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# Effects of Probucol in Hyperlipidemic Rabbit Liver: A Preliminary Ultrastructural Study

By

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**Key Words:** Probucol, Liver, Ultrastructure

**Summary: Probucol is a lipid-lowering agent with an antioxidant effect; however, its influence on the liver remains unclear. The effects of probucol on hyperlipidemic rabbit liver are investigated to add a structural data on its therapeutical profile. Local albino rabbits were divided into three groups. 1) Hyperlipidemic group: fed with 1% cholesterol (150 g/kg/day) enriched chow for 2 months. 2) Probucol treated group: group 1 + intraperitoneal probucol (10 mg/kg/day) administration for 15 days. 3) Control group fed with normal chow. The blood lipid profile was investigated biochemically. Liver samples were examined electronmicroscopically. Within the parenchymal cells of group 1, the amount of rough surfaced endoplasmic reticulum was increased, its cisterna was dilated displaying a moderately electron dense substance in it and showed close apposition with the condensed mitochondria. In group 2, smooth surfaced endoplasmic reticulum was in extensive amounts filling almost all of the cytoplasm, displayed a reticular, degenerated appearance and was in close relation with the condensed, degenerated mitochondria. Probucol may cause degenerative changes on the liver parenchyme at the subcellular level. It alters the structure of these cells mainly acting on the smooth surfaced endoplasmic reticulum and the mitochondria that are known to be involved in cellular detoxification.**

Many agents used in human therapy have adverse effects. Hypolipidemic drugs that are currently used to normalise hyperlipoproteinemia in long-term therapy have been sometimes suspected of inducing hepatotoxicty[1].

Probucol is a cholesterol-lowering agent used in the treatment of hypercholesterolemia, atherosclerosis and some cardiovascular diseases. The mechanism by which probucol lowers the plasma cholesterol is not clearly established[2–4]. The liver is known to be the most important organ for cholesterol and lipoprotein metabolism. The effects of probucol and other hypolipidemic agents on the liver were evaluated histologically in a limited number of studies[5,6]. The effects of some hypolipidemic agents on liver cells of male rats, dogs, hamsters and mice were investigated at ultrastructural level and the results were compared to each other[5]. These drugs were reported to increase the peroxisomes at the subcellular level. The effects of probucol on rat liver cell ultrastructure were comparatively studied with two other hypolipidemic drugs as described by Barnard *et al.*[6]. In this study, probucol caused no significant changes at the subcellular level apart from an occasional increase in smooth-surfaced endoplasmic reticulum when compared to the control group. In particular, no increase in the number of peroxysomes was noted.

There is still a controversy on the effects of probucol on liver morphology. The aim of this study is to investigate the effects of therapeutic doses of probucol on the liver parenchyme at the ultrastructural level in hyperlipidemic rabbits.

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Abbreviations: Low density lipoprotein (LDL), very low-density lipoprotein (VLDL), high-density lipoprotein 2 and 3 (HDL2, HDL3), sodium phosphate buffer.(PBS).

## Materials and Method

*Experimental protocol*

Two-month old local albino rabbits of both sexes were used. The animals were allocated to one of the following three groups:

Group 1 Hyperlipidemic group (n = 5): Rabbits were fed with 1% cholesterol enriched chow for 2 months.

Group 2 Probucol treated hyperlipidemic group, (n = 5): Being fed with 1% cholesterol enriched chow for 2 months, probucol (10 mg/kg/day) was administered intraperitoneally for 15 days.

Group 3 Control group (n = 3): Rabbits were fed with normal chow.

Water was allowed ad libitum. Animals were housed under stable and controlled environmental conditions (temperature, humidity, and feeding time and 12 hours light-dark periods). At the end of the experimental period, the rabbits were sacrificed by sodium pentobarbital (30 mg/kg) and their livers were extracted and preserved in 2.5% gluteraldehyde solution. Animals belonging to Group 1 and Group 2 were all found to be hyperlipidemic at the end of the 2 months follow-up period (Table 1). Atherosclerotic plaques were observed in most of the aortic samples in light-microscopic examination (data not presented). Cholesterol and probucol were purchased from Sigma (St. Louis, U.S.A).

