Effects of hypertension and ovariectomy on rat hepatocytes. Are amlodipine and lacidipine protective? (A stereological and histological study)

Hipertansiyon ve overektominin sıçan hepatositlerine etkisi. Amlodipin ve lasidipin sıçan hepatositlerini korur mu? (Histolojik ve stereolojik çalışma)

Hakan DURSUN[1], Fatih ALBAYRAK[1], Abdullah UYANIK[2], Nuri Osman KELEŞ[3], Polat BEYZAGÜL[4], Ednan BAYRAM[5], Zekai HALICI[4], Zuhal Berrin ALTUNKAYNAK[3], Halis SÜLEYMAN[4], Nihat OKÇU[1], Bünyamin ÜNAL[3]

Departments of, [1]Gastroenterology, [2]Nephrology, [3]Histology and Embryology, [4]Pharmacology, Atatürk University, School of Medicine, Erzurum
Department of [5]Cardiology, Government Hospital, Erzurum

Background/aims: *Calcium channel blockers are increasingly used for the treatment of hypertension. Menopause and hypertension are both important risk factors for liver damage and several other circulatory abnormalities. The aim of this study was to determine the effects of amlodipine and lacidipine in an ovariectomy-induced postmenopausal period model and a deoxycorticosterone acetate-salt-induced hypertensive model in rats.* ***Methods:*** *In this study, animals were divided into six groups as follows: control (Group 1), hypertension (Group 2), ovariectomy (Group 3), ovariectomy and hypertension (Group 4), ovariectomy, hypertension and amlodipine-treated (Group 5), and ovariectomy, hypertension and lacidipine-treated (Group 6). At the end of the experiment, the livers were removed and tissue samples were histologically and stereologically examined.* ***Results:*** *The numerical densities of the hepatocytes according to group were 0.000422, 0.00329, 0.000272, 0.00259, 0.00374 and 0.000346 μm³, respectively. Significant differences were found between values of all groups (p<0.01, Mann-Whitney U test). According to histopathological investigation, Group 3 and particularly Group 4 showed some microscopic abnormalities such as dilatation in sinusoids - central veins and branches of portal vein, irregularities of the hepatocyte columns, significant mononuclear cell infiltrations, and unstained vacuoles in the cytoplasm of the hepatocytes. Histological structure was protected from the destructive effects of ovariectomy and hypertension in Groups 5 and 6.* ***Conclusions:*** *Our experimental results show that both hypertension and the postmenopausal period have negative effects on the number of hepatocytes and histological structure of the liver. Both amlodipine and lacidipine appear to ameliorate the hypertension- and/or postmenopausal period-related decrease in hepatocyte number. We thus suggest that lacidipine and particularly amlodipine have important protective and recovering effects on the liver.*

Key words: Hypertension, ovariectomy, liver, stereology, amlodipine, lacidipine

Amaç: *Kalsiyum kanal blokerleri, hipertansiyon (HT) tedavisinde sıkça kullanılmaktadır. Menapoz ve hipertansiyonun her ikiside karaciğer hasarı ve çeşitli dolaşım bozuklukları için risk faktörleridir. Çalışmamızın amacı overektominin indüklediği postmenapozal dönem ve/veya deoksikortikosteron asetat-tuz modeli ile oluşturulan hipertansiyona amlodipin ve lasidipinin etkilerini belirlemektir.* ***Yöntem:*** *Çalışmamızda hayvanlar kontrol grubu (Grup 1), hipertansiyon (Grup 2), overektomi (Grup 3), overektomi ve hipertansiyon (Grup 4), overektomi, hipertansiyon ve amlodipin verilen grup (Grup 5) ve overektomi, hipertansiyon ve Lasidipin verilen grup (Grup 6) olmak üzere 6 gruba bölündü. Deneyin sonunda karaciğerler ayrılarak histolojik ve stereolojik çalışmaya alındı.* ***Bulgular:*** *Grupların hepatosit sayısal yoğunluğu sırasıyla 0.000422, 0.00329, 0.000272, 0.00259, 0.00374 ve 0.000346 μm³ olarak tespit edildi. Mann Whitney U testi ile peşpeşe gelen her grup arasında belirgin fark tespit edildi (p< 0.01). Grup 3 ve özellikle grup 4'ün histopatolojik incelenmesinde sinusoidler-santral venlerde genişleme ve portal venle köprüleşme, hepatosit kolonlarında düzensizlik, hepatosit stoplazmalarında boyanmamış vakuoller ve belirgin momonükleer hücre infiltrasyonu gibi bazı mikroskopik anormallikler izlendi. Grup 5 ve 6'nın histolojik yapıları, overektomi ve hipertansiyonun tahrip edici etkilerinden korunmuştu.* ***Sonuç:*** *Deneysel çalışmamız hipertansiyon ve postmenapozal dönemin hepatosit sayısı ve karaciğerin histolojik yapısını olumsuz yönde etkilediğini göstermiştir. Gerek lasidipin gereksede amlodipin postmenapozal dönem veya hipertansiyon ile ilişkili olarak azalan hepatosit sayısını iyileştirebilir. Bu nedenle, lasidipin ve özellikle amlodipinin karaciğeri koruyucu etkisinin olduğunu düşünmekteyiz.*

