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From Cell Biology to Tissue Engineering

4-(3,4-dihydroxybenzoyloxymethyl)phenyl-O-β-D-glucopyranoside effect in liver regeneration

Tülin Firat[1], Gizem Söyler[2], Fatma Töre[3], Mustafa Şit[4], Aysu Kiyan[5], Ufuk Özgen[6] and Aysel Kükner[2]

[1]Department of Histology and Embryology, Faculty of Medicine, Abant Izzet Baysal University, Bolu, Turkey, [2]Department of Histology and Embryology, Faculty of Medicine, Near East University, Nicosia, Cyprus, [3]Department of Physiology, Faculty of Medicine, Biruni University, Istanbul, [4]Department of Surgery, Faculty of Medicine, Abant Izzet Baysal University, [5]Department of Public Health, Faculty of Medicine, Abant Izzet Baysal University, Bolu and [6]Department of Pharmacognosy, Faculty of Pharmacy, Karadeniz Technical University, Trabzon, Turkey

Summary. Following an injury or resection, the mammalian liver has the capacity to regain its former volume and functioning by restoring itself. Studies have demonstrated that antioxidants play a role in hepatic regeneration. This study investigated the effect of 4-(3,4-dihydroxybenzoyloxymethyl)phenyl-O-ß-D-glucopyranoside (PG) obtained from *Origanum micranthum* on liver regeneration. Sixty Wistar Albino rats were used. In the sham-operated group, a midline abdominal laparotomy was performed without hepatectomy. In the partial hepatectomy (PHx) group, the median and left lateral lobes were removed. Rats in the PHx group received 20 mg/kg/day PG intraperitoneally before being sacrificed at 24, 48, and 72 hrs, and 7 days later. Liver tissues were collected for immunohistochemical analysis and electron microscopic evaluation. We found an increase in mitotic index, and the numbers of Ki-67 stained hepatocytes in all PHx early stage groups (24 hr, 48hr, 72 hr), but not in 7-day groups. The regeneration mediators eNOS, iNOS, TNF-α and NF-κB were shown to increase in PHx groups. This increase was more prominent dependening on time. In the PHx treatment (PHx+PG) groups, while eNOS was still high, iNOS, TNF-α and NF-κB had decreased. The apoptotic index was markedly high in the PHx groups; this was prevented by PG treatment. These findings were supported by the ultrastructural results. Our findings indicate that PG supports liver regeneration, hepatocyte proliferation, reduced liver damage, and inflammatory mediators following PHx.

Key words: Partial hepatectomy, Liver regeneration, Origanum micranthum

Offprint requests to: Aysel Kükner, Department of Histology and Embryology, Faculty of Medicine, Near East University, Nicosia, Cyprus.
e-mail: akukner@hotmail.com

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Introduction

The only mammalian organ with the capacity to regain its former volume and functioning by restoring itself following an injury or resection is the liver (Qiu et al., 2012). Mitosis is rare under normal conditions, and hepatocyte regeneration is rather slow, whereas mitosis in hepatocytes increases after toxic or viral injury or after partial hepatectomy. Hepatocytes then proliferate, restoring both normal hepatic mass and functional capacity (Michalopoulus, 2007; Batusic et al., 2011; Stanger, 2015). Liver regeneration is a multifactorial event in which cytokines, growth factors, hormones, intracellular signaling pathways, and transcriptional factors each play a role. Three molecular pathways in liver regeneration are β-catenin, Hippo/YAP, 3 kinase/Akt/mammalian target of rapamycin signaling cascade. (Fausto et al., 2006; Gilgenkrantz and Collin de l'Hortet, 2018).

The most popular experimental procedure for liver regeneration is partial hepatectomy (PHx). The Higgins and Anderson method (Higgins and Anderson, 1931) is one of the experiemental approaches used, in which the left lateral and median lobes are resected; thus, 70% of

the liver (2/3) is removed. PHx surgery is advantageous, as no massive necrosis occurs in the remaining tissue, the surgical area remains clean, no acute inflammation is observed, and postoperative regeneration begins immediately. DNA synthesis (mitosis) reaches its highest level in rats at 24 hr and in mice at 36 hr. Regeneration is complete within 7-10 days in rodents and in 3-4 months in humans (Michalopoulus, 2010). The hour after PHx is defined as the "priming" phase. During this phase, >100 genes become activated in a few minutes after PHx (Gilgenkrantz and Collin de l'Hortet, 2018) The second phase is the activation of the growth factor receptor, and the termination phase including transforming growth factor-b and integrin signaling (Wang et al., 2015). The tumor necrosis factor-α (TNF- α) and interleukin-6 (IL-6) are secreted from Kupffer cells, trigger the G0/G1 phase, and cell proliferation is regulated within 2 hrs (Carnovale and Ronco, 2012). Inflammatory mediators such as TNF-α and Nuclear Factor-kappa B (NF-kappa B) appear during liver regeneration. In liver regeneration after PHx, NF-kappa B is believed to play a pivotal role depending on the cell-type (Wajant et al., 2003; Black et al., 2004). In Kupffer cells, activation of NF-kappa B is essential to trigger hepatocyte proliferation (Kirillova et al., 1999; Salazar-Montes et al., 2006; Cook et al., 2015), and the activation in hepatocytes is required to protect them against apoptosis during TNF-α stimulation. The NO has a crucial role in liver regeneration and NO depletion could impair IL-6 induction after partial hepatectomy (Yu et al., 2017). It has been reported that the level of iNOS has increased during liver regeneration and eNOS is a potent apoptotic regulator (Mei and Thevananther, 2011). A low concentration of Nitric oxide (NO) may be cytoprotective and necessary for liver regeneration, but a high concentration of NO may be toxic to hepatocytes (Wang and Lautt, 1998; Liu et al., 2000; Hsu et al., 2002).

Experimental studies have suggested that antioxidants play a role in liver regeneration (Gonzalez et al., 2012; Yormaz et al., 2012; Ekici et al., 2018).

Oregano is native to the Mediterranean, Europe and south and central Asia, and is cultivated elsewhere. The genus includes the important group of culinary herbs: marjoram (*Origanum majorana*) and oregano (*Origanum vulgare*). Oregano contains polyphenols, including numerous flavones. Over 60 different compounds have been identified, with the primary ones being carvacrol and thymol, ranging to over 80%, while lesser abundant compounds include p-cymene, γ-terpinene, caryophyllene, spathulenol, germacrene-D, β-fenchyl alcohol and δ-terpineol (Teixeira et al., 2013). The genus Origanum L. consists of 43 species and 18 hybrids most of which are distributed through the eastern Mediterranean region (Tepe et al., 2016). 21 taxa of them are endemic for Turkish flora. *Origanum* species are known as "Thyme, Marjoram" in Turkey. It is commonly used as a spice and also in the form of tea or oil. *O. micranthum* Vogel grows in Adana Province in Turkey (Ietswaart, 1982).

