bortezomib + β-1,3-D-glucan-treated (hour 48) group, **D-** Degenerative and binucleate *(black arrow)* hepatocytes and migration and adhesions of inflammatory cells (mono and polymorphonuclear leukocytes) within sinusoids in livers from Bortezomib + β-1,3-D-glucan-treated (hour 72) group. Scale bars in A, B, C, and D, 30 µm

Şekil 2. A- Bortezomib ile tedavi (saat 48) grubunda karaciğer parankimasında hipertrofik Kupffer hücreleri *(beyaz oklar)*, Councilman cisimcikleri *(siyah ok)* ve inflamatuvar hücre infiltrasyonları ile düzensiz kalınlaşmış hepatosit kordonları, **B-** Bortezomib ile tedavi (saat 72) grubunda karaciğer parankimasında Councilman cisimcikleri *(küçük kare)* ve hepatosit debrisleri ile şiddetli inflamatuvar hücre infiltrasyonları **C-** Bortezomib+ β-1,3-D-glukan (saat 48) ile tedavi grubunun karaciğer dokusunun midzonal ve sentrilobuler bölgelerinde orta düzeyde hipertrofik ve iki nükleuslu hepatositler ve hepatositlerde nekrozis *(beyaz ok)*, **D-** Bortezomib + β-1,3-D-glukan (saat 72) ile tedavi grubunun karaciğerlerinde sinüzoidler içinde inflamatuvar hücrelerin (mono ve polimorfonükleer lökositler) adhesyonu ve göçü ve dejeneratif ve iki nükleuslu hepatositler *(siyah oklar)*. A, B, C ve D'deki ölçek barlar, 30 um

of the bortezomib + β-1,3-D-glucan-treated group at hour 72 had no Councilman bodies, only mild level necrotic and degenerative cells within midzonal and centrilobular zones, and minimal level inflammatory areas (mono and polymorphonuclear leukocytes) within and around sinusoids, minimal level hypertrophic Kupffer cells (*Fig. 2D* and *Table 1*). The bortezomib + β-1,3-D-glucan-treated group at hour 48 showed no Councilman bodies or inflammatory cells and had minimal level necrotic and mild level degenerative cells, moderate level binucleate

hepatocytes within midzonal and centrilobular zones, and normal appearance Kupffer cells. The general liver histological structures of the Bortezomib + β-1,3-D-glucan-treated group at hour 48 were close to the control group (*Fig. 2C* and *Table 1*).

Biochemical Results for Oxidant and Antioxidant Parameters

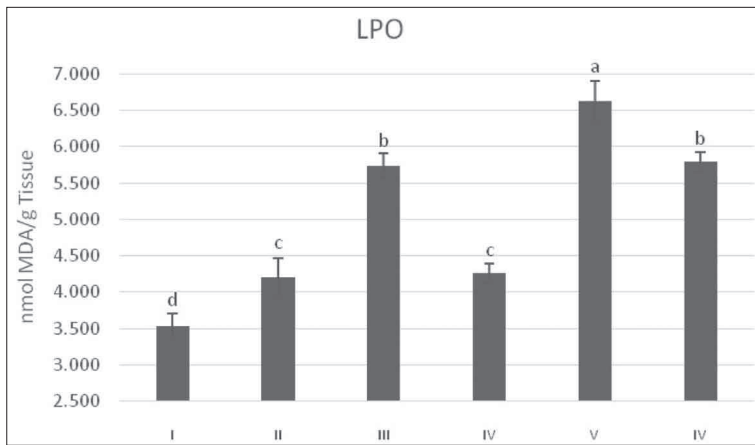The lipid peroxidation amounts (as indicators of oxidative stress of livers) for the treatment and control

Fig 3. Effects of bortezomib, β-1,3-D-glucan, and bortezomib + β-1,3-D-glucan treatments in levels of lipid peroxidation (LPO) in rat livers (mean ± S.E.M.) [I; control, II; β-1,3-D-glucan, III; bortezomib (hour 48), IV; bortezomib + β-1,3-D-glucan (hour 48), V; bortezomib (hour 72), VI; bortezomib + β-1,3-D-glucan (hour 72)].