Anahtar kelimeler: Hipertansiyon, overektomi, karaciğer, stereoloji, amlodipin, lasidipin

**The abstract of this study was presented at the 25th National Gastroenterology Congress, 12-16 November 2008, Adana, Turkey, and published in the Turkish Journal of Gastroenterology Supplement data (poster no:19).*

Address for correspondence: Fatih ALBAYRAK
Department of Internal Medicine, Division of Gastroenterology
Ataturk University, Medical School
TR 25240 Erzurum, Turkey
Phone: + 90 442 231 72 10
E-mail: fatihalbayrakerz@gmail.com

Manuscript received: 04.09.2009 **Accepted:** 04.02.2010

doi: 10.4318/tjg.2010.0125

INTRODUCTION

Calcium channel blockers (CCB) are commonly used in clinical medicine, and more is being learned about their new effects and indications every day. Although they were first used as vasodilator agents, further studies have proven their usefulness in the treatment of a number of disorders, including arrhythmia, angina pectoris, left ventricular diastolic dysfunction, myocardial infarction, Raynaud syndrome, migraine, esophageal spasm, mesenteric ischemia, and even subarachnoid bleeding (1-4). Recent studies have verified that these drugs can be used experimentally for several new indications. Two of the most commonly prescribed CCBs, amlodipine and lacidipine, are blockers of L-type calcium channels. Both have been proven effective in treating a number of different diseases, in addition to having antihypertensive effects.

Hypertension is recognized as a risk factor for several cardiovascular diseases, including atherosclerosis and myocardial infarct (5). Both of these conditions involve production of reactive oxygen species (ROS) by vascular cells, as they contribute to decreased nitric oxide (NO) availability, promote vascular smooth muscle proliferation, and induce neutrophil infiltration (5, 6). Alterations in antioxidant enzymes have been implicated in the pathogenesis of vascular diseases such as hypertension, but the effects of CCBs at this level are not yet known.

In previous studies, amlodipine and lacidipine were shown to exert anti-osteoporosis, anti-hyaluronidase, and anti-inflammatory effects (7-9), as well as anti-atherogenic effects (10, 11). Amlodipine and lacidipine have many other properties that provide beneficial effects in different indications, including antioxidant and NO modulation (12, 13). In addition, they improve many inflammatory parameters mediated through chronic defense mechanisms at the cellular level (14, 15). Aging, an inevitable situation for all living organisms, is also associated with inflammatory processes. The aging process causes many problems, such as deregulation of normal bodily functions, which leads to many chronic disorders, such as damage to the vascular endothelium or atherosclerosis, and will often produce hypertension (16, 17). Old age combined with hypertension may increase the risk of hypertension-related complications (18).

Because the liver has a rich vascular supply, it is subject to vascular disorders such as hypertension. Although healthy liver tissue has a great ability to regenerate itself after hepatotoxic events, vascular disorders caused by aging such as hypertension may no longer be easy to manage. The literature about the effects of hypertension on the liver is still inadequate because research has been more heavily focused on the effects of hypertension on the kidney and the heart. Damage to these organs incurs a high mortality risk.

In an experimental study, hypertension was shown to decrease the number of nephrons, while amlodipine had a protective effect on the number of nephrons (19). In light of these data, the present study aimed to determine the effects of hypertension and the postmenopausal period on the liver, both together and separately, using stereological methods. The study also compared the effects of amlodipine and lacidipine on liver tissue, by exploring the effects of these drugs on both hypertensive and postmenopausal rat livers.

