Physiol. Res. 47: 241–251, 1998

Effects of Ethinylestradiol and Levonorgestrel on Morphology, Ultrastructure and Histoenzymatic Activity of Rat Kidney

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Received December 1, 1997
Accepted March 9, 1998

Summary

Sexually mature female Wistar rats were given daily intragastric doses of ethinylestradiol (EE) and levonorgestrel (LE) used normally in women: (1) 0.03 mg EE and 0.05 mg LE; (2) 0.04 mg EE and 0.075 mg LE; (3) 0.03 mg EE and 0.125 mg LE. All groups were treated for 6 months in 5-day cycles (four-day treatment with a one-day break), i.e. for 36 sexual cycles. In rat kidneys, the activity of succinic dehydrogenase, NADPH-tetrazolium reductase, Mg^{2+}-ATPase and alkaline phosphatase were decreased, while those of lactate dehydrogenase, acid phosphatase and glucose-6-phosphatase were enhanced. We have found a correlation between enzymatic changes and ultrastructural changes in epithelial renal cells. These changes may reflect: (1) inhibited oxidative processes associated with the mitochondrial and microsomal systems of electron transport; (2) a compensatory increase in anaerobic processes; (3) increased glyconeogenesis; (4) inhibited transport processes and increased cellular catabolism. The kidney cortex and medulla did not show any significant morphological changes after 6 months of treatment. The study has shown that EE/LE combinations produce histochemical and ultrastructural changes in the kidney, which are dependent on the doses of gestagens.

Key words
Oral contraception – Ethinylestradiol – Levonorgestrel – Kidney – Rats

Introduction

Contraceptive-induced adverse effects depend on both the route of administration and the proportion of estrogens to progestogens (Baciewicz 1985, Skouby *et al.* 1985). It has been proven that the doses of steroids in oral contraceptives positively correlate with disturbances of blood coagulation, glucose and lipid metabolism (Gaspard *et al.* 1985, Burkman 1988, Kjaer *et al.* 1989, Kose *et al.* 1993).

Ultrastructural studies revealed changes in the number or structure of liver and kidney cell nuclei, detachment of ribosomes from rough endoplasmic reticulum (RER), accumulation of secondary lysosomes, damage to the structure of mitochondria and the smooth endoplasmic reticulum (SER). Estrogens and their derivatives are known to be mitogenic (Perez *et al.* 1969, Gonzalez *et al.* 1989, Oberley *et al.* 1991). The increased risk of blood coagulation disturbances and thrombotic complications during oral contraception is related to estrogens. This effect can be balanced by activation of the fibrinolytic system and can be modified by gestogens (Bonnar 1987, Daly and Bonnar 1990, Norris and Bonnar 1994, 1996). Progestogens increase the risk of cardiovascular complications (Fotherby 1985) and alter

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carbohydrate metabolism (Wynn and Godsland 1986). The incidence of these complications depends on the dose and potency of the steroids used (Dorflinger 1985). Therefore, low-dose preparations of estrogen and progestogen are vitally needed (Ramos *et al.* 1989, Kuhl 1990).

Relatively new three-phase oral contraceptives contain the lowest effective doses of ethinylestradiol and levonorgestrel (Apelo and Suplido 1985, Spellacy *et al.* 1989, Fotherby 1992). These are preferred because they effectively control the cycle and show low pregnancy rates combined with a minimal risk of metabolic alterations. Levonorgestrel can be used in three increasing doses: 0.05 mg for 6 days, 0.075 mg for 6 days, and 0.125 mg for 9 days, whereas estrogen is given in doses of 0.03, 0.04 and 0.03 mg for the same periods of time (Tyrer 1985, Ramos *et al.* 1989). The preparations provide a woman with low doses of progestogens in the first phase of the menstrual cycle, and slightly higher doses of estrogens from day 7 to day 11 of the cycle, thus imitating physiological variations in sex steroid levels in a women.

The aim of our study was to evaluate the effects of long-term administration of the above mentioned EE/LE combinations on the morphology, ultrastructure, and histoenzymatic activity of the rat kidney.

Material and Methods

The study was performed on 50 sexually mature female Wistar rats weighing 160±15 g. All rats were fed on a standard diet with water *ad libitum* throughout the experiment. They were kept in plastic and metal cages (5 animals per cage) under standard conditions (20 °C, 60 % humidity, day-night cycle 12:12). Animals were divided into three experimental and two control groups, 10 animals in each group.

EE and LE were dissolved in a 0.4 % aqueous solution of sodium carboxymethylcellulose prepared *ex tempore*, and then they were administered separately in 1 ml portions through a stomach tube. The hormones were given in the following doses: group 1: 0.05 mg LE and 0.03 mg EE; group 2: 0.075 mg LE and 0.04 mg EE; group 3: 0.125 mg LE and 0.03 mg EE.

Groups 1–3 were given steroids in 5-day cycles with 1-day breaks in between. Control groups K1 and K2 were given 1 ml of 0.4 % aqueous solution of sodium carboxymethylcellulose and 1 ml of physiological saline, respectively, according to the same schedule.

The treatment was started on the first day of the ovulatory cycle. The whole experiment lasted for 180 days. The phases of ovulatory cycle was determined by analyzing epithelial cells obtained from vaginal swabs. All animals were decapitated 24 hours after the last dose of the preparation or the vehiculum. Tissue sections were excised from the left kidney always at 09:00 h to avoid variations in enzymatic activities related to the diurnal rhythm (Czekaj *et al.* 1994).