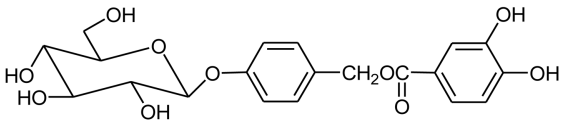
Origanum species are antifungal, antibacterial, antiinflammatory, antioxidant, antispasmodic, immunostimulant, antimutagenic, anticancer, insecticidal and nematicidal (Lin et al., 2008; Rodríguez-García et al., 2015; García-Beltrán and Esteban 2016; Leyva-López et al., 2017; Karaman et al., 2017; Semeniuc et al., 2017). *Origanum Vulgare* has shown protective effects on liver toxicity induced by various agents (Baser, 2002; Botsoglou et al., 2008; Sikander et al., 2013; Habibi et al., 2015).

The mechanism of the inflammation that occurs after regeneration remains unclear. 4-(3,4 Dihydroxy-benzoyloxymethyl)phenyl-O-ß-D-glucopyranoside (PG) are isolated from *Origanum micranthum* (Gogus et al., 2005). *Origanum micranthum* is supposed to have antioxidant and antiinflammatory effects. Nakatani and Kikuzaki (1987) isolated this substance for the first time and investigated its anti-oxidant activity with ferric thiocyanate (Nakatani and Kikuzaki, 1987). In this study, we aimed to evaluate the potential regenerative effects of PG in partially hepatectomized rats by analysing the mediators that impact liver regeneration by biochemical, histochemical and ultrastructural methods using light and transmission electron microscopy.

Materials and methods

Isolation of 4-(3,4-dihydroxybenzolyxymethyl)phenyl-O-ß-D-glucopyranoside (PG)

4-(3,4-Dihydroxybenzoyloxymethyl)phenyl-O-ß-D-glucopyranoside (PG) was isolated from the aerial part of *Origanum micranthum*, which is an endemic species of Turkey. The aerial parts of *O. micranthum* were collected from Kozan District (Adana province in Turkey). It was identified by Dr. Mehmet Koyuncu. A voucher specimen is deposited at the Ankara University Pharmacy Faculty Herbarium (AEF 25873). They were dried and then powdered. The powdered plant (410 g) was extracted by refluxing with methanol (3 L x 3). The methanolic extract was concentrated and dried under reduced pressure to give a residue (98 g). The methanolic extract was partitioned with chloroform and ethyl acetate. Several chromatographic studies were used to isolate PG from ethyl acetate fraction. The structure of the substance was eluciated using spectroscopic methods such as ^{1}H NMR, ^{13}C NMR, and ESI-MS. In addition, 1-tetradecanol, ursolic acid, oleanolic acid, rosmarinic acid, 3-(3,4-dihydroxyphenyl)-2-hydroxypropionic acid (Danshensu) were isolated in the same study.



(3,4-Dihydroxybenzoyloxymethyl)phenyl-O-β-D-glucopyranoside

Animals and experimental methods

All experimental protocols were performed according to the guidelines for the ethical treatment of experimental animals and were approved by the Animal Care and Use Local Ethics Committee of Abant Izzet Baysal University. Eight–week old female Sprague-Dawley rats (120-160g) were housed at a constant room temperature (22±2°C) under a 12-hr light/dark cycle. They were fed standard rat chow (210 kcal/100 g/day) and drank tap water ad libitum. All the surgical interventions were performed between 9:00 AM and 12:00 PM to minimize diurnal effects. Partial hepatectomy was performed on the rats according to the procedure originally described by Higgins and Anderson (1931). Anesthesia was induced by intramuscular injection of ketamine hydrochloride (50 mg/kg, Ketalar®; Parke Davis, Eczacibasi, Istanbul, Turkey) and xylazine (10 mg/kg, Rompun®; Bayer AG, Leverkusen, Germany), and all procedures were performed without mechanical ventilation. The animals were placed on the operating table in the supine position, immobilized at four points, and a midline abdominal laparotomy with exposure of the abdominal cavity, the median, and left lateral lobe were ligated and resected under aseptic conditions. 1 ml NaCl 0.9% was administered intraperitoneally, and the abdomen was closed. Rats were placed under heat-producing lamps to recover from anesthesia.

Animals were randomly divided into four groups.

1-Sham group (n=6): Composed of rats subjected to the laparotomy surgical procedure, without hepatectomy, a so-called sham operation.

2-PHx group (n=24): Composed of partially-hepatectomized rats. The median and left lateral lobes of livers were resected (Fig. 1). The subgroups of these rats were euthanized by exsanguination at 24, 48, and 72 hrs, and 7 days after the procedure, and tissue samples were obtained.

3-DMSO group (n=6): In order to minimise the bias, a group of rats received dimethysulfoxide (DMSO) at 0.1 % only, which is the solvent of the PG solution.

4-PHx + PG group (n=24): Composed of partially-hepatectomized rats that received an intraperitoneal injection of PG (20 mg/kg, daily). The subgroups of these rats were euthanized by exsanguination at 24, 48, and 72 hrs, and 7 days after the procedure, and tissue samples were obtained.

Prior to, and at end of the experiment, the body weights of all the test subjects were measured. At the end of each experimental period, livers were weighed as well. The liver weight was calculated as a percentage of body weight.

Immunohistochemistry

The liver tissue specimens were fixed in neutral formalin for 48 hrs, embedded in paraffin, and cut into 5 μm sections. Hematoxylin - eosin, Masson's trichrom and PAS staining were carried out to assess the general structure of the liver.

Immunohistochemical evaluation of the liver samples were performed by the following stains:

•TUNEL (Universal Apoptosis Detection Kit GenScript Corp. Lot: R50311201)
•Ki-67 (anti-Ki-67 Antibody Rabbit Polyclonal ab 15580)
•TNF-alpha (Anti-TNF alpha antibody ab183896, abcam)
•NF-kappa B p65 (Anti-NF-kappa B p65 antibody - ChIP Grade ab7970, abcam)
•iNOS (Anti-iNOS antibody ab15323, abcam)
•eNOS (Anti-eNOS antibody ab66127, abcam)

TUNEL-positive apoptotic cells and Ki-67 positive proliferating cells were counted in 20 areas in different high power fields. The mitotic index was calculated per 2000 hepatocytes after counting the number of mitosis in 20 randomly-selected areas . The cells stained positively with TNF-α, NF-kappa B, iNOS, eNOS were counted in a light microscope with a magnification of 40x in 20 different areas.