MATERIALS AND METHODS

Animals

Thirty-six, eight-week-old female albino rats were obtained from the Animal Care Center at Ataturk University. The animals were housed in plastic cages at 21°C, with humidity at 50%, with a 12-hour light-dark cycle. The rats were fed a standard commercial rat diet. The animals used in this study were cared for in accordance with institutional guidelines.

Chemicals

In this experiment, deoxycorticosterone acetate (DOCA) was purchased from MP Biomedicals LLC (101497), amlodipine [Norvasc 5 mg tablet (tb)] was purchased from Pfizer (Istanbul, Turkey), lacidipine (Lacipil 4 mg tb) was purchased from GlaxoSmithKline; Beecham (Istanbul, Turkey), and thiopental sodium (Pentothal sodium, 1 g) was purchased from Abbott (Istanbul, Turkey).

Ovariectomy Surgery

During the acclimatization period, the rats were fed a standard commercial rat diet. One week before starting the experiment, four groups of animals, or 24 rats in total, underwent bilateral ovariectomy (Groups 3-6). For this procedure, the animals were anesthetized with 25 mg/kg thiopental sodium, injected intraperitoneally. A longitudinal incision (0.5-1 cm) was made in the midline area of the lower abdomen and the ovaries were removed.

Hypertension Modeling

Hypertension was induced using the DOCA-salt treatment. Groups 2, 4, 5 and 6 received biweekly (subcutaneous, s.c.) injections of 30 mg/kg DOCA dissolved in vehicle (soybean oil, 0.25 ml per animal) for six weeks, plus drinking water containing 1.0% NaCl and 0.03% KCl.

Experimental Design

Group 1 rats served as controls. After ovariectomy and hypertension-generating processes, the groups including ovariectomized and/or hypertensive rats (Groups 2-6) were divided as follows:

Group 2: only DOCA treatment (hypertension model); Group 3: only ovariectomy (postmenopause model); Group 4: DOCA treatment + ovariectomy; Group 5: DOCA treatment + ovariectomy + oral administration of amlodipine at a dose of 3 mg/kg (dissolved in 1 ml of distilled water) for six weeks; Group 6: DOCA treatment + ovariectomy + oral administration of lacidipine at a dose of 4 mg/kg (dissolved in 1 ml of distilled water) for six weeks. Additionally, the rats in the Groups 1, 2, 3 and 4 were orally administrated 1 ml of distilled water for six weeks.

Histological Procedures

Light Microscopy

Each liver was post-fixed in a 10% formalin solution for 48-55 hours (h), dehydrated in a graded alcohol series, embedded in paraffin wax, and serially sectioned using a Leica RM2125RT microtome. Serial sections at 40 µm thickness were mounted onto glass slides and used for stereological analyses. For histopathological investigation, light microscopic sections of 5 µm thickness were obtained from the same blocks. For both estimating the number of hepatocytes and histopathological analysis, all slides were stained with hematoxylin-eosin (H-E). Histopathological analysis was performed under light microscope (Olympus BX 51; Tokyo, Japan) with camera attachment.

Stereological Method

For stereological application, livers were serially cut in sections of 40 µm thickness. All sections obtained from each block were uniform and randomly sampled for our study. The hepatocytes in the stained sections were counted using optic dissector counting method at a stereology workstation for stereological analyses as described previously (20,21). For this stereological analysis, an image analysis system was used. The system consisted of a color camera (Optronics MicroFire, Optronics; USA), personal computer and computer-controlled motorized specimen stage (BioPrecision MAC 5000 controller system, Biovis; USA), and a light microscope (Leica DM4000 B, Gaitenbein; Turkey). Hepatocytes were counted under a 20x Leica Plan Apo objective (NA = 1.40). Total magnification was M, which allowed for accurate recognition. Each hepatocyte was counted by software of the image analysis system (Stereo Investigator 7.0, MicroBrightField; USA) according to the unbiased counting rules of the optical dissector (22). All details of the microscopy stages for samples belonging to our study are summarized in Table 1.

Microscopy Stages

According to the pilot work, for microscopic sampling, suitable step size (X x Y) was found as 3.240.000 μm^2 (in X, 1.80 mm; in Y, 1.80 mm) in our study. In all the steps of unbiased counting, the size of the used frame was 4900.00 μm^2 (0.070 mm X 0.070 mm). Thus, for this analysis, the area of sampling fraction (frame size/step size) was 4900.00 / 3.240.000.

