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Ginkgo biloba L. extract and flunixin meglumine attenuate sepsis–associated liver injury, oxidative stress, inflammation and apoptosis in rats

El extracto de *Ginkgo biloba* L. y la flunixina meglumina atenúan la lesión hepática, el estrés oxidativo, la inflamación y la apoptosis asociados a la sepsis en ratas

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ABSTRACT

Lipopolysaccharide (LPS), known as a stimulant of inflammation, causes acute liver injury by inducing the production of inflammatory mediators and oxidative stress. The purpose of this study is to determine whether of a nonsteroidal anti–inflammatory drug (NSAID) Flunixin meglumine (FM) and herbal an medicine *Ginkgo biloba* L. extract (GBE) show antioxidative, anti–inflammatory or antiapoptotic effects in liver tissue in LPS–induced hepatotoxicity. Animals were separated to 6 groups as control, sepsis (1 mg·kg^{-1}, 7th day single dose, intraperitoneal (ip)), sepsis + FM (1 mg·kg^{-1}, 7th day single dose, ip + 2.2 mg·kg^{-1} day, ip), sepsis + GBE (1 mg·kg^{-1}, 7th day single dose, ip + 50 mg·kg^{-1} day, gavage), FM and GBE and the study continued for 7 days. Liver tissues taken from rats sacrificed were analyzed biochemically, histologically and immunohistochemically. Accordingly, LPS caused liver function markers alteration, inflammation, oxidative stress, and apoptosis, as well as histopathological changes in liver tissue. However, it was observed that LPS–induced changes were regulated by FM and GBE application. FM and GBE was demonstrated to have antioxidant, antiinflammatory and anti–apoptotic properties in LPS–induced hepatotoxicity.

Key words: Flunixin meglumine; *Ginkgo biloba* L. extract; liver damage; sepsis

RESUMEN

El lipopolisacárido (LPS), conocido como un estimulante de la inflamación, causa daño hepático agudo al inducir la producción de mediadores inflamatorios y estrés oxidativo. El propósito de este estudio es determinar si un fármaco antiinflamatorio no esteroide (AINE) Flunixin meglumine (FM) y un agente natural extracto de *Ginkgo biloba* L. (GBE) muestran efectos antioxidantes, antiinflamatorios o antiapoptóticos en el tejido hepático en la hepatotoxicidad inducida por LPS. Los animales se separaron en 6 grupos como control, sepsis (1 mg·kg^{-1}, dosis única el séptimo día, intraperitoneal (ip)), sepsis + FM (1 mg·kg^{-1}, dosis única el séptimo día, ip + 2,2 mg·kg^{-1} día, ip), sepsis + GBE (1 mg·kg^{-1}, dosis única el séptimo día, ip + 50 mg·kg^{-1} día, sonda), FM y GBE y el estudio continuó durante 7 días. Los tejidos hepáticos extraídos de ratas sacrificadas se analizaron bioquímicamente, histológicamente e inmunohistoquímicamente. En consecuencia, el LPS provocó alteración de los marcadores de la función hepática, inflamación, estrés oxidativo y apoptosis, así como cambios histopatológicos en el tejido hepático. Sin embargo, se observó que los cambios inducidos por LPS fueron regulados por la aplicación de FM y GBE. Se demostró que FM y GBE tienen propiedades antioxidantes, antiinflamatorias y antiapoptóticas en la hepatotoxicidad inducida por LPS.

Palabras clave: Flunixina meglumina; extracto de *Ginkgo biloba* L.; daño hepático; septicemia

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INTRODUCTION

Lipopolysaccharide (LPS), known as a stimulant of inflammation, is the main constituent of Gram-negative bacteria and induces the manufacture of uncontrolled inflammatory mediators and oxidative stress concluded acute hepatic injury [1]. LPS indicates a pro-oxidative effect on this damage by activating liver macrophages that produce inflammatory cytokines inclusive tumor necrosis factor alpha (TNF-α), interleukin-1ß (IL-1ß) and interleukin-6 (IL-6) [2] and inducing excessive production of reactive oxygen species (ROS) [3]. Therefore, it is important to develop the diversity and currency of preferred anti-inflammatory agents for the inhibition of these cytokines.

Nonsteroidal anti-inflammatory drugs (NSAIDs) have the capability to scavenge free radicals and play as robust antioxidants. Flunixin meglumine (FM), an NSAID, is proven to have antitoxemic mechanisms and is frequently used in the treatment of endotoxemia [4]. FM strongly obstructs cyclooxygenase (COX) and the synthesis of eicosanoids and attunes acute hemodynamic alterations throughout endotoxemia [5]. However, such drugs have diverse side effects with long-term use [6]. For this reason, it is noteworthy that natural produce are discovered in the improve of new treatments that are more attractive and have minimal toxicity [7] and they are the focus of recent research in the phytochemical-based management of diseases [6].