Morphological examination

One part of the material was fixed in 20 % formalin for 14 h, dehydrated in increasing concentrations of ethanol and embedded in paraffin. Specimens were then prepared and stained with haematoxylin and eosin.

Histochemical examination

The second part of tissue sections was frozen with carbon dioxide and cut into 7 μm thick sections in which the following enzyme activities were evaluated:

1. NADPH-tetrazolium reductase, with Nitro BT (Sigma) according to Faber *et al.* (1956). The incubation time was 65 min at 37 °C.
2. Succinic dehydrogenase (SDH), E.C.1.3.99.1. with Tetra Nitro BT according to Nachlas *et al.* (1957). The incubation time was 8 min at 37 °C.
3. Lactate dehydrogenase (LDH), E.C. 1.1.1.27. with Tetra Nitro BT according to Pearse (1961). The incubation time was 30 min at 37 °C.
4. Glucose-6-phosphatase (G-6-Pase), E.C. 3.1.3.9. with G-6P disodium salt (Reanal), according to Wachstein and Meisel (1956). The incubation time was 18 min at 37 °C.

The third part of the material was fixed in cold 4 °C Baker's fluid for 16 h, frozen with carbon dioxide (−19 °C), cut into 7 μm thick sections and mounted on slides. Then the following reactions were performed:

5. Mg^{2+}-dependent adenosine triphosphatase (Mg^{2+}-ATPase), E.C.3.6.1.4. with bisodium ATP salt (Wachstein and Meisel 1957). The kidney was incubated at 37 °C for 15 min.
6. Acid phosphatase (AcP), E.C.3.1.3.2. with sodium glycerophophate by Gomori's (1953) method. The kidney was incubated at 37 °C for 30 min.
7. Alkaline phosphatase (AP), E.C. 3.1.3.1. with naphthol AsBi phosphate (Sigma), according to Burstone (1958). The incubation time was 12 min at 20 °C.

Specimens were photographed with a Polyvar-Reichert light microscope.

Ultrastructural analysis

Three rats from each group were weighed and anaesthetized with a 20 % aqueous solution of urethane given intraperitoneally. Twenty minutes before anaesthesia rats were injected with intraperitoneal heparin (750 U/100 g b.w.). Rats were perfused with a 0.1 M cacodylic buffer containing 3.5 % glutaraldehyde (pH 7.4). The left kidneys were then excised and postfixed with 2.5 % glutaraldehyde for 2 h. After fixation, tissue specimens were rinsed for 24 h in 7.5 % saccharose dissolved in 0.1 M cacodylic buffer. The tissue blocks were placed in a 2 % OsO_4 solution, dehydrated through a graded alcohol-

propylene series and embedded in Epon 812 (Reimer 1967). The fixed blocks were cut into semithin and ultrathin sections with a Reichert ultramicrotome. Ultrathin sections were stained with lead citrate and uranyl acetate and analyzed with a JEM 100 CX transmission electron microscope (voltage 80 kV). The ultrastructure of proximal convoluted renal tubules was analyzed.