Statistical Analysis

To evaluate the significance of the observed differences, the LSD test (least significant difference)

Table 1. A sampling strategy used for stereological analysis for a healthy (control) group

Healthy (control) group **Group 1 – Animal 1**	
Number of sampling sites for microscopic stages	29
Counting frame area (XY) (μm^2)	4900 (70 µmX70 µm)
Dissector height (Z) (µm)	14
Dissector volume (XYZ) (μm^3)	88200 (70 µmX70 µmX18 µm)
Sampling grid area (XY) (μm^2)	3240000 (1800 µmX1800 µm)
Section thickness (µm)	30
Section periodicity	1
The number of dissector particles	734
Hepatocyte numerical density formulas	The number of dissector particles/counting frame area x dissector height x number of sampling sites
Hepatocyte numerical density value	741/4900 μm^2 X 14 µm X 29 = 0.0003725 N/μm^3

was used. The significance level (α) was 0.05 in this test. All statistical calculations were performed using SPSS 13.0 software for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Stereological Results

All stereological results are summarized in Figure 1. The numerical density of hepatocytes (number of hepatocytes per mm^3) was 422,000 in the healthy control rats (Group 1). The numerical density of hepatocytes was found as 329,000 in hypertensive rats (Group 2) and as 272,000 in the ovariectomy group (Group 3). This decrease in the numerical density of hepatocytes in both Group 2 and Group 3 was significant compared to the control group ($p<0.01$). In the ovariectomized + hypertensive group (Group 4), numerical density of hepatocytes was 259,000. After induction of hypertension and ovariectomy and treatment with either amlodipine (Group 5) or lacidipine (Group 6), the numerical density of hepatocytes was determined as 374,000 and 346,000, respectively. When statistically evaluating the results, it was found that the numerical density of hepatocytes significantly decreased in Groups 5 and 6 when compared to the control group ($p<0.001$). There was a significant difference in terms of numerical density of hepatocytes between the DOCA-given group (Group 2) and the amlodipine-treated group (Group 5), in favor of Group 5 ($p<0.001$). The numerical density of hepatocytes in the ovariectomized group (Group 3) was significantly lower than those of the drug-administered groups (Groups 5, 6) ($p<0.001$). Numerical density of hepatocytes of the ovariectomized + DOCA-given group (Group 4) was statistically lower than that of the control group ($p<0.001$) and of the drug-administered groups (Groups 5, 6). The numerical density of hepatocytes in the lacidipine group was significantly lower than that of the amlodipine group ($p<0.001$).

Histopathological Results

For conventional histopathological examination under a light microscope, all slides were stained with H-E to define routine histological structures.

In the control group (Group 1), recognized healthy liver histology was seen (Figure 2A, B). In the livers of the hypertensive group, thin hepatocyte cords were seen and sinusoids between these cords