Ginkgo biloba L. extract (GBE), which is very widely used universally, has diverse pharmacological properties [8]. The beneficial influences of Ginkgo *biloba* extract 761 (EGb 761) obtained from the leaves based on these pharmacological properties are due to its active ingredients consisting of flavonoids (24%) and terpene lactones (6%) [9]. It has been reported that GBE, which has antioxidant, anti-inflammatory, anti-apoptotic and antigenotoxic effects, is effective in various toxicities [10], and especially prevents oxidative stress and decrease in antioxidant defense in hepatotoxicity [11]. It is known that the antioxidant capacity of Ginkgo biloba, which causes this hepatic renovation, is related to increasing glutathione content, reducing lipid peroxidation and hydroperoxide levels, and restoration of antioxidant enzyme activity [12]. EGb 761 has been reported to decrease LPS-induced oxidative stress, especially through ROS and nitric oxide (NO) [13]. Also, the robust anti-inflammatuar characteristics of GBE components occur through their inhibitive effects on TNF-α, IL-6, prostaglandin E2 (PGE2), inducible nitric oxide synthase (iNOS) mRNA and COX-2 mRNA values in LPS-induced macrophages [14]. This work designed to specified the ameliorative activities of FM and GBE in LPS-induced liver injury by reducing some inflammatory mediators, preventing the formation of oxidative stress and inhibiting cell apoptosis.

MATERIALS AND METHODS

Chemicals and animals

Lipopolysaccharide (*Escherichia coli*, 055:B5, Sigma, USA), FM (Vilsan Pharmaceuticals, TR) and EGb 761 (Abdi İbrahim İlaç, TR) were acquired from the indicated companies. The rest agents were provided by Sigma-Aldrich Company (USA). Thirty-six male Spraque-Dawley rats (*Rattus norvegicus*) (male, 6-8 weeks old, 280-300 g weight) were acquired from Firat University Experimental Research Center (Elazig, Turkey). The animals were provided with optimal situation (50-60% humidity, 22-24°C temperature, feed and optional water, 12h light/12-h cycle) throughout the experimental applications. This study was approved by Firat University Animal Experiments Local Ethics Committee (20.11.2013/09-127).

Experimental design

Animals were randomly separated to six groups (n=6) and the study continued for 7 days (d). The average solution volume applied to all groups was determined as 0.5 mL. For the control group, physiological saline solution was received through intraperitoneally (ip) injection for 7 d. For the sepsis group, 1 mg·kg^{-1} LPS solved in saline was received through ip injection only on the 7th day [15]. For the FM group, 2.2 mg·kg^{-1} d FM was received through ip injection for 7 d [4]. For the GBE group, 50 mg·kg^{-1} day EGb 761 was received through gavage for 7 d [16]. For the sepsis + FM group, 1 mg·kg^{-1} LPS was received only on the 7th d following 2.2 mg·kg^{-1} d FM application for 7 d. For the sepsis + GBE group, 1 mg·kg^{-1} LPS was received only on the 7th d following 50 mg·kg^{-1} d EGb 761 application for 7 d. The rats were sacrificed under anesthesia then blood specimens and liver tissues were taken and evaluated for biochemical, histological and immunohistochemical examinations.

Serum analysis

Serum TNF-α (E-EL-R0019, Elabscience, USA; pg·mL^{-1}), PGE2 (E-EL-0034, Elabscience, USA; pg·mL^{-1}) and IL-1ß (E-EL-R0012, Elabscience, USA; pg·mL^{-1}) levels were specified by commercial enzyme linked immunosorbent assay (ELISA) kit and studied in accordance with the specified procedures. All process were applied according to Ilhan *et al.* [13]. Serum glucose, albumin, globulin, total protein, aspartate aminotransferase (AST), alanine transaminase (ALT), total cholesterol, triglyceride, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and very low-density lipoprotein (VLDL) analyzes were determined with Olympus AU 600 (Optical Co., Ogaki, JAPAN) autoanalyzer.

Biochemical analysis

Malondialdehyde (MDA) and glutathione (GSH) levels and superoxide dismutase (SOD) and catalase (CAT) activities were determined spectrophotometrically in homogenate of liver tissues. MDA levels were expressed as nmol·ml^{-1} [17], GSH levels as nmol·mg^{-1} protein [18], SOD levels percent inhibition·mg^{-1} protein [19], CAT levels as k·g^{-1} protein [20].