Transmission electron microscopy

The liver tissues were fixed in 2.5% glutaraldehyde in a 0.1 M sodium phosphate buffer (pH 7.2). Tissues were postfixed with 2% osmium tetraoxide in a sodium phosphate buffer. Dehydration was accomplished by a gradual ethanol series, and tissues were embedded in epoxy resin. Semi-thin sections were stained with toluidine blue. Ultrathin sections (100 nm) were stained with uranylacetate and lead citrate. Sections were then viewed and photo-graphed with a Zeiss 9EM.

Statistics

All the statistical analyses were performed with SPSS 17.0. Data was confirmed as non-parametric by the Kolmogorov-Smirnov test. Data obtained from the groups were compared using the non-parametric Kruskal-Wallis test within each group, whereas the groups were compared with each other using the Mann-Whitney U test and Bonferroni correction. The relevance of the treatment and time was analysed with a Two-Way Anova test.

Results

In all groups, there was no statistically significant difference between the DMSO and sham groups.

Liver weight

Liver weights in the 24- and 48-hr PHx groups decreased ($p=0.011$, $p=0.014$, respectively) when

compared to the sham group, then tended to increase over longer time periods. The weight in the 7-day treatment group was almost equal to sham group, and PG caused significant increase in liver weight when compared to the 7-day PHx group (p=0.043) (Fig. 2).

Mitotic index

The mitotic indices differed among the sham, PHx, and treatment groups. The most prominent mitotic index was observed in the 24 hrs after PHx and PHx+PG when compared to the sham group (p=0.018). These indices were gradually decreased by the time course. In addition, 7 days after PHx, in PG treatment was observed as higher mitotic index than that of the 7-day PHx group (p=0.020) (Fig. 3).

Ki-67

Ki-67 positive stained hepatocytes increased

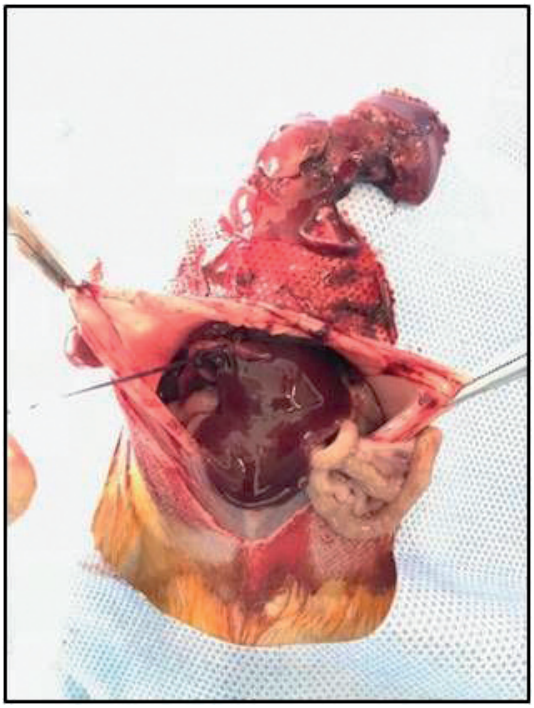


Fig. 1. Photo of PHx surgical procedure.

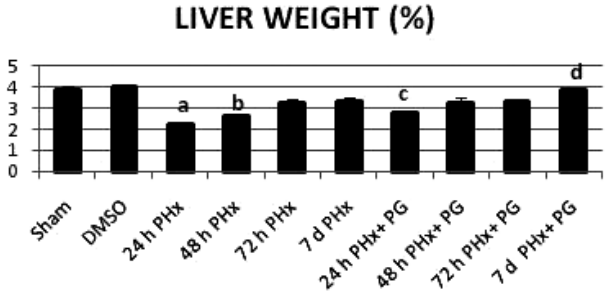


Fig. 2. The liver weight in percentage. a, b, c: Versus sham group p=0.011, p=0.014, p=0.021. d. Versus 7 d PHx group p=0.043.

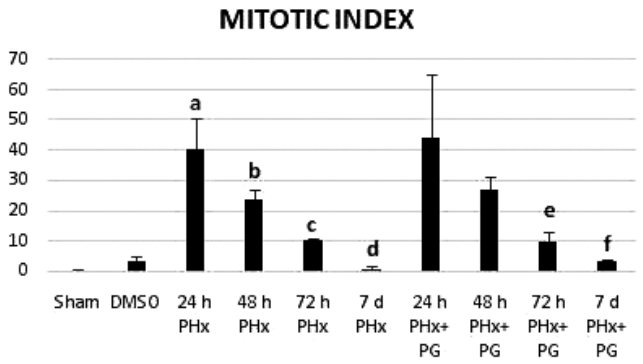


Fig. 3. Mitotic index of hepatocytes. a-d. Versus sham group p=0.018, p=0.018, p=0.017, p=0.032. e. Versus 72hr PHx group p=0.013. f. Versus 7d PHx group p=0.020.

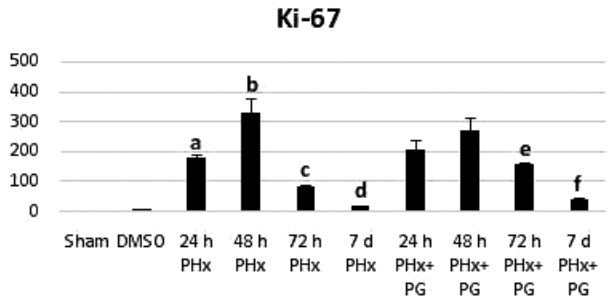


Fig. 4. Number of the Ki-67 positive hepatocytes. a-d- Versus sham group p=0.019. e. Versus 72hr PHx group p=0.021. f. Versus 7d PHx group p=0.020.

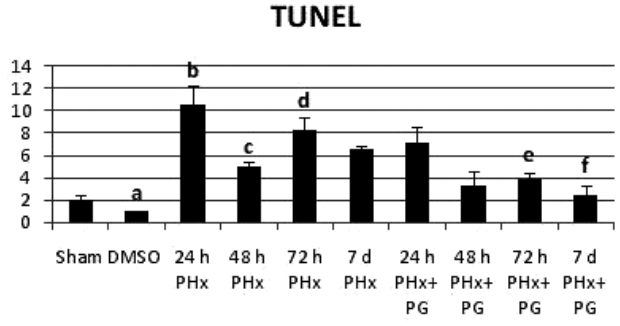


Fig. 5. Number of TUNEL (+) hepatocytes. a-d. Versus sham group p=0.020, p=0.020, p=0.019, p=0.019. e. Versus 72hr PHx group p=0.020. f. Versus 7d PHx group p=0.036.