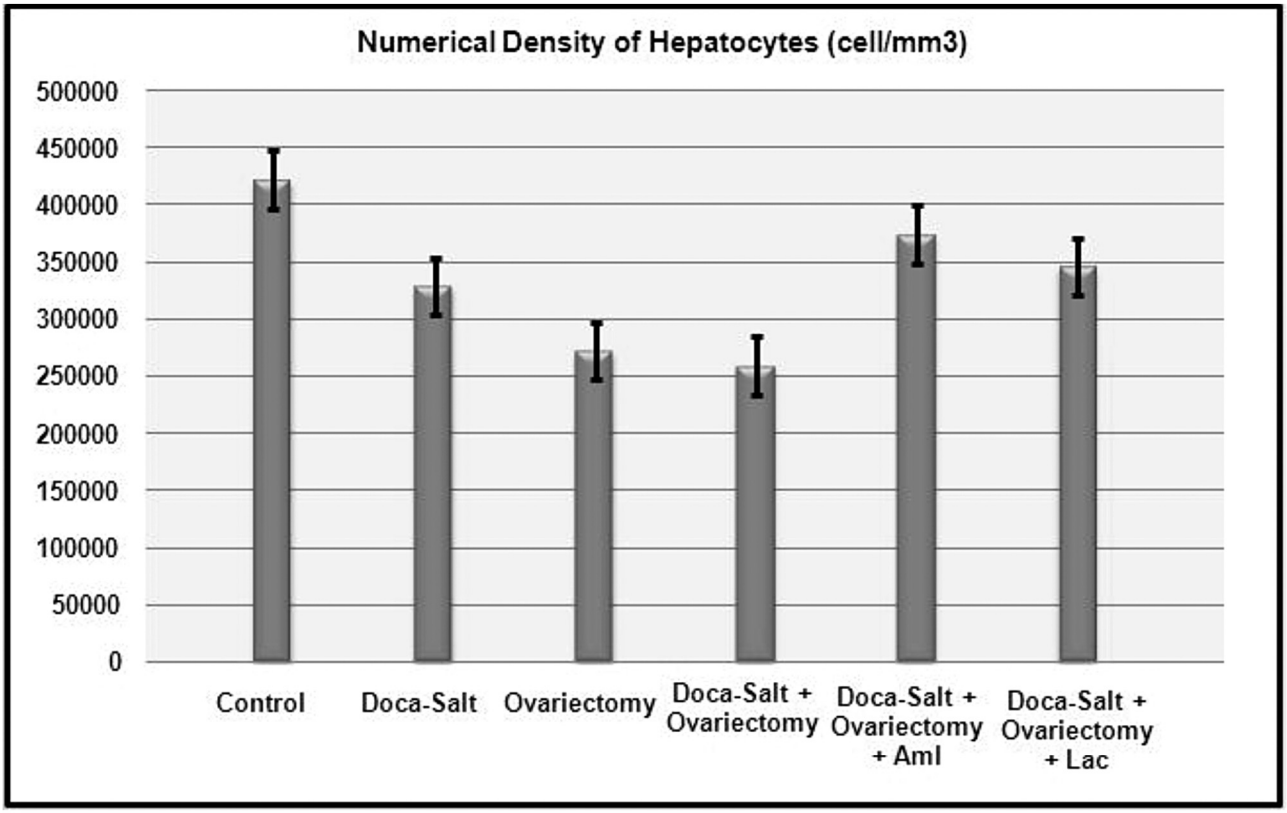

Figure 1. Numerical density of hepatocytes in all groups (± SEM).

were dilated (Figure 2C). The hepatocytes of the hypertensive group had more acidophilic and granular cytoplasm than those of the control group (Figure 2D). They had uncolored chromatin contents and irregular contours (Figure 2D). Their cytoplasm was dispersed to the lumen of sinuso-