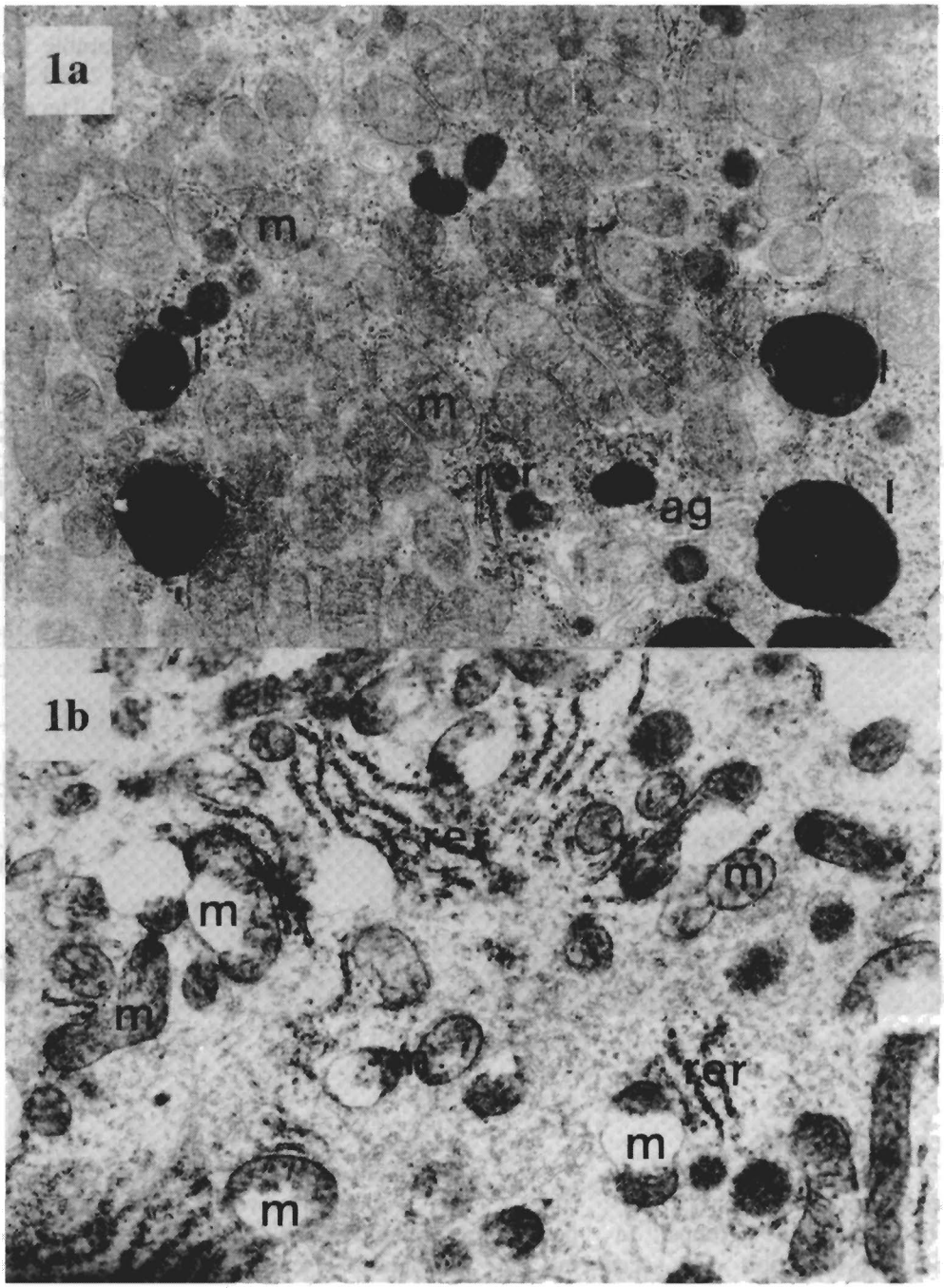

Fig. 1. *Ultrastructure of epithelial cells of proximal convoluted tubules in EE/LE-treated rats in group 2 (Fig. 1a; 8300x) and group 3 (Fig. 1b; 10 000x). Golgi apparatus (ag), lysosomes (l), mitochondria (m), rough endoplasmic reticulum (rer).*

Results

Morphological evaluation

There were no significant differences in the morphological structure of the kidney cortex and medulla between experimental and control groups. The structure of renal parenchyma was regular both in the cortical and medullary sections. Numerous normal renal glomeruli were accompanied by remnants of nephrons. The proximal tubules were lined with a

simple cuboidal epithelium with a high brush border. The remaining parts of the nephrons were also normal.

Electron microscopy evaluation

In group 1, there were no alterations in the ultrastructure of epithelial cells of proximal convoluted tubules. The apical surface of the cells contained normal high brush border, while the basal cytoplasm contained the labyrinth with elongated mitochondria perpendicular to the base of the cell. Another group of round or oval mitochondria was located in the periapical cytoplasm. The mitochondria contained numerous conspicuous cristae. Close to the mitochondria lay aggregates of short SER channels and short RER cisterns with irregularly located ribosomes. The basal cytoplasm contained a large oval nucleus. In the equator zone an ill-developed Golgi was present. The apical cytoplasm contained sparse lysosomes and peroxisomes.

We observed a considerable increase in the number of lysosomes in groups 2 and 3 in both apical and peribasal cytoplasm (Fig. 1a). The amount of RER decreased markedly. In both these groups (especially in group 3) mitochondria exhibited blurred cristae, disrupted membranes and clear areas in the matrix (Fig 1b).

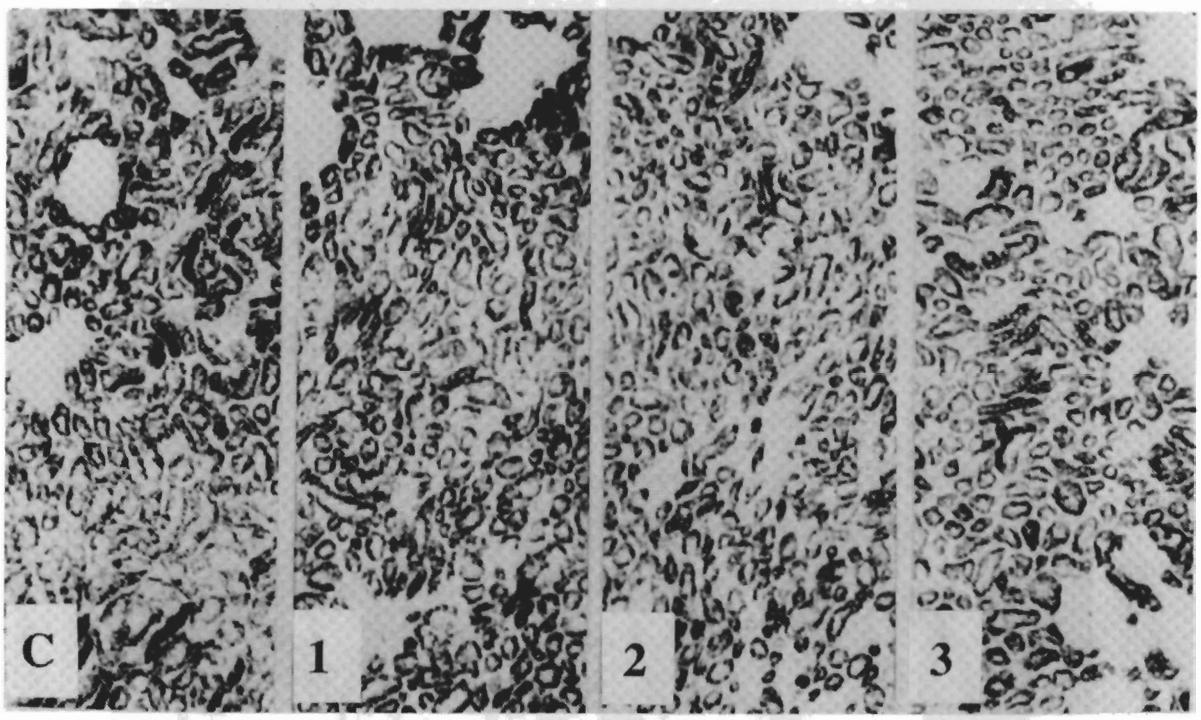

Fig. 2. *SDH reaction in rat kidney of control (C) and EE/LE-treated groups (1–3); 100x.*

Histochemical evaluation

Succinic dehydrogenase (SDH)

In both control groups, a positive microgranular-diffuse reaction was seen in the cytoplasm of epithelial cells of the proximal and distal tubules. In other segments of the nephron, the SDH reaction, though weaker, was similar in character and cellular distribution.