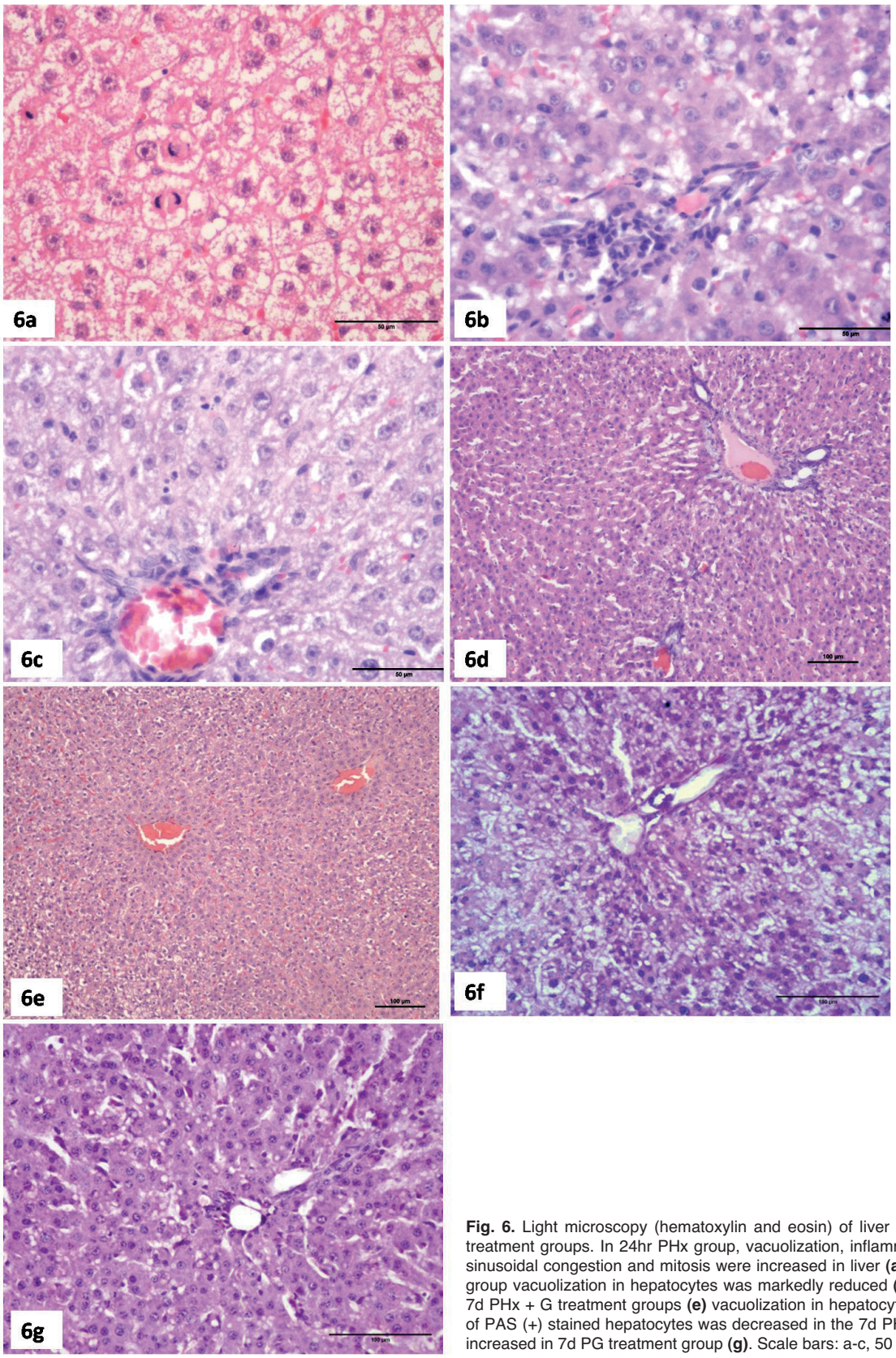


Fig. 6. Light microscopy (hematoxylin and eosin) of liver tissue PHx and PHx+ PG treatment groups. In 24hr PHx group, vacuolization, inflammatory cells in portal area, sinusoidal congestion and mitosis were increased in liver **(a, b)**, in 24hr PG treatment group vacuolization in hepatocytes was markedly reduced **(c)**. In the 7 d PHx **(d)** and 7d PHx + G treatment groups **(e)** vacuolization in hepatocytes were reduced. Number of PAS (+) stained hepatocytes was decreased in the 7d PHx group **(f)**, and markedly increased in 7d PG treatment group **(g)**. Scale bars: a-c, 50 μm; d-g, 100 μm.

significantly in the PHx and treatment groups as compared to the sham group (p=0.019). The greatest increase was observed at 48 hrs, and numbers then decreased gradually up to 72 hrs and 7 days (Fig. 4).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)

The numbers of TUNEL-positive stained hepatocytes significantly increased in the 24-, 48-, 72-hr, and 7-day PHx groups, compared to the numbers in the sham group (p=0.020, p=0.019, p=0.020, Pp=0.019, respectively). The numbers of apoptotic cells decreased in all PG treatment groups; nevertheless, in the 72-hr and 7-day treatment groups this decrease was striking (p=0.020, p=0.036, respectively) (Fig. 5).

Histopathological evaluation

Numerous vacuoles were observed in hepatocytes around the portal area in the 24-hr PHx group when compared to the sham group, whereas this vacuolization was decreased in the treatment groups (Fig. 6a-c). Decreased vacuolization was very prominent in the 7-day PHx (Fig. 6d) and PHx+PG groups (Fig. 6e).

Table 1. eNOS, iNOS, NF-κB and TNF-α expressions in experimental groups. Values are expressed as median ± standard deviation p<0.05.