Histological evaluation

The tissues were paraffinized after 10% formalin fixation and blocks were prepared. For histological examination, hematoxylin-eosin (H&E) applied sections were photographed with Olympus BX-51 microscope. (Olympus Optical Co., Ltd., Tokyo, JAPAN). Different histological fields were appreciated semi-quantitatively at 20x magnification to determine the extent of histopathological alterations in the tissues of all groups. The analysis scores of histopathological changes was calculated according to the criteria of none (0), slight (1), medium (2) and severe (3) [21].

Immunohistochemical examination

Caspase-3 (Casp 3) (#RB-1197-B, Thermo Fisher Scientific Co., USA) immunohistochemistry reactivity in the tissues was defined by the Avidin-Biotin-Peroxidase Complex method [22]. Background staining was performed with haematoxylin. Immunoreactivity was computed by the formula area x intensity (intensity; none (0), very little (0.5), little (1), moderate (2), severe (3) x area; 0.1 (<25%), 0.4 (26-50%), 0.6 (51-75%), 0.9 (76-100%) [22].

Statistical analysis

SPSS 21.0 (SPSS Inc., Chicago, IL, USA) software was preferred. Differences between groups were carried out using one-way analysis of variance subsequently the posthoc Tukey test. The data were presented as mean ± standard deviation format. The values with differences of $P<0.05$ were deemed statistically significant.

RESULTS AND DISCUSSIONS

Sepsis is a complicated pathophysiological process involving an excessive inflammatory and immune response that cause multiorgan failure. It is a global health problem that continues to cause large numbers of deaths today. LPS is considered a extremely pathogenic endotoxin responsible for these dysfunctions in sepsis. Therefore, studies are still ongoing to identify new therapeutic drugs for effective sepsis treatment [23]. Many studies have shown that natural and medicine produces such as curcumin [24], metformin [25], aminoguanidine [26] have a protective and therapeutic effects on LPS induced hepatotoxicity. However, there is a lack of data regarding the effectiveness of FM and GBE.

In this study, the liver function marker levels of all groups are presented in TABLE I. It was determined that the glucose, total cholesterol, triglyceride, HDL, LDL, VLDL levels ($P<0.05$), AST and ALT levels ($P<0.01$) significantly increased in the sepsis group in comparison to the control. On the contrary, it was defined that significant for albumin and total protein levels ($P<0.05$) decreased in sepsis group than control. Additionally, it was observed that the AST and ALT levels ($P<0.01$) and other liver function marker levels ($P<0.05$) a significantly decreased in the sepsis + FM and sepsis + GBE groups in comparison to the sepsis. In particular, triglyceride and HDL levels were found to reach the control group levels, while albumin, globulin and total protein levels were higher than in the sepsis group. Some researchers have stated that this markers increase with LPS administration [6, 25, 26, 27]. It has been stated that FM [4] and GBE [28, 29] treatments significantly decreased the LPS-induced increase in AST and ALT levels similar to this study. There are also studies in the literature in which this increases caused by LPS is decreased with different agents [24, 25, 26]. It is reported that LPS-induced sepsis causes tissue damage due to mitochondrial dysfunction and cell rupture [30], and the decrease in increased AST and ALT levels indicating this tissue damage may be attributed to the physiological defense mechanism of FM and GBE through tissue regenerative effects.

LPS severely stimulates immune cells and triggers extreme inflammation by increasing the synthesis and release of proinflammatory components including [2]. In an experimental with LPS, it was reported that the levels of proinflammatory cytokines such as TNF-α, IL-1ß and IL-6 levels increased significantly [31]. Our study results showed that TNF-α, PGE2 and IL-1β levels increased significantly in the sepsis group ($P<0.001$) compared to the control group with LPS administration. It was also determined that these values were decreased in the sepsis + FM and sepsis + GBE groups ($P<0.001$) compared to the sepsis group. These values of all groups are presented FIG. 1. In some LPS-induced studies, increased proinflammatory cytokine levels have been shown to be regulated using different agents [6, 25, 26]. In our study, LPS-induced increased TNF-α and IL-1β levels were recorded to be decreased by FM and GBE administration, similar to hepatotoxicity studies [28, 32]. It has also been recorded that GBE decreases the values of these cytokines in liver ischemia/reperfusion [8]. Therefore, it can be said that FM and GBE exhibit anti-inflammatory effects in sepsis by regulating the production and release of various inflammatory mediators.