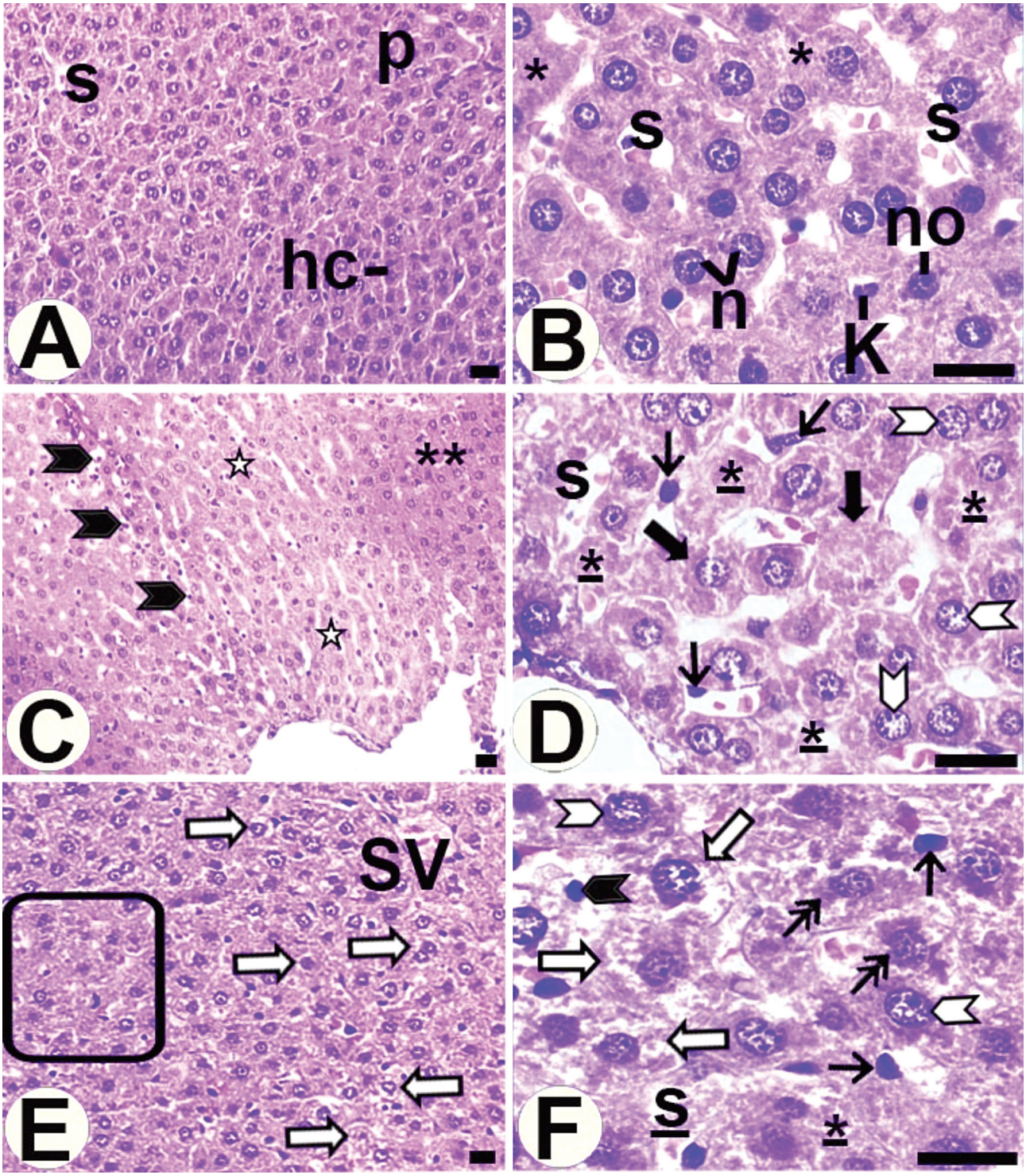

Figure 2. Light microscopic photographs of Groups 1 (A, B), 2 (C, D), and 3 (E, F).

s: Sinusoid. p: Parenchyma. hc: Hepatocyte cord. n: Nucleus. no: nucleolus. K: Kupffer cell. Asterisk: Ergastoplasm. Double asterisk: Hepatocytes with healthy appearance. Black arrowhead: mononuclear cells in sinusoids. Asterisk with white filling: Area containing thin hepatocyte cords and dilated sinusoids. Underlined asterisk: Cytoplasm with granular appearance. Thick arrow: Hepatocytes with irregular membrane boundaries. Thin arrow: Hypertrophied Kupffer cells. Arrowhead with white filling: Nucleus with non-dyed chromatin contents. Arrow with white filling: Hepatocytes with hydropic degeneration. Double-headed arrow: Hepatocytes with dense perinuclear cytoplasm. Underlined s: Sinusoid with irregular contours and eosinophilic contents. Black box shows hepatocytes with undefined boundaries. Magnification Bars: 30 μm.

ids. In the light microscopic sections of the hypertensive rats, hypertrophic Kupffer cells were seen (Figure 2D).

In the hepatocytes of the hypertension + ovariectomy group (Group 4), hydropic degeneration was found (Figure 2E, F), perinuclear cytoplasm of these hepatocytes was dense or cloudy, and the nucleus was abnormally shaped and basophilic stained (Figure 2F). In some hepatocytes, the nucleus was peripherally localized and contained distinct nucleoli (Figure 2F). Moreover, in the light microscopic sections of the hypertension + ovariectomy group, there were abundant Kupffer cells with acidophilic cytoplasm and dense, basophilic nuclei (Figure 2F). Between the cell plates, mild mononuclear cell infiltration was observed in slides of Groups 2 and 3 (Figure 2C, F).

Histological structure of rats subjected to both hypertension and ovariectomy (Group 4) was more corrupted than that of Group 2 and Group 3 rats (Figure 3A, B). Cytoplasm of hepatocytes was completely clear and some hepatocytes were atrophied (Figure 3B). When evaluating the structure at low magnification, smaller hepatocytes than those of controls and dilated sinusoids were detected (Figure 3A). At high magnification, important findings were identified as: nuclei were smaller than those of control rats, perinuclear cytoplasm was dense, and cell membranes were abundant (Figure 3B).

The histological views of the amlodipine- (Group 5) and lacidipine- (Group 6) treated rats (Figure 3C, D, and E, F) were similar to those of the control group (Figure 2A, B).