Groups	eNOS (median ± SD)	iNOS (median ± SD)	NF-kB (median ± SD)	TNF-α (median ± SD)
Sham	0±0,3	0±0,3	0±0	0±0
DMSO	0±0,3	0±0,3	0±0	0±0
PHx 24hr	6,5±4,7[a]	23±7,2[a]	15±7[a]	44±19,6[a]
PHx 48hr	9±5,4[b]	15±4,9[b]	11,5±4,1[b]	17±3,3[b]
PHx 72hr	19±7,1[c]	19±5,1[c]	5,5±3,6[c]	13±5,8[c]
Phx 7d	15,5±7,4[d]	11±5[d]	7±2,7[d]	9,5±3,6[d]
Phx+PG 24hr	16,5±5,3[e]	13,5±4,5[e]	17,5±6,6[e]	15±4,2[e]
Phx+PG 48hr	14,5±4,4[f]	12±3,8[f]	11,5±6,4[f]	10±2,4[f]
Phx+PG 72hr	10,5±2,6[g]	10,5±2,1[g]	13±5,4[g]	7,5±2,3[g]
Phx+PG 7d	7±3,5[h]	8±2,4[h]	7,5±4,3[h]	6±1,4[h]

[a,b,c,d,e,f,g,h] compared with sham and DMSO groups; [a,e]: eNOS, iNOS and TNF-α expression difference between PHx 24 hr and PHx+PG 24 hr; [b,f]: eNOS, iNOS and TNF-α expression difference between PHx 48 hr and PHx+PG 48 hr; [c,g]: eNOS, iNOS and TNF-α expression difference between PHx 72 hr and PHx+PG 72 hr; [d,h]: eNOS, iNOS and TNF-α expression difference between PHx 7 d and PHx+PG 7 d; [g,h]: eNOS, iNOS, NF-κB and TNF-α expression difference between PHx+PG 72 hr and PHx+PG 7 d; [a,b,c,d]: NF-κB and TNF-α expression difference between PHx 24 hr and PHx 48 hr, PHx 72 h, PHx 7 d groups; [b,c,d]: eNOS, iNOS, NF-κB and TNF-α expression difference between PHx 48 hr and PHx 72 hr and PHx 7 d groups; [c,d]: TNF-α expression difference between PHx 72 hr and PHx 7 d; [e,f]: NF-κB and TNF-α expression difference between PHx+PG 24 hr and PHx+PG 48 hr; [e,g]: eNOS, NF-κB and TNF-α expression difference between PHx+PG 24 hr and PHx+PG 72 hr; [e,h]: eNOS, iNOS, NF-κB and TNF-α expression difference between PHx+PG 24 hr and PHx+PG 7 d; [f,g]: eNOS, NF-κB and TNF-α expression difference between PHx+PG 48 hr and PHx+PG 72 hr; [f,h]: eNOS, iNOS and TNF-α expression difference between PHx+PG 48 hr and PHx+PG 7 d.

Kupffer cell activation was detected in the 24- and 48-hr PHx groups. The numbers of PAS-positive cells around the portal area decreased, particularly in the 7-day PHx groups (Fig. 6f), whereas PAS staining was more visible in the 7-day treatment group (Fig. 6g).

Table 1 shows median numbers of immune-stained cells with TNF-α, NF-κB, eNOS and iNOS. The results of statistical evaluation are given in a detailed manner in Table 1.

TNF-α, NF-kappa B, eNOS and iNOS expressions were significantly increased on a time dependent gradual manner in the PHx groups, compared to the sham group (p<0,01). These parameters were decreased by PG treatment in all time periods (p<0,01).

TNF-α immune staining significantly increased in PHx groups in 24-hr, 48-hr, 72-hr and 7-day groups when compared to that of the sham group. NF-kappa B and iNOS stainings were similar to TNF- α expressions. NF-kappa B and iNOS immune staining significantly increased in 24 hr PHx groups. This increase was prevented by PG treatment, in the 24-, 48-, 72-hr, and 7-day groups. Interestingly, eNOS expression was increased significantly in 24-hr PHx+PG groups compared to the PHx group. It was still high in the PHx+PG 7d group (Fig. 7a,b).

In transmission electron microscopic evaluation, PHx 24-hr rats' hepatocytes had many lipid droplets and necrotic hepatocytes (Fig. 8a), PHx 48-hr rats' hepatocytes had large autophagic vacuoles (Fig. 8b). PHx 7-day rats' hepatocytes had an enlarged space of Disse (Fig. 8c). PG treament prevented the vacuolization and lipid accumulation in all time periods in 24-, 48-, 72-hr and 7-day PHx+PG groups (Fig. 8d). Ultrastructural findings showed similarity between sham and PHx+PG 7-day groups.

Our findings suggested that PG could promote liver regeneration, hepatocyte proliferation, reduced liver damage, and inflammation following experimental PHx.

Discussion

Although the physiopathology of liver regeneration is not fully known, what was known is that the mitotic activity begins in hepatocytes after liver injury, the number of cells increases, and regeneration begins. The resulting Phx liver damage triggers hypertrophy, hyperplasia, or reprogramming of a specific cell compartment, depending on the size of the remaining liver mass (Gilgenkrantz and Collin de l'Hortet, 2018). A 70% partial hepatectomy (PHx) is a commonly performed experimental model in rodents to assess liver regeneration and parenchymal cell repair mechanisms, and is well tolerated. Following resection, rat and mouse livers regenerate within 5-7 days and regain their former masses without requiring treatment. Hypertrophy of the remnant liver occured rapidly, followed by hyperplasia, with the entry into S phase of almost all hepatocytes (Miyaoka et al., 2012). Hepatocyte proliferation has been reported as concentrated on the periportal zone; no