In the experimental groups, a slight (groups 1 and 2) or marked (group 3) decrease in the intensity of SDH reaction was observed mostly in the epithelial cells of the proximal and distal renal tubules (Fig. 2). In the remaining segments of the nephron the character and intensity of the reaction did not differ from that of the control group.

NADPH-tetrazolium reductase

We observed a positive microgranular diffuse reaction which was strongest in the parabasal cytoplasm of epithelial cells lining the proximal, thin and thick tubules. The reaction was weaker in the remaining segments, but its distribution and character did not change. The reaction was also weaker in Bowman's capsule and the walls of both glomerular capillaries and large blood vessels (Fig. 3).

A slight decrease in the intensity of the reaction was found in group 1. In groups 2 and 3 the decrease was greater and was found mostly in the proximal and thick tubules. In group 3, a decrease in the reaction intensity was also found in the glomeruli.

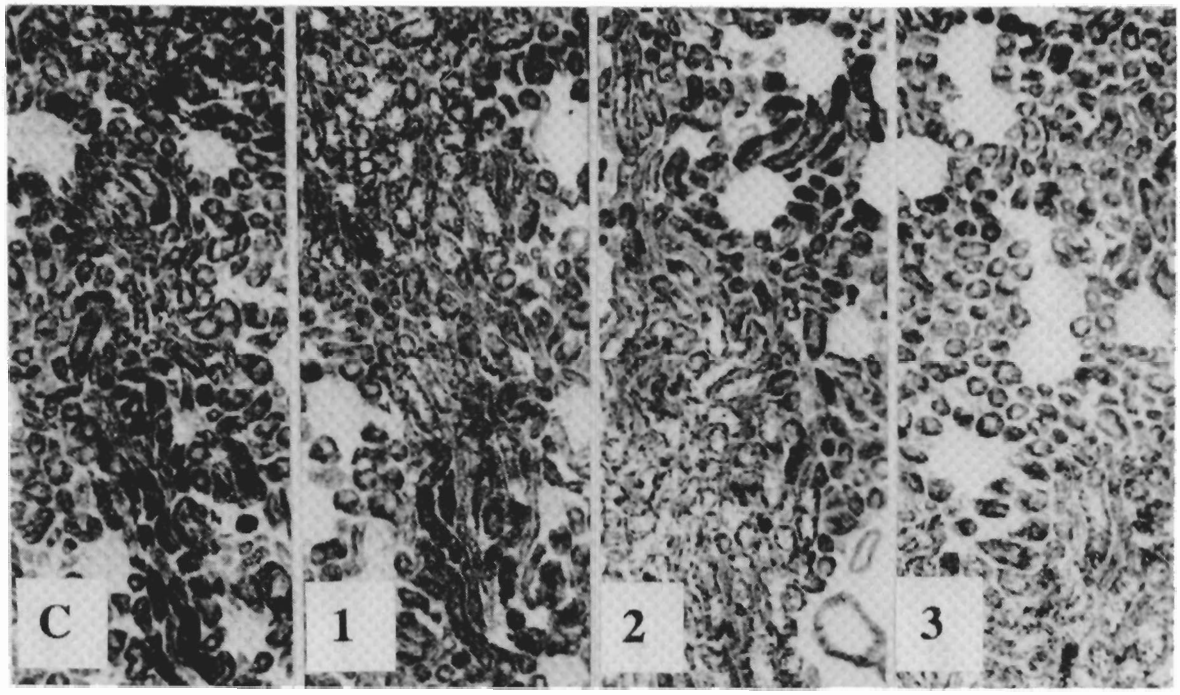

Fig. 3. *NADPH-tetrazolium reaction in rat kidney of control (C) and EE/LE- treated groups (1–3); 100x.*