Oxidative stress activates a number of transcription factors and induces the expression of many genes, containing various proinflammatory cytokines [21]. It has been determined that TNF-α and IL-1ß, which are proinflammatory cytokines, induce the production of ROS and this damage further increases the production of these cytokines [33]. LPS induced sepsis models, it has been shown by many studies that ROS levels increases, especially in the liver [24, 34]. In our study, MDA and GSH levels and SOD and CAT activities of all groups are presented in FIG. 2. MDA levels showed a significant increase in the sepsis group compared to the control group ($P<0.01$). Also, a decrease in GSH levels, SOD ($P<0.01$) and CAT activities ($P<0.05$) was detected. These data are consistent with previous studies

TABLE I
Effect of FM and GBE on LPS-induced liver function biomarkers

Parameter	Control	Sepsis	FM	GBE	Sepsis+FM	Sepsis+GBE	*P*
Glucose (mg·dl^{-1})	78.83 ± 2.24^{a}	113.80 ± 3.12^{c}	79.03 ± 2.03^{a}	80.01 ± 2.60^{a}	88.62 ± 2.30^{b}	89.38 ± 3.01^{b}	*
Albumin (g·dl^{-1})	3.45 ± 0.06^{a}	2.98 ± 0.08^{b}	3.43 ± 0.06^{a}	3.41 ± 0.07^{a}	3.22 ± 0.08^{a}	3.23 ± 0.06^{a}	*
Globulin (g·dl^{-1})	2.36 ± 0.03	2.30 ± 0.02	2.35 ± 0.03	2.35 ± 0.04	2.41 ± 0.03	2.47 ± 0.01	NS
T.protein (g·dl^{-1})	5.81 ± 0.03^{a}	5.28 ± 0.05^{b}	5.78 ± 0.04^{a}	5.76 ± 0.05^{a}	5.63 ± 0.05^{a}	5.70 ± 0.03^{a}	*
ALT (U·L^{-1})	77.30 ± 2.30^{a}	107.50 ± 2.70^{c}	77.10 ± 2.10^{a}	76.40 ± 3.20^{a}	90.10 ± 2.80^{b}	89.20 ± 2.60^{b}	**
AST (U·L^{-1})	192.10 ± 8.70^{a}	289.00 ± 7.20^{c}	189.70 ± 5.10^{a}	190.00 ± 6.30^{a}	234.10 ± 3.10^{b}	232.90 ± 4.60^{b}	**
T. cholesterol (mg·dl^{-1})	43.00 ± 1.13^{a}	74.80 ± 2.07^{c}	43.80 ± 2.12^{a}	44.30 ± 1.62^{a}	58.50 ± 1.80^{b}	57.80 ± 1.37^{b}	*
Triglyceride (mg·dl^{-1})	47.30 ± 3.5^{a}	86.40 ± 4.71^{b}	47.60 ± 5.01^{a}	48.20 ± 3.67^{a}	59.30 ± 4.02^{a}	57.10 ± 3.80^{a}	*
HDL (mg·dl^{-1})	12.90 ± 0.45^{a}	29.46 ± 0.60^{b}	10.81 ± 0.91^{a}	10.50 ± 0.74^{a}	16.90 ± 0.86^{a}	17.20 ± 0.67^{a}	*
VLDL (mg·dl^{-1})	9.46 ± 0.97^{a}	15.23 ± 1.25^{c}	10.40 ± 0.82^{a}	10.80 ± 0.93^{a}	11.60 ± 0.79^{b}	11.80 ± 0.87^{b}	*
LDL (mg·dl^{-1})	5.50 ± 0.73^{a}	9.66 ± 0.90^{c}	7.21 ± 0.18^{b}	7.30 ± 0.74^{b}	6.71 ± 0.52^{b}	6.72 ± 0.91^{b}	*

^{a,b,c}: Different superscripts in the same row indicate the significant difference, NS: Non-significant, * $P<0.05$: statistically significant, ** $P<0.01$: statistically significant, AST: aspartate aminotransferase, ALT: alanine transaminase, HDL: high-density lipoprotein, LDL: low-density lipoprotein, VLDL: very low-density lipoprotein