Şekil 3. Bortezomib, β-1,3-D-glukan, ve bortezomib + β-1,3-D-glukan tedavilerinin sıçan karaciğerlerinde lipid peroksidayon (LPO) seviyelerine etkileri (ortalama ± S.E.M.) [I; kontrol, II; β-1,3-D-glukan, III; bortezomib (saat 48), IV; bortezomib + β-1,3-D-glukan (saat 48), V; bortezomib (saat 72), VI; bortezomib + β-1,3-D-glukan (saat 72)]

Fig 4. Effects of bortezomib, β-1,3-D-glucan, and bortezomib + β-1,3-D-glucan treatments in activity changes of super-oxide dismutase (SOD) in rat livers (mean ± S.E.M.) [I; control, II; β-1,3-D-glucan, III; bortezomib (hour 48), IV; bortezomib+β-1,3-D-glucan (hour 48), V; bortezomib (hour 72), VI; bortezomib + β-1,3-D-glucan (hour 72)]

Şekil 4. Bortezomib, β-1,3-D-glukan, and bortezomib + β-1,3-D-glukan tedavilerinin sıçan karaciğerlerinde süperoksit dismutaz (SOD) aktivite değişimlerine etkileri (ortalama ± S.E.M.) [I; kontrol, II; β-1,3-D-glukan, III; bortezomib (saat 48), IV; bortezomib + β-1,3-D-glukan (saat 48), V; bortezomib (saat 72), VI; bortezomib + β-1,3-D-glukan (saat 72)]
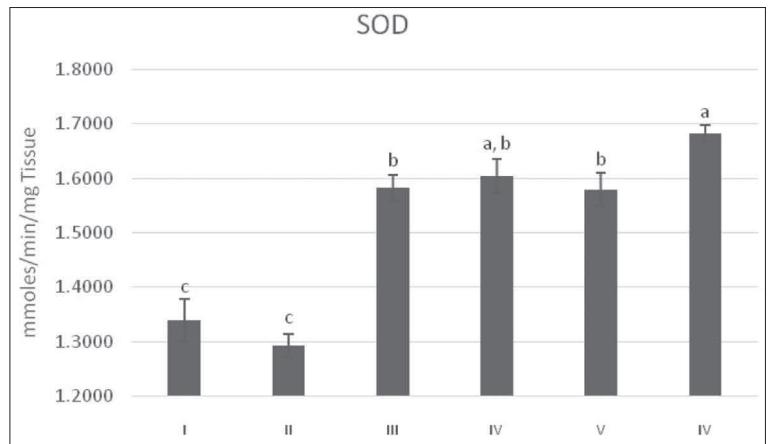
groups are shown in *Fig. 3*. Hepatic MDA levels in the bortezomib treatment groups (hours 48 and 72) with the increasing influence of time-dependence was significantly higher than the control group (P<0.05). Administration of β-1,3-D-glucan significantly reduced the tissue MDA levels (26.7%) at hours 48 (25.7%) and 72 in hepatic MDA levels increased by bortezomib treatment.

The hepatic SOD enzyme activities for all treatment and control groups were measured to understand the behavior of the antioxidant defense mechanism and were shown in *Fig. 4*. In both bortezomib treatment groups (hours 48 and 72), SOD activity was higher than the control group (P<0.05). Administration of β-1,3-D-glucan significantly elevated the tissue SOD activity at hours 48 and 72 in hepatic SOD activity comparing all groups.