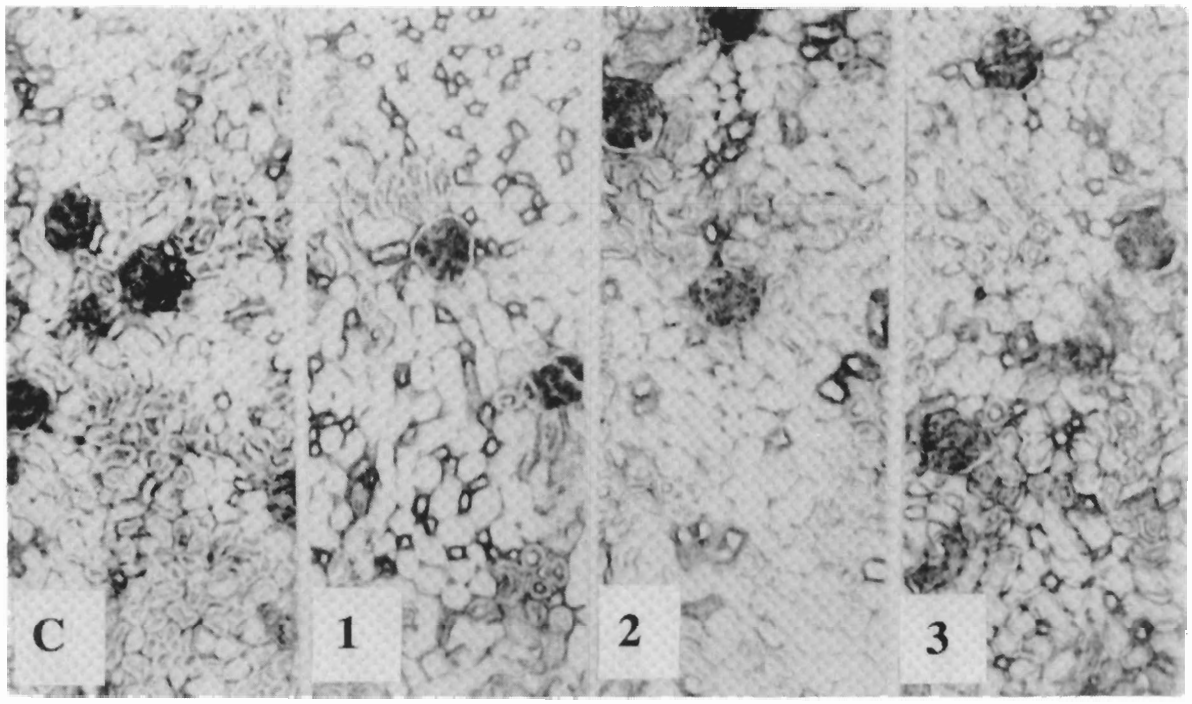

Fig. 4. *Mg^{2+}-ATPase reaction in rat kidney of control (C) and EE/LE-treated groups (1–3); 100x.*

Mg^{2+}-ATPase

In the control groups, a strong diffuse reaction was found in the glomerular capillaries and in the walls of large blood vessels. A slightly weaker diffuse reaction occurred in the brush border of proximal tubules. There was no positive reaction in the other segments of the nephron.

EE/LE groups showed either a slight (groups 1 and 2) or a marked decrease in the intensity of the reaction (group 3) (Fig. 4). In group 1, the decrease in the intensity of the reaction was observed only in the glomerular capillaries, and in groups 2 and 3 also in the brush border of proximal tubules.

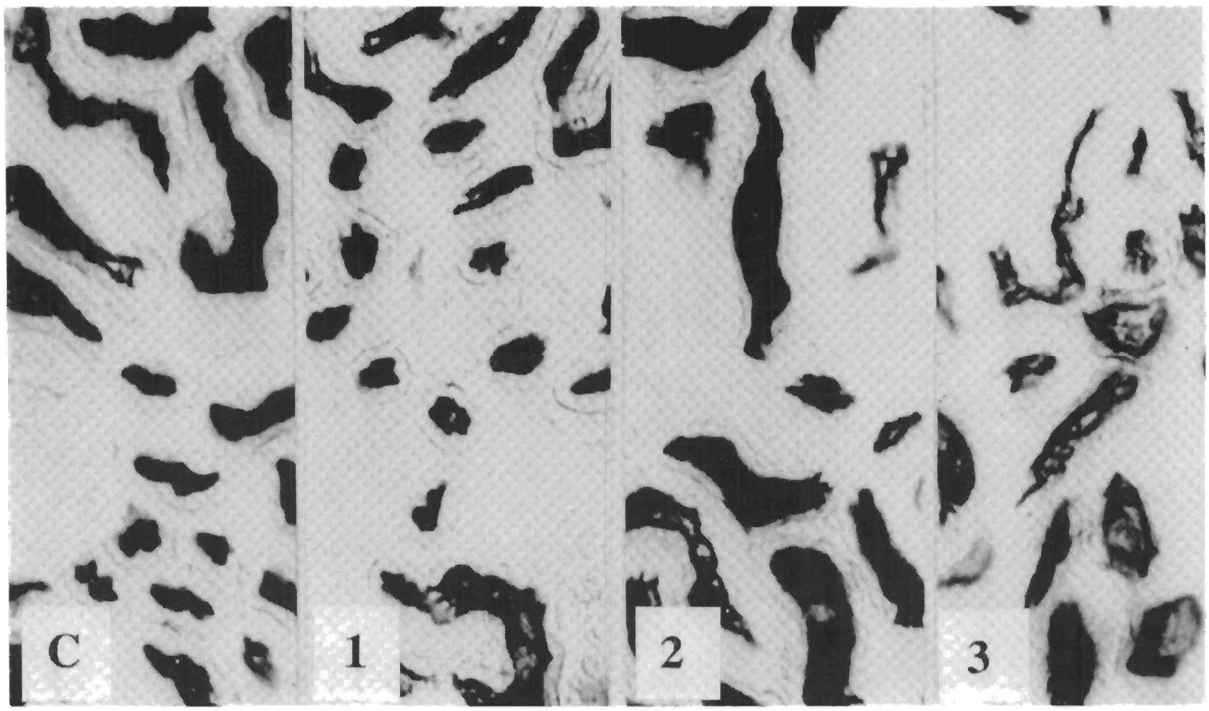

Fig. 5. *AP reaction in rat kidney of control (C) and EE/LE-treated groups (1–3); 400x.*