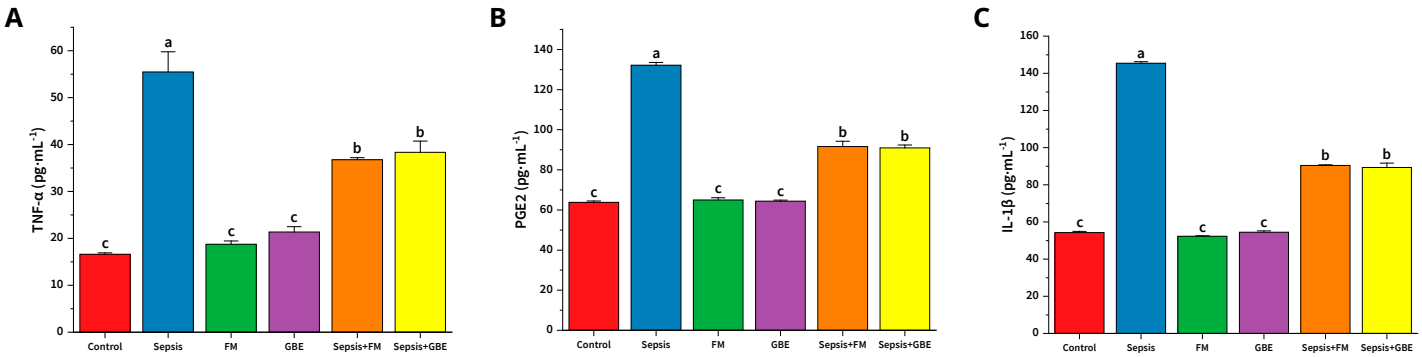

FIGURE 1. Serum levels of TNF–α (A), PGE2 (B) and IL–1ß (C) analyzed by ELISA method. Data are stated as mean ± standard deviation, *P*<0.001. TNF–α:tumor necrosis factor alpha, PGE2: prostaglandin E2, IL–1ß: interleukin–1ß, ELISA: enzyme–linked immunosorbent assay

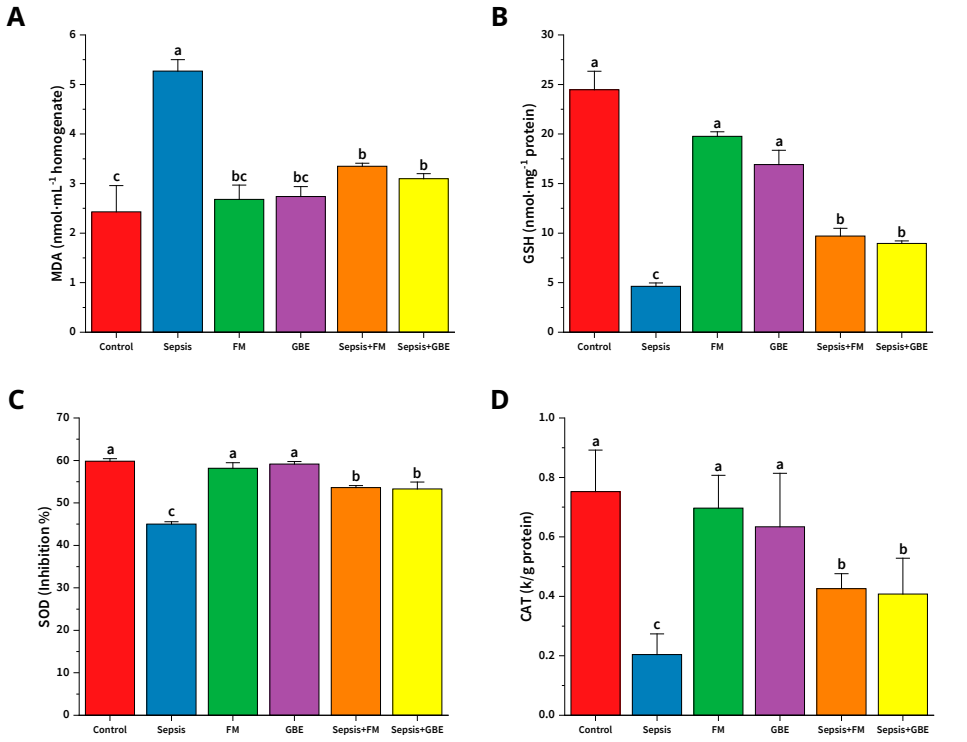

FIGURE 2. Liver levels of MDA (A) and GSH (B) and activities of SOD (C) and CAT (D) analyzed by spectrophotometrical method. Data are stated as mean ± standard deviation, *P*<0.01 for all analyzes apart from CAT activitiy (*P*<0.05). MDA: malondialdehyde, GSH: glutathione, SOD: superoxide dismutase, CAT: catalase