Immunohistochemistry Results for p65 (RelA) Activity in Kupffer Cells

Kupffer cells are specialized macrophages located in the walls of the sinusoids and play an important role in late-phase hepatotoxicity. Early-phase hepatic injury-induced (xenobiotics dependent) Kupffer cells produce cytokines and growth factors via canonical NF-κB pathway. Activated Kupffer cells increase other inflammatory cell migration from the liver microcirculation to the parenchyma and lead to hepatic cell death by uncontrolled

inflammation. NF-κB activation is measured by p65 (NF-κB subunit) migration to the nucleus in Kupffer cells. Thus, this study investigated the numerical density of nuclear p65 localization of Kupffer cells in liver tissues of the control and treatment groups using immunohisto-chemical staining and stereological methods *(Fig. 5)*. The numerical density of nuclear p65 (RelA) for treatment and control groups are shown in *Fig. 6*.

The immunohistochemistry results showed no statistically significant differences between the control group and β-1,3-D-glucan, Bortezomib + β-1,3-D-glucan (hour 48) groups in numerical density of p65, as determined by stereological examination of the liver tissues (P>0.05). However, this data was high at hour 48 and very high at hour 72 after bortezomib treatments (P<0.05). Bortezomib treatment-induced increased nuclear p65 localization was decreased via β-1,3-D-glucan treatments at hours 48 and 72 (P<0.05).

DISCUSSION

Hepatotoxicity associated with chemotherapeutic agents is of interest to clinicians and researchers. The chemotherapeutic agents are among the more commonly used drugs associated with hepatotoxic effects ranging from acute to chronic damage, hepatitis, steatosis
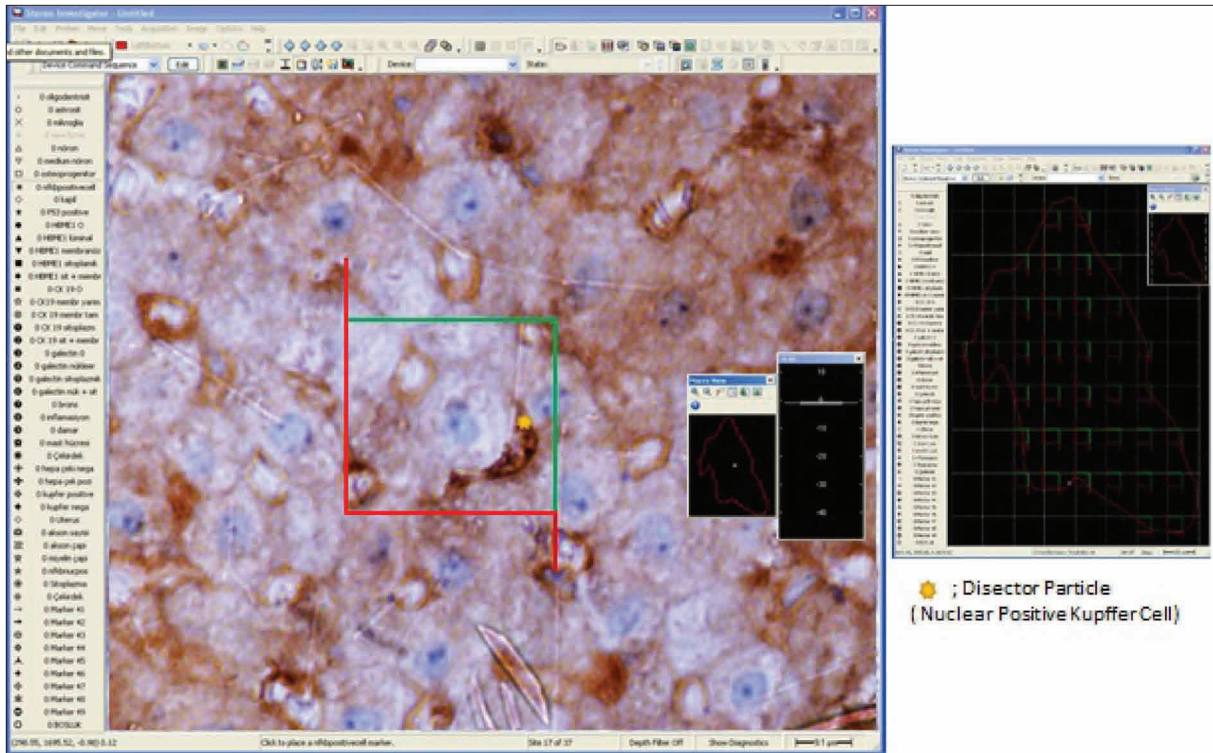
Fig 5. Unbiased counting frames used for numerical calculation of nuclear p65 localization by fractionator method (63×). Nuclear p65 hitting inclusion lines were calculated [Bortezomib + β-1,3-D-glucan group (hour 48)]