DISCUSSION

In this study, the effects of aging and/or hypertension on the numerical density of hepatocytes and histological structure of livers were determined using stereological and routine histochemical methods. The effects of amlodipine and lacidipine, commonly used L-type CCBs, were also investigated. DOCA-induced hypertension significantly decreased the numerical density of hepatocytes in rats. Similarly, the numerical density of hepatocytes in the ovariectomized rat group was statistically lower than that of controls. Comparison of the numerical density of hepatocytes in the DOCA group to that in the ovariectomy group indicated a greater decrease in hepatocyte numerical density in the postmenopausal period (ovariectomized) rat group. However, the numerical density of hepatocytes was lowest in rats subjected to both ovariectomy and DOCA. These data suggest that hypertension, the postmenopausal period, and both together could all decrease the number of hepatocytes in the rat liver.

Estrogen production stopped in the ovariectomized rats. Estrogen and estrogen-related receptors are known to increase the bioavailability of endogenous NO (23,24). Estrogen has also been shown to inhibit circulating renin and angiotensin converting enzyme (ACE) levels, to decrease angiotensin-2 (ATE-2) levels and to down-regulate angiotensin receptors-1 (ATE-1) in many tissues (25-27). In contrast, an increase in ATE-2 dependent aortic vasoconstriction and ATE-1 gene expression has been observed in ovariectomized rats (28), and estrogen deficiency was shown to increase renal ATE-1 receptor and related oxidative stress (29).

ATE-2 induces endothelial growth and is one of the most important factors in angiogenesis (30-32). Endothelial growth factor significantly increased hepatic fibrosis in experimental models (33). In addition, ATE-2 causes overexpression of the transforming growth factor beta (TGF-β), a potent fibrotic agent, in hepatocytes (34,35). In the present study, the decrease in the hepatocyte number in ovariectomy-induced old age may be the result of an increase in ATE-2 and a related increase in ATE-1 receptors. These changes may then cause an increase in oxidative stress, vascular endothelial growth factor, TGF-β, and many other fibrotic agents. The same mechanism may be responsible for the hypertension-induced decrease in hepatocyte numbers. Hypertension is known to increase ATE-2, which is responsible for many types of organ damage and complications (36).

According to our histopathological investigation, both ovariectomy and hypertension may cause pathological changes in the liver. Our findings showed that hydropic degenerations, nuclear abnormalities, atrophy of hepatocytes, sinusoidal dilatation, and mild mononuclear cell infiltration occur in response to both of these situations. At this point, we should add that ovariectomy appeared to be more destructive than hypertension; furthermore, it was not surprising that the two situations together could lead to even more severe liver damage than either treatment alone.

When our experimental results were evaluated, it was clear that amlodipine and lacidipine both exerted positive protective effects on the hepatocy-

tes in hypertensive and/or postmenopausal rat livers. Both drugs prevented the decrease in hepatocyte numerical density and the pathological changes associated with either hypertension or old age. The protective effect of amlodipine on the hepatocyte numerical density in hypertensive and/or postmenopausal period rats was greater than that of lacidipine. The protective effect of either drug on rat livers may be dependent on their antihypertensive effect.