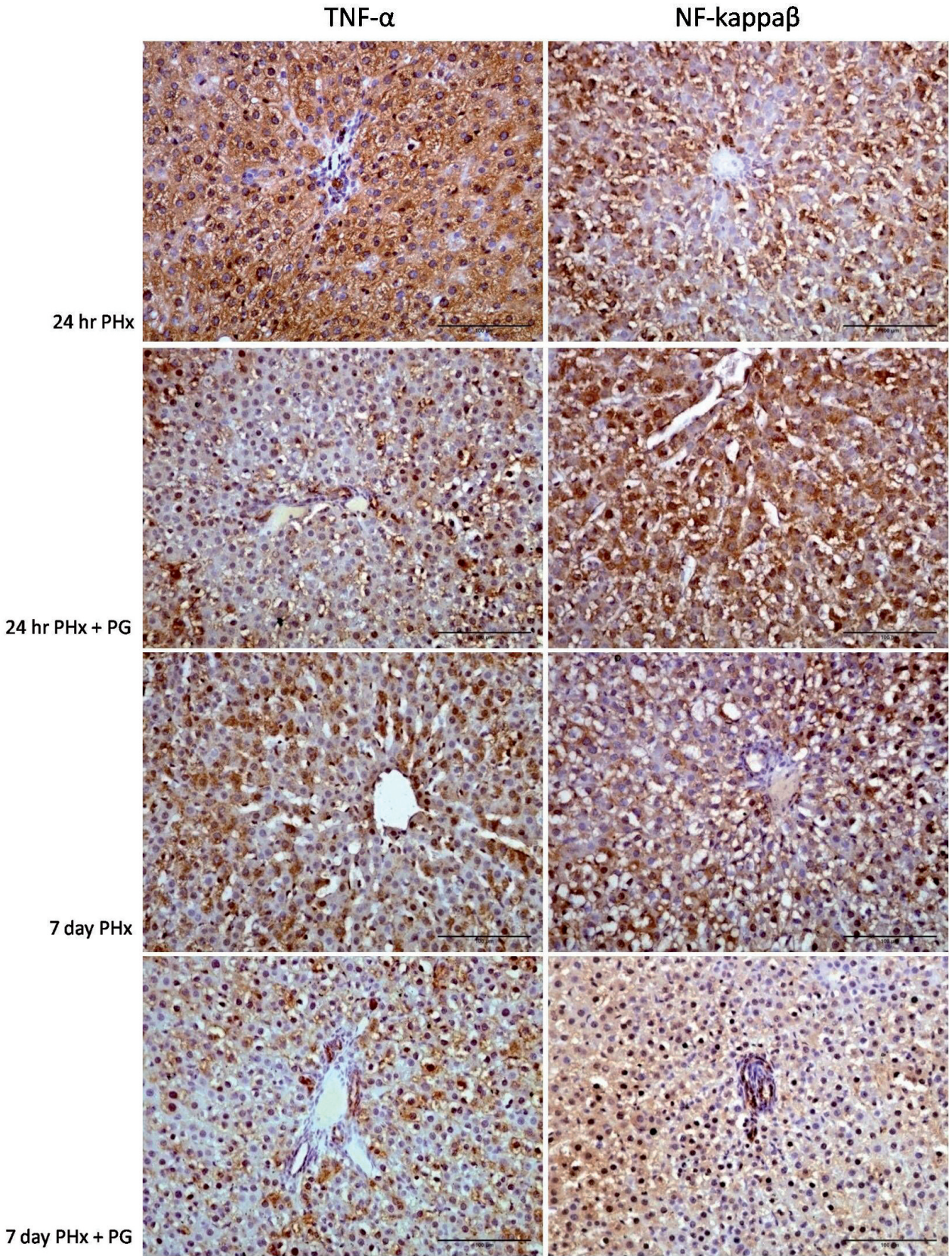


Fig. 7. a. Immunostained TNF-α and NF-kappa ß expressions. Scale bars: 100 μm.

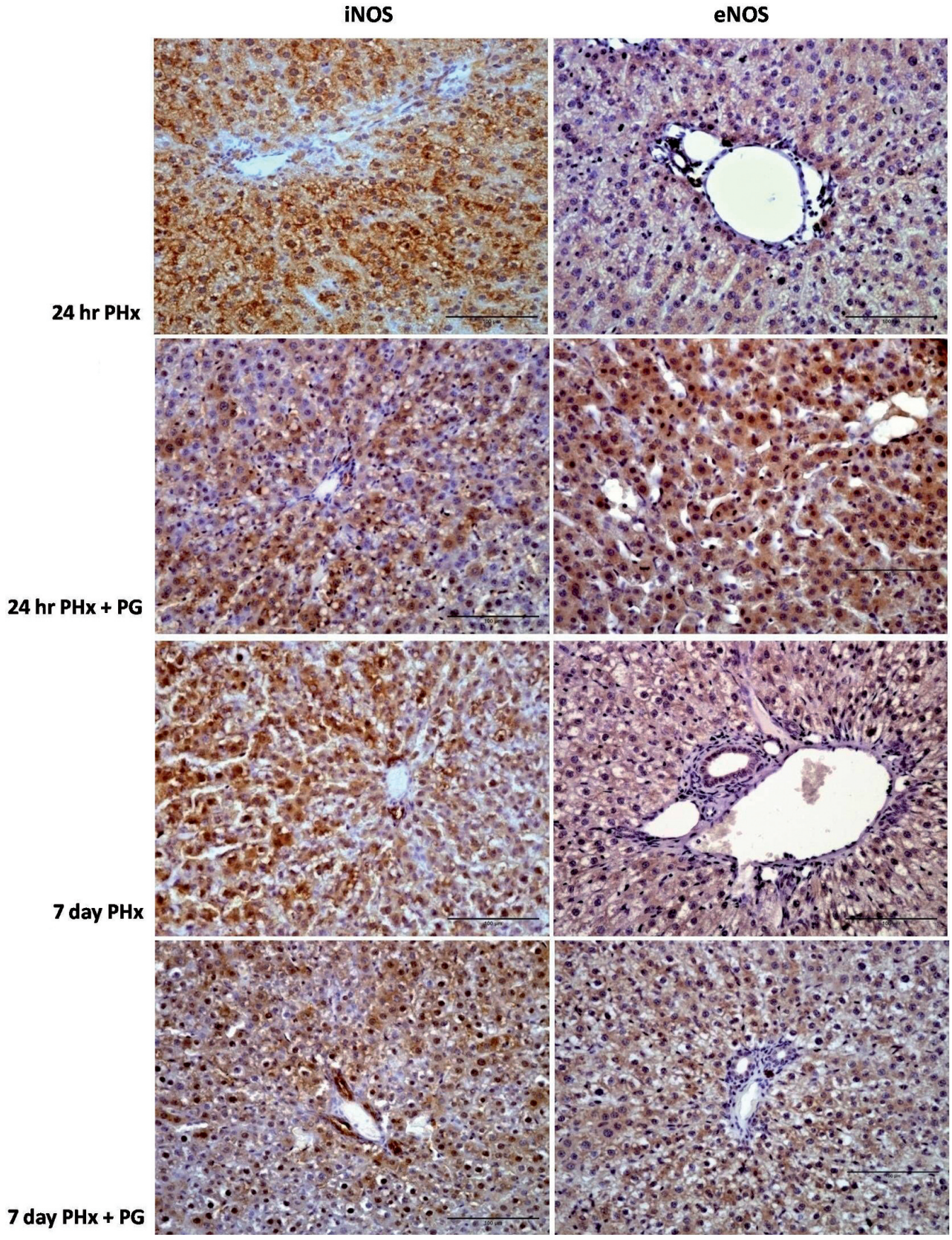


Fig. 7. b. Immunostained iNOS and eNOS expressions. Scale bars: 100 μm.