*Histological Study*

Tissue specimens were fixed with immersion in a solution of 2.5 percent gluteraldehyde in 0.1 M sodium phosphate buffer (PBS) for 3 hours at 4°C at a pH of 7.4. After washing with PBS, they were postfixed in 1 percent osmium tetroxide in 0.1 M PBS for 1 to 2 hours at 4°C. Specimens were then dehydrated in graded series of ethanol up to absolute ethanol to prepare for embedding in araldite. The araldite (epoxy resin kit) was purchased from Agar (Germany). The specimens were incubated in araldite at 60°C for 24–48 hours. Semithin sections were stained with methylene blue-AzurII and photographed with an Olympus BH2 microscope.

Thin sections were stained with uranyl acetate and lead citrate respectively; they were then examined and photographed with a Carl Zeiss EM 9S-2 electron microscope.

*Biochemical Study*

Blood lipid profile was demonstrated by evaluating plasma cholesterol, triglyceride and HDL levels. Data were presented as means ± s.e.m. Statistical significance was examined by Kruskal-Wallis of variance then post hoc Dunn test. A p value of $<0.05$ was considered as indicative of statistical significance (Table 1).

## Results

According to the biochemical results all the animals of groups 1 were hyperlipidemic at the end of 2 months. Blood lipid concentration significantly decreased in the probucol treated hyperlipidemic animal group (Table 1).

*Group 1 (Hyperlipidemic group)*

Semi-thin sections of the hyperlipidemic group displayed normal lobular integrity. The most prominent alteration was the dilated and lipid-droplet-filled sinusoids (Fig. 1). The parenchymal cells displayed abundant cytoplasmic basophilic bodies (Fig. 1). These basophilic bodies were lesser in amount in the vicinity of the portal area and normal parenchymal cells were observed occasionally. In examination of thin sections, the ultrastructure of parenchymal cells appeared almost normal. An increase in rough-surfaced endoplasmic reticulum was observed (Fig. 2). This organelle was dilated, filled with a homogenous, moderately electron-dense substance and gave the appearance of anastomosing tubules (Fig. 3). Mitochondria were condense, their crista were undetectable and surrounded by the anastomosing tubular arms of the rough-surfaced endoplasmic reticulum (Figs. 2, 3). The smooth surfaced endoplasmic reticulum appeared normal and was in close relation with the

Table 1. Blood lipid profile

| | Hyperlipidemic group (Group 1) | Probucol treated hyperlipidemic group (Group 2) | Control group (Group 3) |
|---|---|---|---|
| Cholesterol (mg/ml) | 166.5 ± 29.3 (*) | 91.7 ± 21.8 (δ) | 55.5 ± 3.4 |
| Triglicerid (mg/ml) | 92.6 ± 9.3 | 168.2 ± 41.4 | 90.1 ± 17.6 |
| HDL (mg/ml) | 29.7 ± 3.3 | 30.5 ± 3.7 | 37.4 ± 3.2 |

mean ± s.e.m.

(*): $p < 0.05$ different from the control group (Kruskal-Wallis, post-hoc Dunn test).

(δ): $p < 0.05$ different from the hyperlipidemic group (Kruskal-Wallis, post-hoc Dunn test).