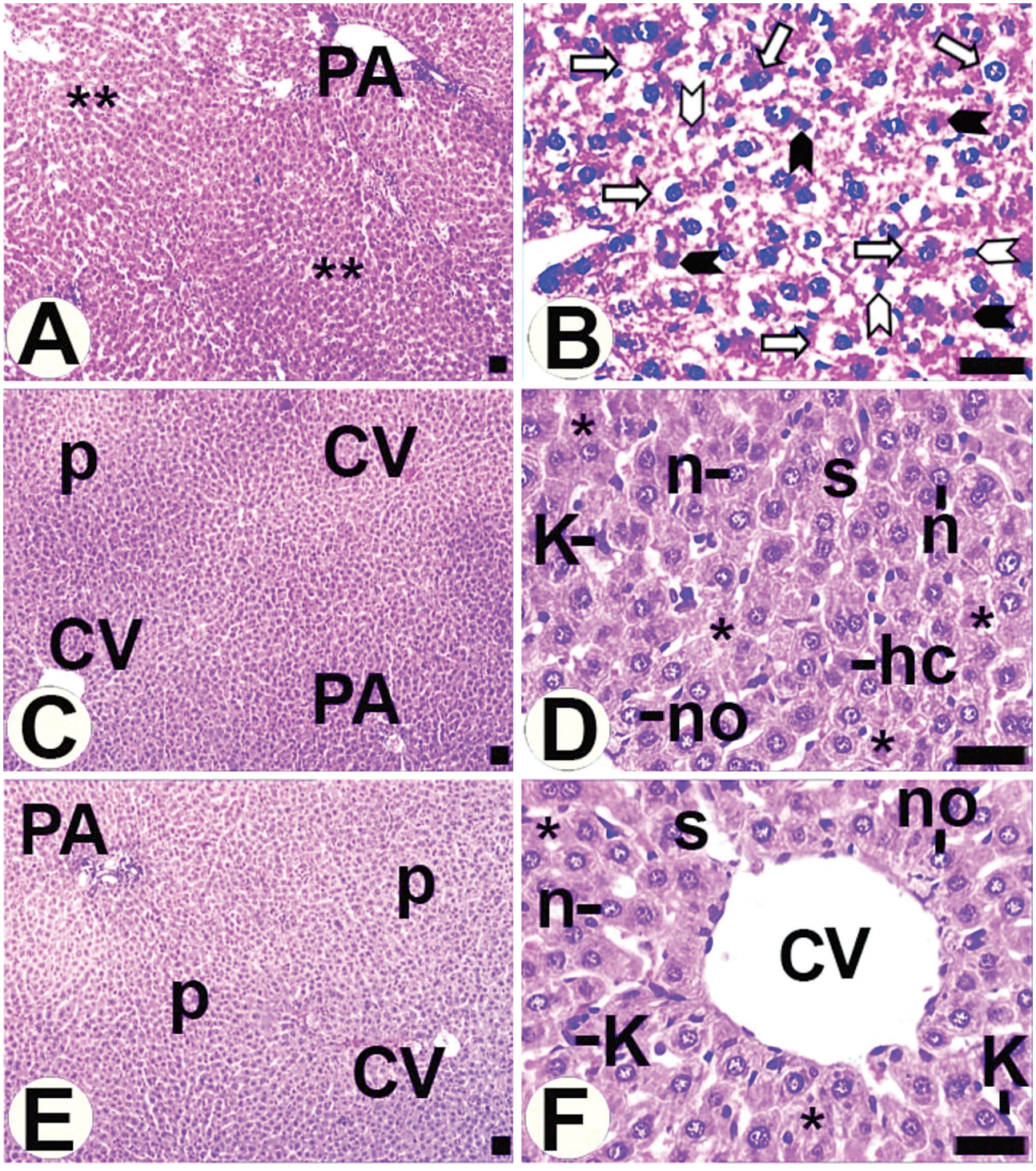

Figure 3. Light microscopic photographs of Groups 4 (A, B), 5 (C, D), and 6 (E, F).

CV: Central veins. PA: Portal area. s: Sinusoid. p: Parenchyma. hc: Hepatocyte cord. n: Nucleus. no: Nucleolus. K: Kupffer cell. asterisk: Ergastoplasm. Double asterisk: Area containing thin hepatocyte cords and dilated sinusoids. Black arrowhead: Hepatocytes with dense perinuclear cytoplasm. Arrow with white filling: Hepatocytes with hydropic degeneration. Arrowhead with white filling shows small and degenerated nuclei. Magnification Bars: 50 μm.

The treatment of hypertension should be improved by reducing the effect of hypertension on hepatocyte numbers in the liver. Nevertheless, both drugs have many different effects accompanying their antihypertensive effect. Each drug exerts anti-inflammatory, antioxidative and antiatherogenic activities (8,11-13,15,37). In a previous study, amlodipine was shown to prevent the decrease in the number of nephrons in response to hypertension (19). Recent studies have also indicated that amlodipine increases the bioavailability of endothelial NO and that the increased eNO has antioxidant properties. Furthermore, it has been shown that amlodipine suppresses proinflammatory cytokines and ROS (38-40). In the present study, both amlodipine and lacidipine were effective in ameliorating the decreases in hepatocyte number caused by either hypertension or postmenopausal period aging.

In conclusion, both hypertension and postmenopausal period aging markedly decreased the numerical density of hepatocytes in the rat liver. We suggest that during treatment of hypertension, the number of hepatocytes should not be overlooked as one of the complications of hypertension. Furthermore, we note that the number of hepatocytes should be considered in the medical treatment of elderly patients, as well as when treating patients with abnormal liver functions. We suggest that amlodipine and lacidipine, but especially amlodipine, should be considered as the primary drugs of choice for the treatment of postmenopausal period aging in patients with hypertension because of the protective effects on the liver observed in the present study.

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