reporting that oxidative stress markers such as MDA increase and GSH, SOD and CAT decrease in LPS-induced hepatotoxicity. Some researchers have associated the irregularity in these levels with LPS-induced liver damage [24, 31, 34]. This study is consistent with other studies in that the imbalance in LPS-induced oxidative stress markers can potentially be regulated by antioxidant agents such as FM and GBE. In the sepsis + FM and sepsis + GBE groups, MDA levels were reduced compared to sepsis group ($P<0.01$), while GSH levels, SOD ($P<0.01$) and CAT activities ($P<0.05$) were significantly increased. Consistent with this study data, it was recorded that FM regulates impaired oxidant and antioxidant systems in LPS-induced sepsis [4]. Moreover, Tatli Seven *et al.* [32] suggested that FM normalizes oxidative stress markers in liver toxicity. Parimoo *et al.* [35] stated that the imbalance in oxidative stress markers was regulated by GBE, an antioxidant agent. In addition, it is stated that GBE attenuates hepatic oxidative stress through modulation of redox imbalance [36]. It has been also shown that the impaired oxidant and antioxidant systems LPS induced are regulated in studies by using different antioxidant agents [26, 37]. It can be predicted that antioxidant agents used against excessive ROS production in LPS-induced sepsis may be a good choice to reverse liver damage.

Endotoxic shock, one of the most critical symptoms of LPS–induced sepsis, causes various pathological changes. It is known that these changes are tissue damage caused by the rising in cytokine release, oxidative stress and mitochondria dysfunction [30]. Some research showed various histopathological alterations such as serious necrosis, hemorrhage, congestion, edema, inflammatory cell infiltration, and significant destruction of hepatolobular structure with LPS application [6, 25]. Similarly, a study revealed that LPS impaired the hepatic architecture along with vacuolization, degeneration, and inflammatory cell infiltration in the liver [24]. In our study, lobuler structure, central vein and portal areas were in normal histological architecture and hepatocytes were radially located in control, FM and GBE groups. In contrast, it was noted significant alterations as disorganization in hepatic cord areas, hyperemia in vena centralis and congestion in sinusoids in the sepsis group. In addition to Kupffer cell activation, karyomegaly and necrosis in hepatocytes were noted. Inflammatory cell infiltration, mainly neutrophil granulocytes, was also detected. Interestingly, it was noted in our study that FM and GBE alleviated these necrotic, hemorrhagic and infiltrative changes caused by LPS. The histopathology and histopathological score of all our study groups are presented in FIG 3. It has been reported that FM improves histopathological damage in the liver by restoring liver function markers that are impaired in sepsis with gluconeogenic and ureogenic activity and by regulating the inflammatory response and oxidative stress status with its anti–inflammatory and antioxidant activities. Also, it was stated that FM application reduced leukocyte infiltration and eliminated the prevalence of necrosis in LPS–induced hepatotoxicity [4]. Similarly, other researchs have reported that FM exhibits hepatopreventive property by reducing degenerative and necrotic changes [32]. The cleavage activity of GBE plays a role in supplying pharmacological preservation of cellular functions in pathological cases [38]. In an experimental hepatoxicity model, it was reported that GBE provided a significant improvement in the histopathological changes [36]. Besides, in another research, it was detected that GBE remarkably inhibited hepatocyte denaturation and necrosis due to toxicity [29]. Therefore, it can be said that FM and GBE improve the histopathological injury produced by LPS by regulating the inflammatory response and strengthening the antioxidant defense system.