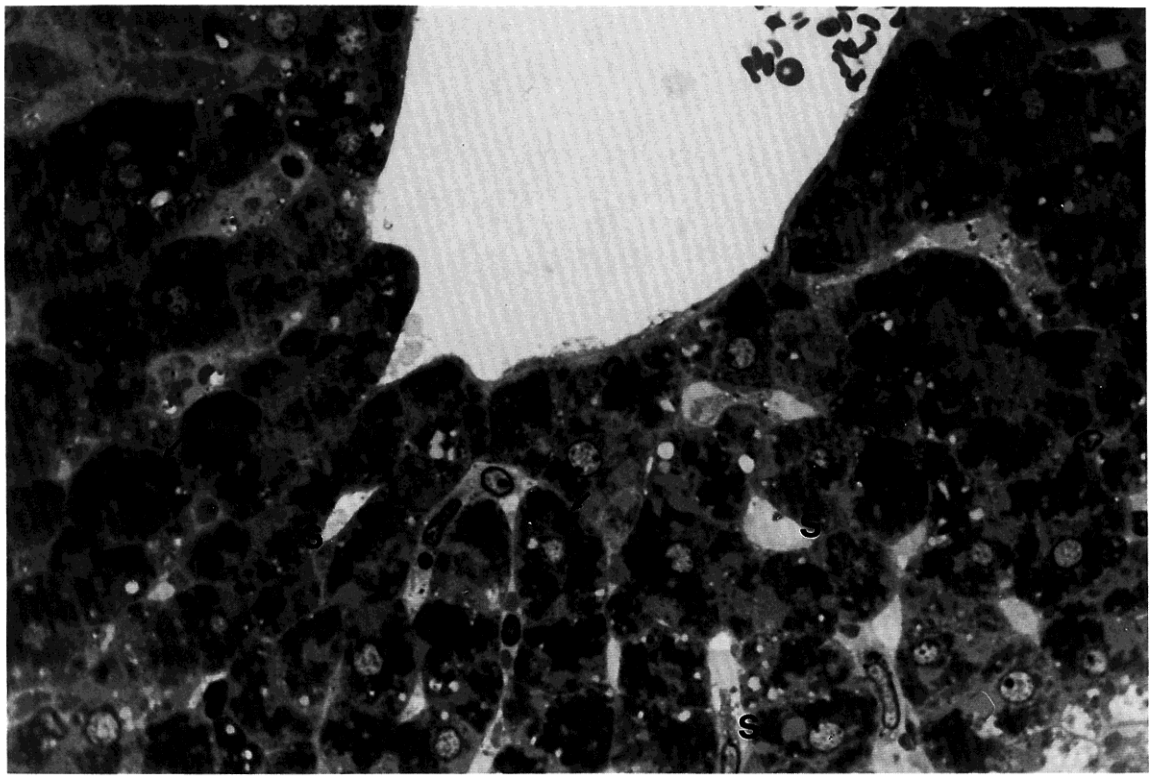


Fig. 1. Micrograph of hyperlipidemic group presents dilated and lipid-filled sinusoids (s). The parenchymal cells appear to be normal apart from their cytoplasmic basophilic bodies (arrow). Methylene blue-Azur II ×40.

rough-surfaced endoplasmic reticulum-mitochondria complexes (Fig. 2 inset). In the cytoplasms of parenchymal cells there were lysosomal bodies formed to metabolise the undigested residues of lipids (Fig. 2). Glycogen was observed in normal amounts (Fig. 3). Kupffer cells were filled with different amounts of lipid droplets.

*Group 2 (Probucol treated hyperlipidemic group)*

Examination of the semi-thin sections revealed degeneration and foamy appearance of the parenchymal cells in differing amounts and localisation in different lobules (Fig. 4). Thus, in an individual lobule, the amount of degeneration changed in different parts of it. As an example, in the vicinity of the central vein, there were dilated and lipid-filled sinusoids opening to this vein and parenchymal cells displayed a vacuolated appearance in the lobule (Fig. 5A), whereas, the same area appeared completely normal in another one (Fig. 5B).

Examination of the ultra-thin sections revealed that organelles of the parenchymal cell were enforced to work against a metabolic overload slightly over-passing the physiological borders. The granulated endoplasmic reticulum gained its normal morphology. The mitochondria were still condensed, their crista were undetectable and showed degeneration (Figs. 6, 7). The smooth surfaced endoplasmic reticulum was increased in amount, had lost its normal appearence, its membranous boundaries were undetectable and displayed a degenerated appearance (Figs. 6, 7). It was in close relation with the condensed mitochondria and accumulated in the cytoplasm as patches filling almost all of it (Figs. 6, 7). They seemed as groups of irregularly anastomosing tubules. Glycogen was not observed in the cytoplasm.

*Group 3 (Control group)*

The control group displayed normal hepatic morphology at light and electron microscopical level with a healthy appearance of the lobules and the stroma.