division occured in >40% of hepatocytes in the first 24 hrs, and the rate of division in hepatocytes after a 70% PHx was 0.7-fold the normal value, and the number of hepatocytes increased only 1.6-fold (Peng et al., 2014). In another study, 60% of hepatocytes underwent mitosis after PHx in the first 24 hrs, and this percentage was 95% at 48 hr (Michalopoulos, 2007). Miyaoka and Miyajima (2013) reported that hepatocyte diameters after PHx increased 1,5-fold within a few hrs after PHx. Liver weight did not change 1-2 days after PHx, but hypertrophy occurred and was followed by proliferation and increased diploid hepatocyte (Miyaoka and miyajima, 2013). Kuramitsu et al. (2011) reported that the hepatocyte size increased significantly 24 and 48 hrs after mice underwent PHx when compared with a control group (Kuramitsu et al., 2011). According to Gentric hepatocyte polyploidization occurred during normal and pathological liver growth (Gentric et al., 2012; Gentric and Desdouets, 2014). Replicated studies suggested that the numbers of Ki-67 stained cells were greatest at 1-3 days after PHx, diminished after day 5, and the mitotic index increased (Nakatani and Kikuzaki, 1987; Gentric et al., 2012; Andersen et al., 2013a). Ki-67 staining was presented as at its maximum in hepatocytes and biliary duct epithelial cells 48 hrs after PHx (Gentric et al., 2012; Lin et al., 2013; Karaca et al., 2014; Wolf et al., 2014). Ki-67 staining began to increase in rats within the first 8 hrs after PHx, peaked at 24 hrs, followed by a second peak at 72 hrs (Batusic et al., 2011).

The effects of PG on hepatocyte proliferation after PHx were investigated in this study. In our study, proliferation of hepatocytes was detected in the experimental groups; however, no increase in the numbers of double nucleus hepatocytes was observed.

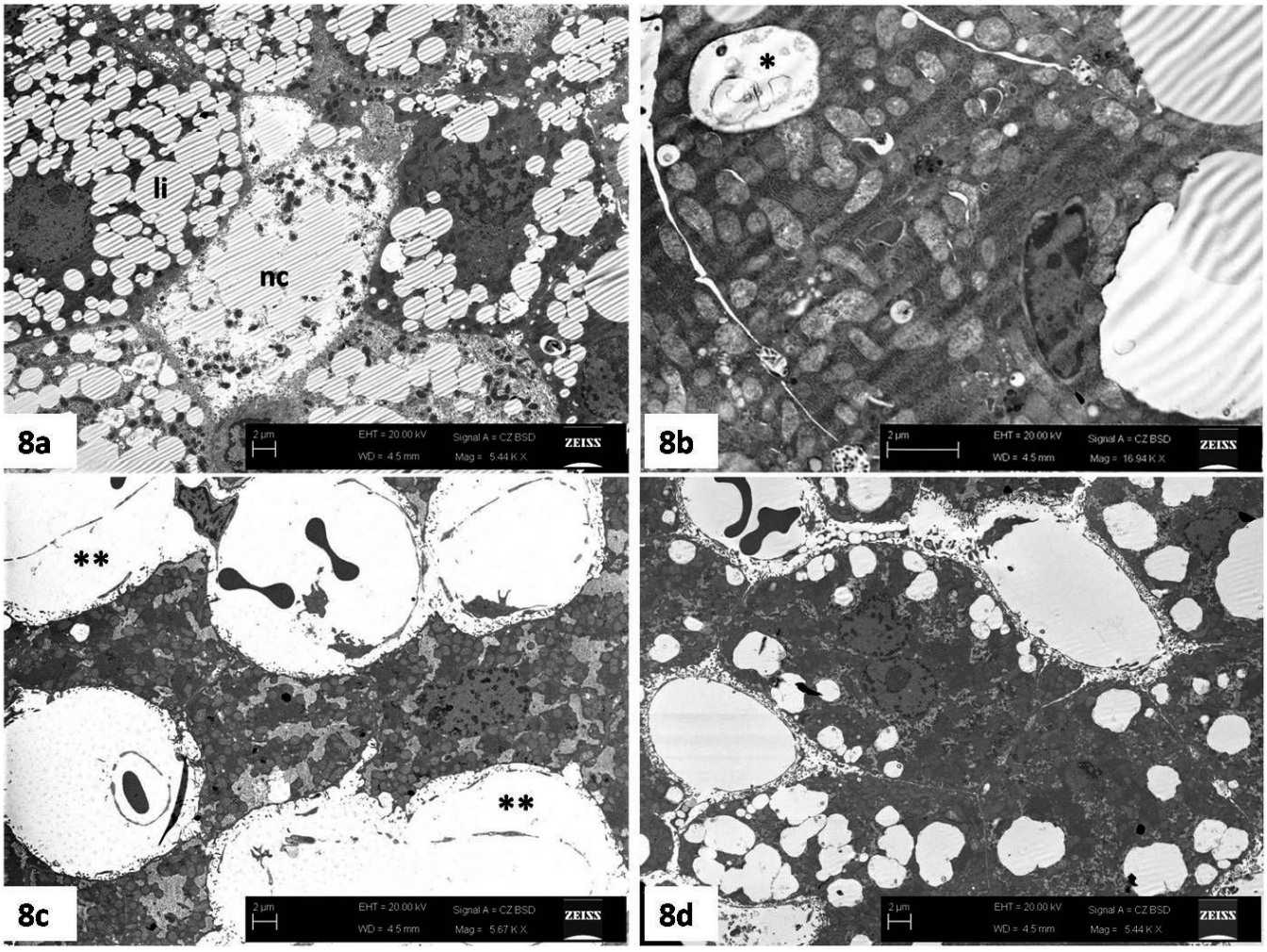


Fig. 8. Transmission electron microscopic images. In the 24-hr PHx group, many lipid droplets (li) and necrotic hepatocytes (nh) (**a**). A big autophagic vacuole (*) in hepatocytes in the 48-hr PHx group (**b**. Widened Disse space (**) in the 7day PHx group (**c**). PG treated group (7 day) showed decreased vacuolization and lipid accumulation (**d**). Scale bars: 2 μm.

Liver weight decreased within the first 24 hrs in the PHx and treatment groups. An increase in liver weight was observed in the 72-hr PHx group, but this increase started at 48 hrs in the treatment groups. The increase in liver weight was greater in the 7-day treatment group than in the PHx group on the same day and was similar to that in the sham group. The mitotic indices differed among the sham, PHx, and treatment groups. The mitotic index peaked after PHx in the 24-hr groups. The number of Ki-67 stained hepatocytes peaked at 48 hrs and decreased at 72 hrs and 7 days in the PHx and treatment groups. The numbers of Ki-67 stained hepatocytes were greater in the 72-hr and 7-day treatment groups than in the PHx groups on the same day. Greater numbers of Ki-67 stained epithelial cells were observed in the biliary ducts of the treatment groups.