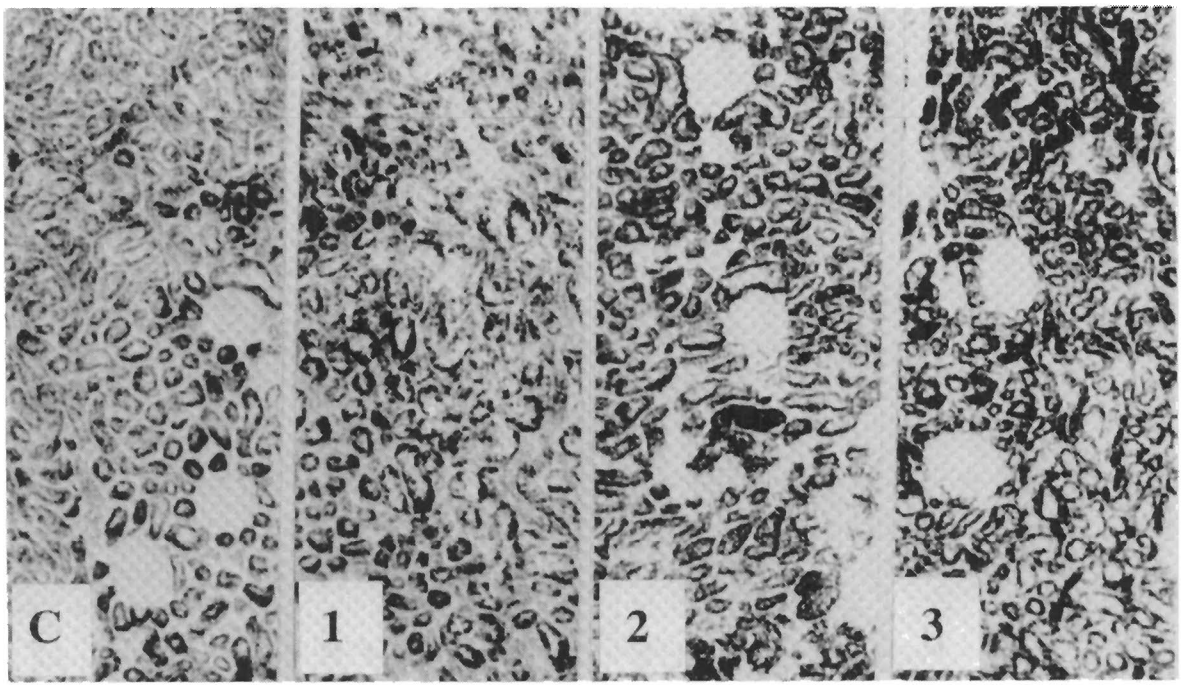

Fig. 6. *LDH reaction in rat kidney of control (C) and EE/LE-treated groups (1–3); 100x.*

Alkaline phosphatase (AP)

A positive diffuse reaction was found in the renal cortex in the brush border of proximal tubules.

The reaction intensity decreased slightly in groups 1 and 2, and markedly in group 3 (Fig. 5).

Lactate dehydrogenase (LDH)

In the control groups, a positive granular diffuse reaction was found in the basal region of the epithelium of proximal convoluted and straight tubules. The remaining segments of the nephron exhibited a

considerably weaker reaction similar in character and cellular distribution. LDH reaction was also weak in the walls of blood vessels and in glomerular capillaries.

The LDH reaction was markedly stronger in group 1, and still stronger in groups 2 and 3 (Fig. 6). In groups 1 and 2 an increased reaction was seen in both proximal convoluted and straight tubules. In group 3, the reaction was also found in the remaining segments of the nephron.

Glucose-6-phosphatase (G-6-Pase)

A positive microgranular reaction was found in the peribasal cytoplasm of epithelial cells of proximal and distal convoluted tubules, straight tubules and in thick tubules.

In groups 1–3, the reaction intensity increased gradually (Fig. 7). The reaction was microgranular diffuse in group 1, while it was medium-grained or medium-grained and diffuse in groups 2 and 3.