Şekil 5. Parçalama yöntemi (63 ×) tarafından nüklear p65 lokalizasyonu sayısal hesaplaması için kullanılan tarafsız sayım çerçeveleri. Dahil hatlara isabet eden nüklear p65 hesaplandı [Bortezomib + β-1,3-D-glukan grubu (saat 48)]
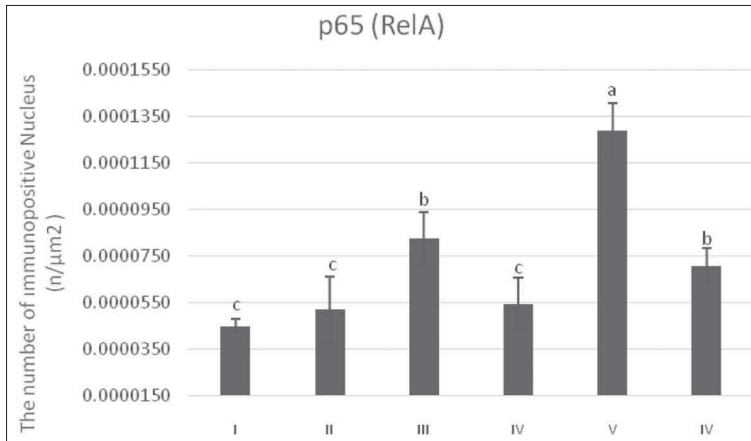

Fig 6. Effects of Bortezomib, β-1,3-D-glucan, and Bortezomib + β-1,3-D-glucan treatments on changes in nuclear p65 localization in Kupffer cells (mean ± S.E.M.) [I; control, II; β-1,3-D-glucan, III; bortezomib (hour 48), IV; bortezomib + β-1,3-D-glucan (hour 48), V; bortezomib (hour 72), VI; bortezomib + β-1,3-D-glucan (hour 72)]

Şekil 6. Bortezomib, β-1,3-D-glukan, and bortezomib + β-1,3-D-glukan tedavilerinin sıçan karaciğerlerinde Kupffer nüklear p65 lokalizasyon değişimlerine etkileri (ortalama ± S.E.M.) [I; kontrol, II; β-1,3-D-glukan, III; bortezomib (saat 48), IV; bortezomib + β-1,3-D-glukan (saat 48), V; bortezomib (saat 72), VI; bortezomib + β-1,3-D-glukan (saat 72)]

cholestasis and granuloma [23]. Bortezomib (PS-341 or Velcade®) is a modified dipeptidyl boronic acid 26S proteasome inhibitor for the treatment of multiple myeloma which is the second most common hematological cancer (after non-Hodgkin's lymphoma) [24]. Although Hideshima et al.[25] reported bortezomib is 1.000-times more effective for triggering apoptosis on myeloma cells than normal plasma cells. There is limited information about its effects on other normal cells because of new drug marketing. Phase studies have shown that the drug had tolerable adverse effects, such as peripheral neuropathy, gastrointestinal symptoms, thrombocytopenia, and hypotension in non-target organs [26]. However, a few case reports clinically determined that bortezomib increased liver enzymes (aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase) levels [27].