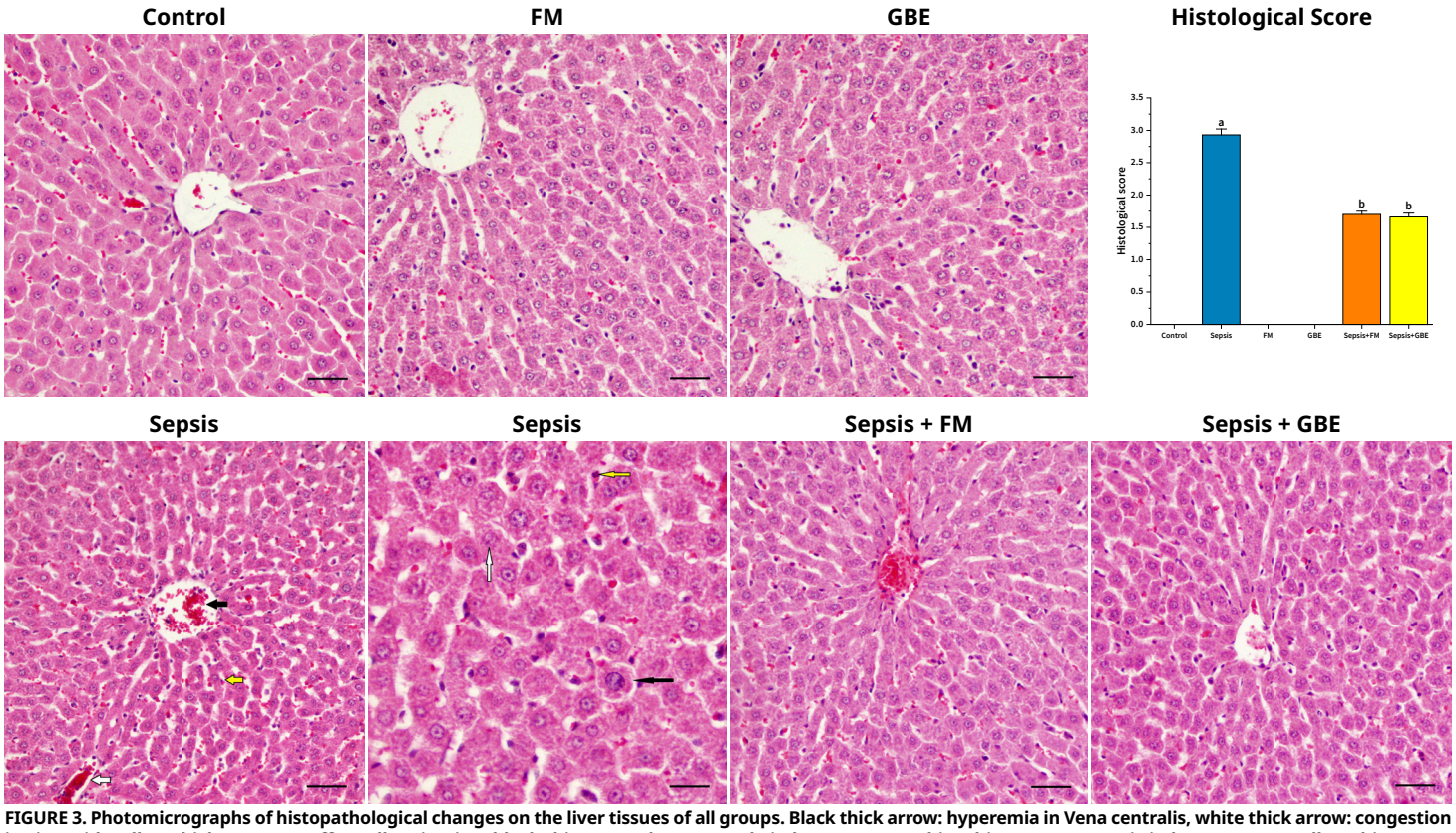

FIGURE 3. Photomicrographs of histopathological changes on the liver tissues of all groups. Black thick arrow: hyperemia in Vena centralis, white thick arrow: congestion in sinusoid, yellow thick arrow: Kupffer cell activation, black thin arrow: karyomegaly in hepatocytes, white thin arrow: necrosis in hepatocytes, yellow thin arrow: inflammatory cell infiltration. Scale bar=100 µm for the groups apart from high magnification (Scale bar=50 µm) of sepsis group. H&E

LPS–induced ROS increase aggravates acute liver injury, leading to apoptotic cell death [39]. Also, Killilea *et al.* [27] reported that the rise in LPS–induced liver proinflammatory mediators causes increased Casp3 activity and expression, apoptosis and aggravated hepatic damage. Another research announced that LPS application rised the protein expression level of the pro–apoptotic gene Casp3 and the number of TUNEL positive hepatocytes [37]. The Casp3 immunoreactivity and the immunohistochemical histoscore of all this study groups are offered in FIG. 4. Similar to the above data, in our study, it was determined that Casp3 immunoreactivity increased