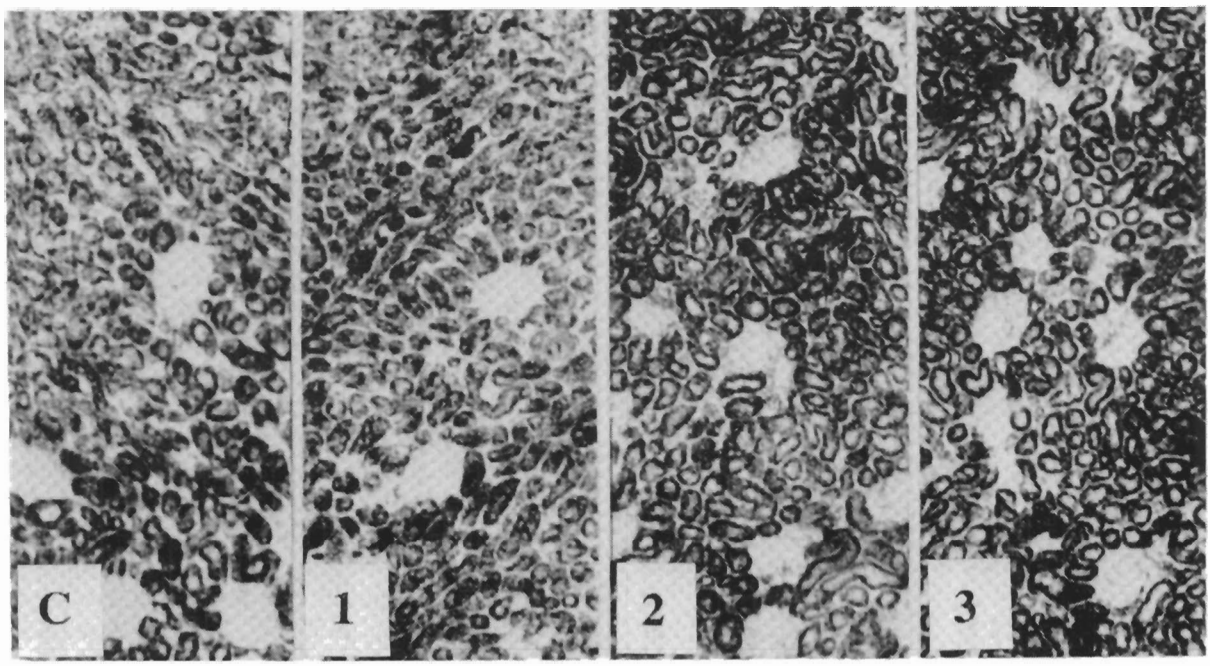

Fig. 7. *G-6-Pase reaction in rat kidney of control (C) and EE/LE-treated groups (1–3); 100x.*

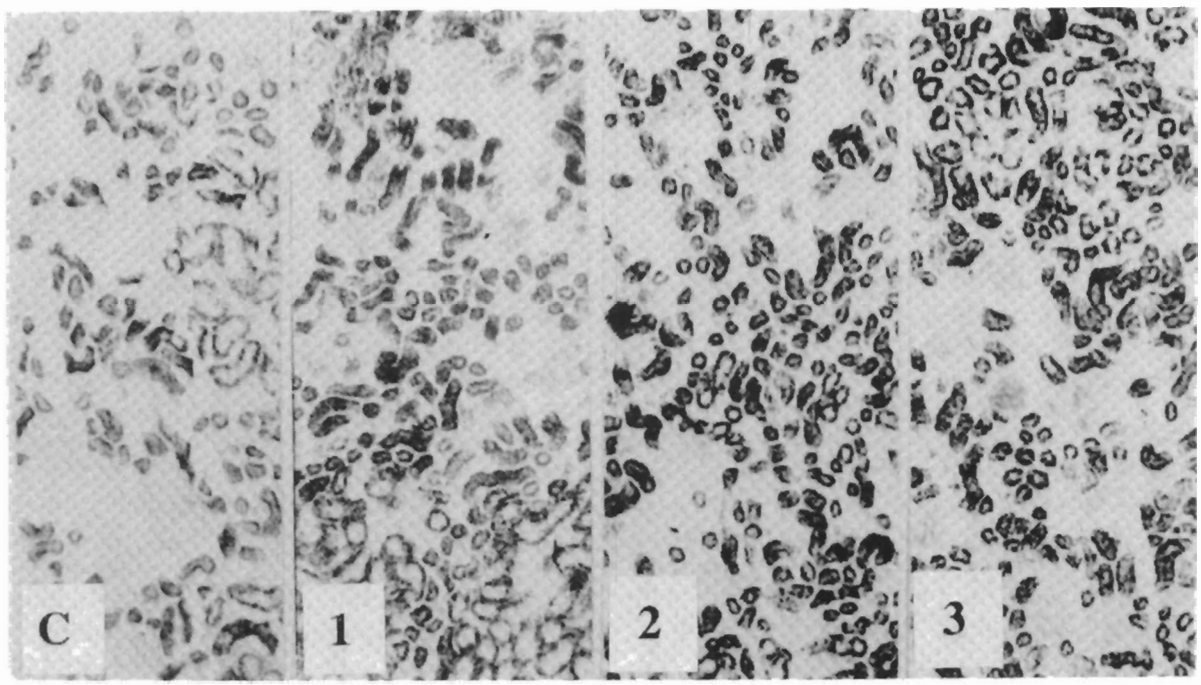

Fig. 8. *AcP reaction in rat kidney of control (C) and EE/LE treated groups (1-3); 100x.*

Acid phosphatase (AcP)

In the control groups, a positive granular or granular-diffuse reaction occurred in the apical cytoplasm of the cells of proximal and distal convoluted tubules. The glomeruli showed a weak, diffuse or granular-diffuse reaction.

We found a marked (groups 1 and 2) or very intensive (group 3) increase in this reaction (Fig. 8). It was seen chiefly in the proximal tubules and, to a lesser extent, in the thick and distal convoluted tubules. We also observed changes in the character of the reaction: the reaction was diffuse-macrogranular in group 3, in which the reaction intensity also increased in the glomeruli.

Discussion

Synthetic estrogens, such as EE, are converted into catechol metabolites by liver and kidney microsome enzymes, and undergo enterohepatic recirculation (Martucci and Fishman 1993, Zhu *et al.* 1993, Shenfield 1993). Progestogens are quickly metabolized in the liver by stereoselective metabolism and do not undergo enterohepatic recirculation (Stanczyk and Roy 1990, Shenfield 1993). The kidney is the ultimate site of their elimination. Reactive metabolites generated in the liver and released into the blood and urine may affect renal functions (Ball and Knuppen 1990).