Swollen hepatocytes and fatty changes were seen in the periportal area during the first hr after PHx, whereas sinusoidal hemorrhage and a few focal necrotic hepatocytes were observed 3 hrs later (Andersen et al., 2013b; Kawaguchi et al., 2013). Congestion in the sinusoids became prominent 6 hrs after PHx, and hepatocyte lipid accumulation continued until day 10, and then decreased gradually (Iguchi et al., 2014). Lipids delivered from the periphery or synthesized in response to partial hepatic resection or other liver injury might serve in the membrane synthesis required for hepatocellular proliferation. Liver tissue loss caused apoptosis in the remaining hepatocytes, which is one of the most important causes of liver failure (Rudnick and Davidson, 2012). Activation and an increase in pro-apoptotic mechanisms affect regeneration in the early period after PHx (Kawaguchi et al., 2013). Oxidative stress influences cell proliferation. Proliferation increases under low stress, whereas it is inhibited under high stress, accompanied by elevated apoptosis and necrosis. An anti- apoptotic effect occurs in normal hepatocytes during liver regeneration after PHx (Xiang et al., 2012). TUNEL-positive cells are identified in the first hr after PHx, multiply at 6 hrs, and then begin to decrease at 24 hrs (Xu et al., 2012). However, in our study, TUNEL-stained hepatocytes were observed primarily 24 hrs after PHx, and the numbers decreased gradually thereafter. Morever, the increase in the number of TUNEL stained hepatocytes at 24 hrs was greater in the treatment groups than in the PHx groups. More prominent decreases were detected at 48 and 72 hrs. The number of stained cells in the 7-day treatment group was similar to that in the sham group. A significant difference was observed in the numbers of TUNEL stained hepatocytes between the 72-hr and 7-day groups within the PHx and treatment groups.

Inflammation played a critical role in liver regeneration, depending on many cytokines production. TNF-α and IL-6 were key molecules involved in regeneration of the liver: mRNA of that interleukins had started to increase from day 1 and day 2, and they reached maximum expression level at day 4 (Gupta and Venugopal, 2018). TNF-α secretion was increased significantly after PHx (Lin and Hua, 2017). Inflammatory cytokines such as IL-6 and TNF-α also activated various cell death signaling pathways leading to apoptosis and/or necrosis. TNF-α secretion from Kuppfer cells increased in liver regeneration after partial hepatectomy (Saito et al., 2014). It has been observed that TNF-α increases 24 hrs after hepatectomy, which is in parallel with our results (Canberk et al., 2017). TLR4/NF-kappa B played an important role in liver regeneration (Maciej et al., 2006; Lv et al., 2018). Nuclear factor kappa B (NF-kappa B), directly antagonized the pro-apoptotic effects of TNF-α and prevented cell death (Ceyhan and Canbek, 2017). TNF-mediated NF-kappa B activation was known to be initiated within 30 minutes after PHx, and maintained up to 4-5 hrs. (Black et al., 2004). Phenolic compounds from oregano, such as flavonoids and phenolic acids, might exert anti-inflammatory properties (Leyva-López et al., 2016).

Regulation of NO signaling in liver regeneration is still under study. NO has a function to regulate lipid metabolism. NO depletion could impair IL-6 induction after partial hepatectomy. NO depletion in PHx mice affected fatty acid β-oxidation in hepatocyte, and excessive lipid accumulation in hepatocyte might impair its proliferation (Hamano et al., 2014; Yu et al., 2017). NO is synthesized by inducible nitric oxide synthase (iNOS) or endothelial nitric oxide synthase (eNOS), which is excreted from hepatocytes, Kupffer and endothelial cells, and participates in inflammation and regeneration. In the liver, small amounts of NO generated by eNOS was believed to mediate liver regeneration, while large amounts of NO generated by iNOS was implicated in liver fibrosis (Iwakiri, 2015). NO protects hepatocytes from apoptosis after PHx. Increased expression of iNOS has been reported to occur in inflammatory processes caused by cytokines such as TNF-α, endotoxins, or bacterial lipopolysaccharides. Neither TNF-α nor IL-6 alone are sufficient to activate iNOS transcription, but when these two cytokines are combined, upregulation occurs. In the liver, eNOS activity was detected in the plasma membrane of rat hepatocytes (Yagmurdur et al., 2017). Diabetes mellitus triggered the activation of NF-kappa B. NF-kappa B activation was responsible for an increase in iNOS expression (DiNaso et al., 2011). Our results demonstrated that NF-kappa B expression was increased in groups with increased TNF-α levels, while NF-kappa B decreased with decrease in TNF-α expression. It was also found that there was an increase in iNOS in the PHx groups with an increase in TNF-α, and a decrease in TNF-α and iNOS in the treatment groups. It was observed that the TNF-α, NF-kappa B, iNOS peaks were detected at PHx 24-hr groups, while the eNOS peak on PHx 72-hr groups. Decreased levels of TNF-α, NF-kappa B, iNOS and eNOS were observed in the 7-day PG group.

PG was shown to reduce inflammation in liver

regeneration after PHx. It was identified that the Disse area was enlarged in electron microscopy sections of the liver tissues of rats with PHx (Qiu et al., 2012). It has been reported that pores in sinusoidal endothelial cells enlarged post-PHx and local environmental stimuli were a dynamic regulator of pores (Wack et al., 2001). Autophagy plays an active role to protect cells from liver physiology and pathogenesis (Gual et al., 2017). The autophagic peak is 6-72 hrs after partial hepatectomy (Lin et al., 2015). In this study, autophagic vacuoles were observed, and particularly in the 48-hr PHx group and in the 7-day PHx group, enlargement was observed in the space of Disse. In the treatment groups, dilatations decreased in space of Disse, whereas openings between endothelial cells were not different between PHx and treatment groups.

O. vulgare is an antithrombotic and antibacterial herb that exerts antihyperglycemic and antioxidant effects (Morshedloo et al., 2018). In the toxic damage of liver tissue, origanum onites have been reported to have antioxidant effects and to increase SOD and GSH levelsn (Cetin et al., 2011). O. vulgare has a protective role in hepatocytes and keratinocytes by inhibiting lipid peroxidation (Liang et al., 2012).

In conclusion, our immunocytochemistry analyses, structural and ultrastructural findings suggest that the original PG substance isolated from Origanum Micranthum has a promoting impact on liver regeneration following partial hepatectomy, reducing structural abnormalities and inflammation. The molecular mechanisms underlying this promoted liver regeneration are further research topics that could potentially help to reveal new therapeutical approaches that involve liver regeneration.

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