According to literature, bortezomib-induced hepatotoxicity has been rarely characterized and or studied. This research evaluated the time-dependent histopathological, biochemical, and immunohistochemical changes of liver tissues in rats given bortezomib. The first observation was that nonspecific reactive hepatitis (NSRH) or focal hepatitis was histologically observed in the liver tissue of rats given bortezomib alone. NSRH is an entity characterized by the presence of Kupffer cell

mobilization, portal inflammatory infiltration, and focal periportal necroinflammatory areas (generally mononuclear cells) with acidophilic cells (Councilman bodies) through the liver parenchyma [28]. Drug-induced NSRH is rare and determined in some drugs such as naproxen, aspirin, and paclitaxel [29]. This present study is the first to report that bortezomib causes drug-induced NSRH. According to these findings, liver tissues 48 h after bortezomib treatment showed a large number of focal necrotic areas with prominent inflammatory responses in all zones of the liver acinus. Inflammatory responses were associated with the relationship between activated (hypertrophic) Kupffer cells and mononuclear cells. This study also showed wide sinusoidal expansion and hypertrophic changes in hepatocytes in liver parenchyma (without focal necroinflammatory areas) in this group. By hour 72, bortezomib-treated livers had noticeably greater numbers and sizes of focal inflammatory cell infiltrations, necrotic areas, and other degenerative changes than those observed at 48 hours.

In summary, this study demonstrated that bortezomib treatment-induced prominent liver degeneration was apparent by hour 48 and gradually increased until hour 72. Drug-induced liver toxicity is a common cause of liver injury and generally occurs two pathophysiological processes. In the first pathway, drugs and their metabolites with possible toxic effects directly affect the biochemistry of the cell primarily due to increased oxidative stress and changes of intracellular signaling pathways associated with apoptotic or necrotic cell death in hepatocytes (mainly), endothelial cells, and cholangiocytes. In the second pathway, these also caused immune response in inflammatory cells and triggered cellular damage and apoptotic or necrotic cell death in parenchymal cells [30]. As a result, these two pathways are related to the occurrence of inflammatory and oxidative products in the liver [31]. Oxidative stress is often implicated in various deleterious processes resulting from an imbalance between pro-oxidants (reactive oxygen species [ROS] and/or reactive nitrogen species [RNS]) and antioxidants in favor of the pro-oxidants [32]. ROS are formed through oxidative processes within the cell but can be produced at elevated rates under pathophysiological conditions. The excessive ROS may attack polyunsaturated fatty acids of the cellular membrane and initiate lipid peroxidation within the cell, which results in the formation of Malondialdehyde (MDA). Malondialdehyde, a reactive aldehyde, is the major product of lipid peroxidation and widely used to indicate LPO level [33]. Antioxidant reactions inhibit the oxidation of cellular compounds via antioxidant enzymes and molecules such as SOD, CAT, GPx, and GR. SOD is the first line in the defense process against superoxide radicals produced by oxidative reactions and is a significant parameter to evaluate cellular antioxidant activity [34]. In the biochemical analysis the terms of SOD and LPO values, this study revealed that bortezomib treatment elevated SOD and LPO values at hours 48 and 72, increasing with time. However, the increase in the LPO values was higher than the increase in the SOD values of livers in the bortezomib groups compared to other groups. This finding suggested that oxidant-antioxidant balance is broken in favor of the oxidants in cells, or insufficient levels of antioxidants and oxidative stress occur. This elevation is probably related to elevated endogenous pro-oxidants associated with first pathway and increased exogenous pro-oxidants associated with second pathway (resident macrophages [Kupffer cells] and infiltrating phagocytes) according to this study's histopathologic findings.