Different doses of EE/LE given to female rats for 6 months significantly altered the activities of kidney marker enzymes, indicating possible metabolic disturbances at the cellular level. We observed mitochondrial destruction and decreased activities of mitochondrial SDH and NADPH-tetrazolium reductases (enzymes related to electron transport chains), which were proportional to the doses of steroids. NADPH-tetrazolium reductase, which participates in electron transport both in mitochondria and the endoplasmic reticulum, is a marker of their biological activity. In ER, NADPH-tetrazolium reductase is an electron donor for the monooxygenase system responsible for metabolism of xenobiotics and steroid hormones; in mitochondria, the activity of this enzyme reflects the rate of oxidation-reduction processes. In our previous study, a combination of 0.125 mg LE and 0.3 mg EE given for 6 months inhibited the activity of liver microsomal enzymes (Czekaj and Nowaczyk-Dura 1996). Our results are in accord with those of other authors who observed a decrease in the activity and amount of mitochondrial and microsomal enzymes in the liver after oral contraception (Perez *et al.* 1969, Mc Kinnon *et al.* 1977, Mc Queen 1978, Kulcsar and Kulcsar-Gergely 1979).

The alterations in the renal cell function may also be caused by the state of cytoplasmic membrane. EE is known to alter the lipid composition of cell membranes and to affect ion pumps such as Na^+,K^+-ATPase and Ca^{2+}-ATPase (Bossard *et al.* 1993, Van Dyke and Root 1993). In our study, we found a decrease in the Mg^{2+}-ATPase reaction intensity in the glomerular capillaries and in the brush border of the proximal tubules. A similar effect was observed for AP in the epithelium of proximal convoluted tubules and straight tubules.

Based on all these findings we suppose that EE/LE, especially LE, may impair energy production and microsomal electron transport (Rush *et al.* 1984) in the epithelial cells of the proximal tubules and other parts of the nephron. The decreased activities of Mg^{2+}-ATPase and AP (involved in active membrane transport) may result from the decrease in energy production reflected by the activity of SDH and/or by damage of mitochondrial membranes. These effects may appear irrespective of estrogen-induced changes in the cytoplasmic membrane. Our results indicate that active reabsorption of some urine components, such as glucose, potassium, sulphate ions, etc, may be disturbed and thus impair both the production and excretion of primary urine.

The impaired activity of mitochondria due to prolonged EE/LE administration was accompanied by an increase in the rate of anaerobic respiration. The intensity of LDH reaction increased gradually in all EE/LE groups. This indirectly proves that when aerobic energy production is inhibited (a decrease in SDH activity), renal cells are able to compensate for this energy deficit by triggering anaerobic processes, especially in the epithelial cells of the proximal tubules.

The EE/LE-induced inhibition of aerobic reactions (decreased activity of SDH and NADPH-tetrazolium reductases) and the intensified anaerobic metabolism (increased LDH activity) may be associated with the intensification of gluconeogenesis because proximal, distal and thick tubules demonstrated an increase in the activity of G-6-P-ase. Several investigators observed increased levels of glucose in blood when a triphasic preparation was used (Skouby *et al.* 1985, Kung *et al.* 1987).

EE/LE combinations given for six months significantly increased lysosomal activity in renal cells. The AcP reaction intensity increased in the epithelium of proximal tubules and, to a lesser degree, in distal convoluted and thick tubules. There was a significant increase in the number of lysosomes. A diffuse AcP reaction may indicate the beginning of lysosomal membrane damage. Several investigators have found that estrogens induce lysosomal enzymes, such as cathepsin D, lysosomal protease, precursors of β-hexosaminidase, cathepsin B and β-galactosidase. Renal lysosomal cathepsin B positively correlates with plasma estradiol levels (Fu and Hornick 1995). Gonzalez *et al.* (1989) and Oberley *et al.* (1991) observed an increased number of secondary lysosomes

in the renal cells of hamsters treated with contraceptives. Similarly EE affected liver functions, increased the release of lysosomal enzymes and lysosomal contents into bile (Lopez del Pino and Larusso 1981), and altered the turnover rate of H^+-ATPase possibly through changing the endosome lipid composition (Van Dyke and Root 1993).

Estrogen-gestagen interactions related to drug doses (Baciewicz 1985) are an important factor influencing the results of this study. Our findings prove that the rate of cellular injury in the kidney depends on the dose of EE/LE (especially on the dose of LE). In women, plasma LE levels remain virtually unchanged within 1 to 2 hours after administration, and then (from 4 to 24 hours post-ingestion) there is a progressive increase in circulating sulphate and glucuronide LE metabolites (Stanczyk and Roy 1990). However, inhibition of liver monooxygenases (Czekaj and Nowaczyk-Dura 1996), may contribute to prolonged circulation of LE in the blood. On the other hand, it should be remembered that the dose of EE in triphasic preparations is relatively constant and minimal, and varied LE doses are used alternately. Since the net dose of LE in triphasic preparations is low, the duration of presence of progestogen component in blood may be shorter and its harmful effect may be weaker. Moreover, it should be taken into account that the effects of chronic administration of EE/LE to rats may also overlap the effects of aging (Czekaj and Plewka 1990).

Our previous report concerning the effects of EE/LE combinations on rat liver (Nowaczyk-Dura and Ciszkowa 1991) and this study, concerning the kidney, indicate that both organs show significant histochemical changes. Furthermore, unlike the kidney, the liver showed slight changes in morphological structure. Morphological changes are the last changes appearing and are often irreversible. After 6-month EE/LE treatment we did not notice any significant morphological changes, such as interstitial nephritis, stromal lymphocytic infiltrations, microhydronephrosis, renal cysts, and other degenerative lesions induced by oral contraceptives, which were reported by other authors (Zaki *et al.* 1979, Layde 1981, Tyrer 1985, Gonzalez *et al.* 1989, Oberley *et al.* 1991).

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