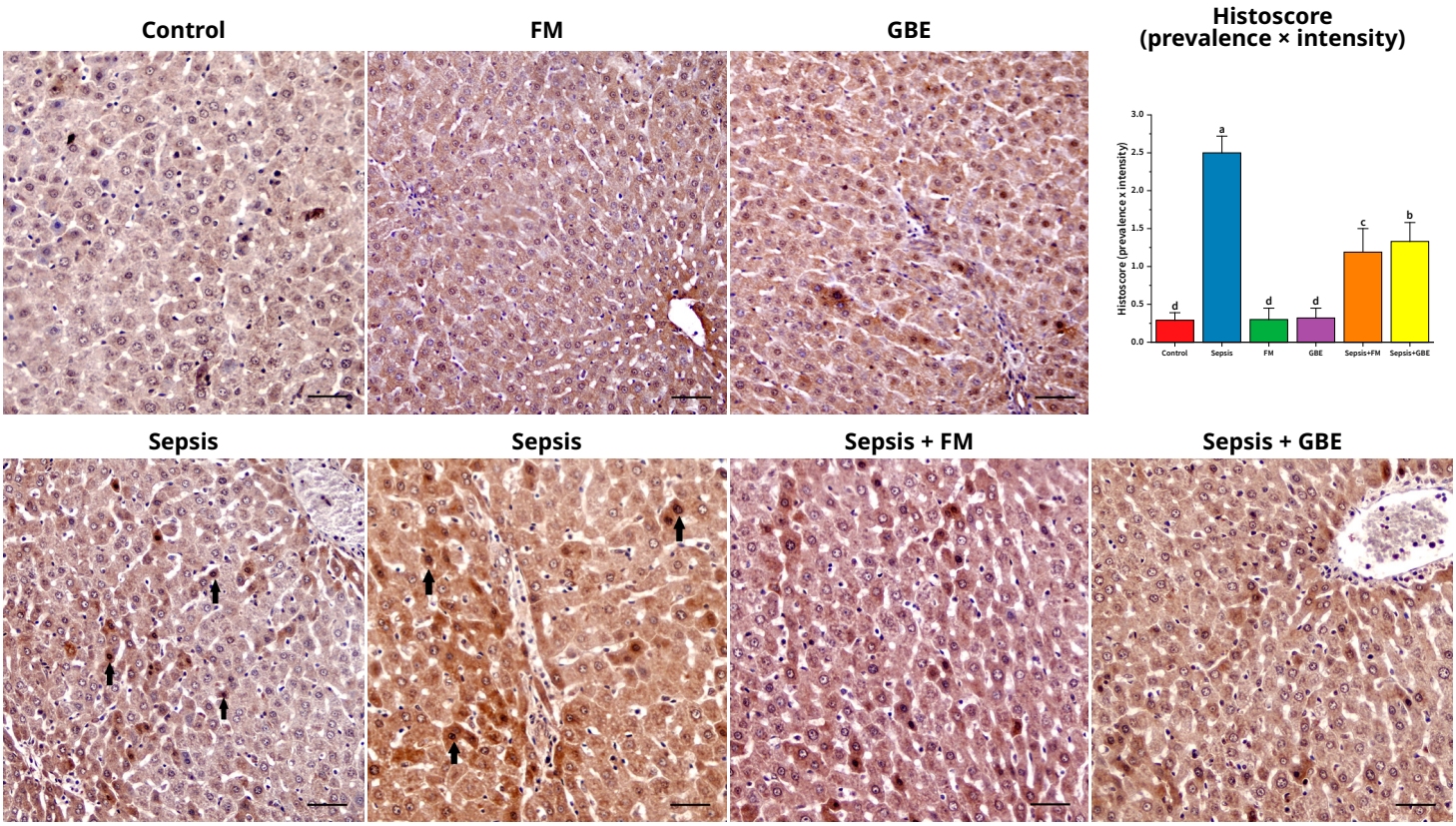

FIGURE 4. Casp-3 immunoreactivity evaluated on the liver tissues of all groups. Arrows: Casp-3 immunoreactive cells. Scale bar=100 µm for the groups apart from high magnification (Scale bar=50 µm) of sepsis group

in the sepsis group due to LPS-induced apoptotic activity compared to the control, FM, and GBE groups. This immunostaining intensity increased in the nucleus and cytoplasm of hepatocytes in the sepsis group than control. Morever, it was observed that FM and especially GBE significantly reduced this increased activity. Casp3 immunoreactivity was found reduced in the sepsis + FM and especially sepsis + GBE groups than sepsis. In this case, the statistical data of Casp3 immunoreactivity was determined to be highest in the sepsis (2.50 ± 0.22, $P<0.05$) compared to the sepsis + FM and sepsis + GBE groups (1.33 ± 0.25 and 1.19 ± 0.31, $P<0.05$, respectively). As expected, the lowest data was in control (0.29 ± 0.10). Avila *et al.* [4] stated that FM application reduced LPS-induced apoptotic cell increase. It has been reported that the increased number of apoptotic cells and staining intensity decreased in studies using FM against other hepatotoxic agents [32]. Furthermore, GBE has been reported to inhibit induced hepatocyte apoptosis [35, 40] and significantly reverse increased Casp3 immunoreactivity in liver toxicity [28]. Considering the anti-apoptotic abilities of FM and GBE, it can be said that they contribute to tissue regeneration by reducing Casp3 immunoreactivity that occurs in liver damage.

CONCLUSION

This study results suggest that FM, and especially GBE, has a ameliorative act on LPS-induced liver toxicity and dysfunction. These effects may be attributed to their capacity to reduce LPS-induced inflammatory responses, oxidative stress and apoptotic activity. The results suggest that GBE, specifically a natural product, may represent a new candidate for ameliorating liver damage. Further researches are required to completely comprehend the molecular mechanism of the potential act of this agent.

Conflict of Interests

The authors declare that there is no conflict of interest.

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