Although bortezomib is a potent apoptotic activator via proteasome inhibition, this research showed that it caused necrotic (hypertrophic changes) and necrotic cell deaths via oxidative stress in focal necroinflammatory and other areas in rat livers. In a preclinical *in vivo* toxicity study in rats, an increase in liver enlargement (35%) caused by the induction of increased peroxisomal acyl-CoA oxidase (60%) activity and after a repeat dose of bortezomib was observed [10]. This data overlaps to hypertrophic changes of hepatocytes in the present study, and increasing LPO amounts may be related to increased peroxisomal acyl-CoA oxidase activity and other oxidases by induced bortezomib treatments. LPO values above vital limits may break down membrane integrity, causing necrotic death [35]. In addition, apoptotic cell deaths (Councilman bodies) were only observed in the focal necroinflammatory areas as well as necrotic cell deaths. Apoptotic cell deaths could result from inflammatory responses of mononuclear cells [36]. Other than direct effects of bortezomib and its metabolites because it was only observed in necroinflammatory areas. It was shown that Kupffer cells have an important role in the initiation and maintenance of this immune response in necroinflammatory areas because of the time-dependent increase in hypertrophic changes and p65 activity. Activated canonical p65 pathway-dependent increased proinflammatory cytokine secretion, such as tumour-necrosis factor (TNF) and interleukin-6 (IL-6) in Kupffer cells induces the activation and migration of mono-nuclear cells [37,38] toward necroinflammatory areas. Furthermore, endothelial cells were shown to have a role in endothelial dilatation-dependent this migration.

In conventional therapies, cancer patients often receive complementary medical treatment to prolong survival and reduce toxicity in non-target organs. It's necessary that the substances used in complementary therapy have protective effects as well as anti-cancer properties [39]. β-1,3-D-glucan has been shown in Japanese medicine, experimental studies, and clinical trials to enhance the effectiveness of chemotherapeutic agents on the neoplastic cells and have protective effects on non-target organs [40-42]. Therefore, this present experiment

examined the effects of β-1,3-D-glucan. Whether β-1,3-D-glucan had adverse effects on the liver compared to the control group was first examined. Delaney et al.[43] reported that β-1,3-D-glucan had no toxic effects on animals. Similarly, this study did not reveal toxic effects in parenchyma or Kupffer cells according to immunohistochemical and histopathologic analyses. However, LPO values showed an increase in physiological levels. Next, this study examined the effectiveness of β-1,3-D-glucan as a protective agent against liver damage induced by bortezomib. β-1,3-D-glucan significantly eliminated bortezomib-induced NSRH findings at both 48 and 72 hours. However, some histopathologic changes were observed in both groups. Diffuse hypertrophic hepatocyte degeneration (an irreversible cell damage), non-focal necrotic deaths and sinusoidal dilatation were observed in the middle-middle, minimal-middle, and minimal-middle levels at 48 and 72 hours respectively. Hypertrophic Kupffer cells and non-focal inflammatory cell infiltrates were also observed at minimal levels at 72 h.

In summary, these findings showed that cellular degeneration and inflammatory response were significantly decreased in both groups. These findings were also parallel with decreasing values of LPO, an important indicator of cellular damage and reduced Rel A activity as an indicator of inflammatory processes. According to our results, β-1,3-D-glucan decreased LPO levels by increasing defense mechanisms such as the activity of SOD enzymes. This may indicate the presence or trend of increasing amounts of superoxide radicals, which could be linked with an enhanced SOD activity [44]. Indeed, the increased SOD activity would also protect tissues from oxidative stress, which reveals that the accumulation of superoxide anion radicals might be responsible for an increased LPO [45]. Descending necrotic deaths and decreased LPO values probably must remain below the threshold value required for the activation of Kupffer cells, so it can cause an inflammatory response is not generated. In addition, β-1,3-D-glucan increased activity on other cellular antioxidative enzymes, and its free radical scavenging activity [46-49] might contribute to the reduction of oxidative stress and cellular degeneration and eliminate NSRH findings. In addition, in this group, absence of apoptotic death, these deaths did not occur with the direct effect of bortezomib and again seems to be associated with the inflammatory process.

To knowledge, this present study is the first to show that, based on biochemical, immunohistochemical, and histopathological findings, the administration of bortezomib causes increased tissue damage and NSRH in rat livers, and β-1,3-D-glucan may decrease the increasingly toxic effects of bortezomib by regulating Kupffer cell activation and suppressing oxidative stress leading to NSRH. In light of this information, β-1,3-D-glucan may be used as a supportive agent for reducing the side effects of bortezomib therapy.

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