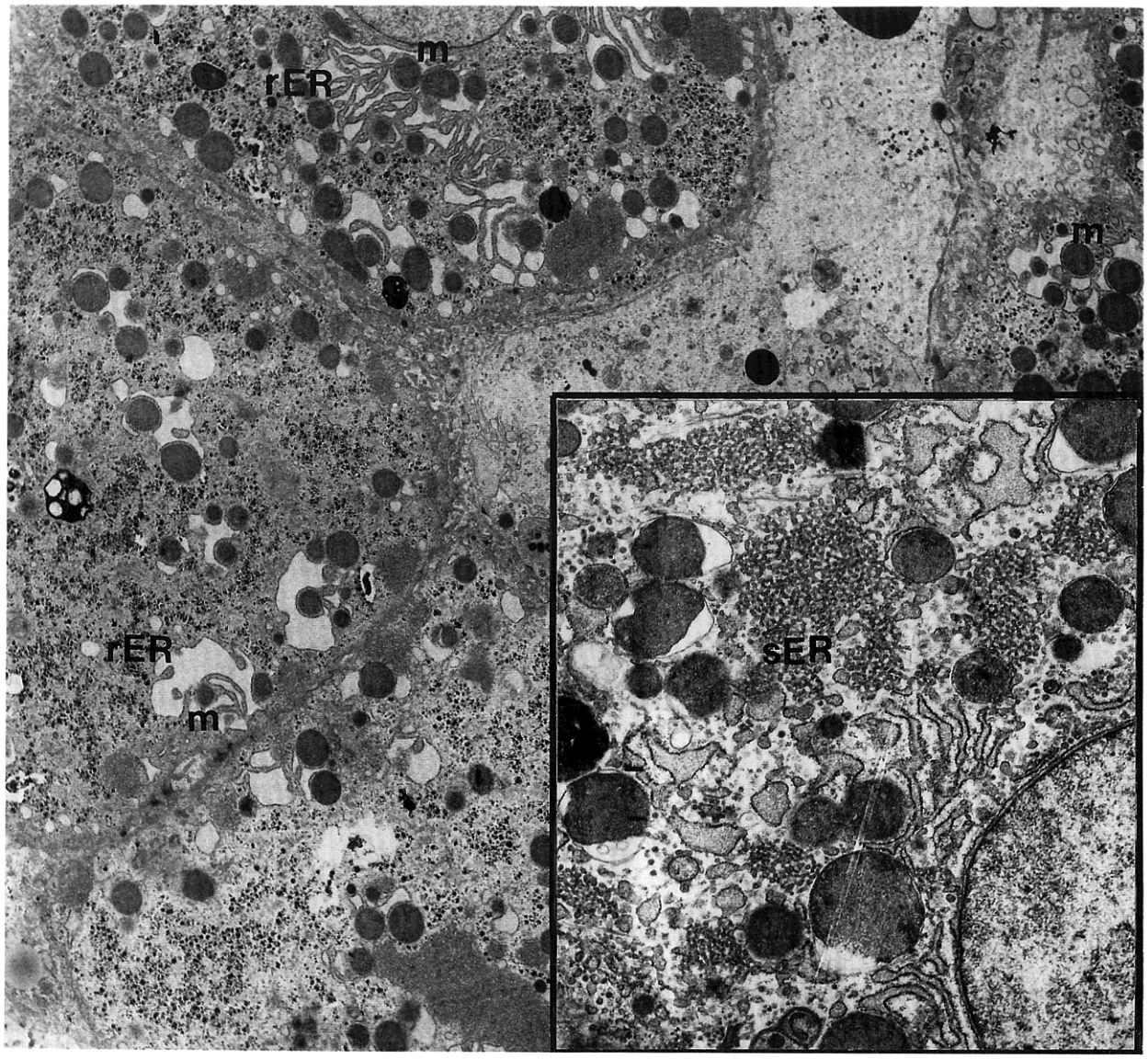



Fig. 2. Electronmicrographs of the hyperlipidemic group Note the increase of the rough-surfaced endoplasmic reticulum (rER), the condensed appearence of mitochondria (M) and their close apposition. Inset: The smooth-surfaced endoplasmic reticulums (sER) appear to be normal. Uranyl acetate lead citrate Uranyl acetate lead citrate ×5700, (inset) ×13500

## Discussion

Probucol is proposed to be a hypolipidemic drug, however, the mechanism of this effect has not been clarified yet[2–4]. It has recently been the focus of attention because of its potency to improve vascular lesions due to its unique pharmacodynamic profile[7–8]. It has been demonstrated that probucol attenuates the progression of atherosclerotic lesions in Watanabe heritable hyperlipidemic rabbits[9,10], hypercholesterolemic rabbits[11], Rhesus monkeys[12], rats[13], and also in humans[2]. The mechanism of this favourable effect has been attributed to its antioxidant properties[2,3,7,14]. And it has been proposed that hypolipidemic and antioxidant actions of probucol are independent of each other[2–4]. Probucol's antioxidant effects are in agreement with the fact that probucol prevents the oxidative modification of both low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL)[7]. It has

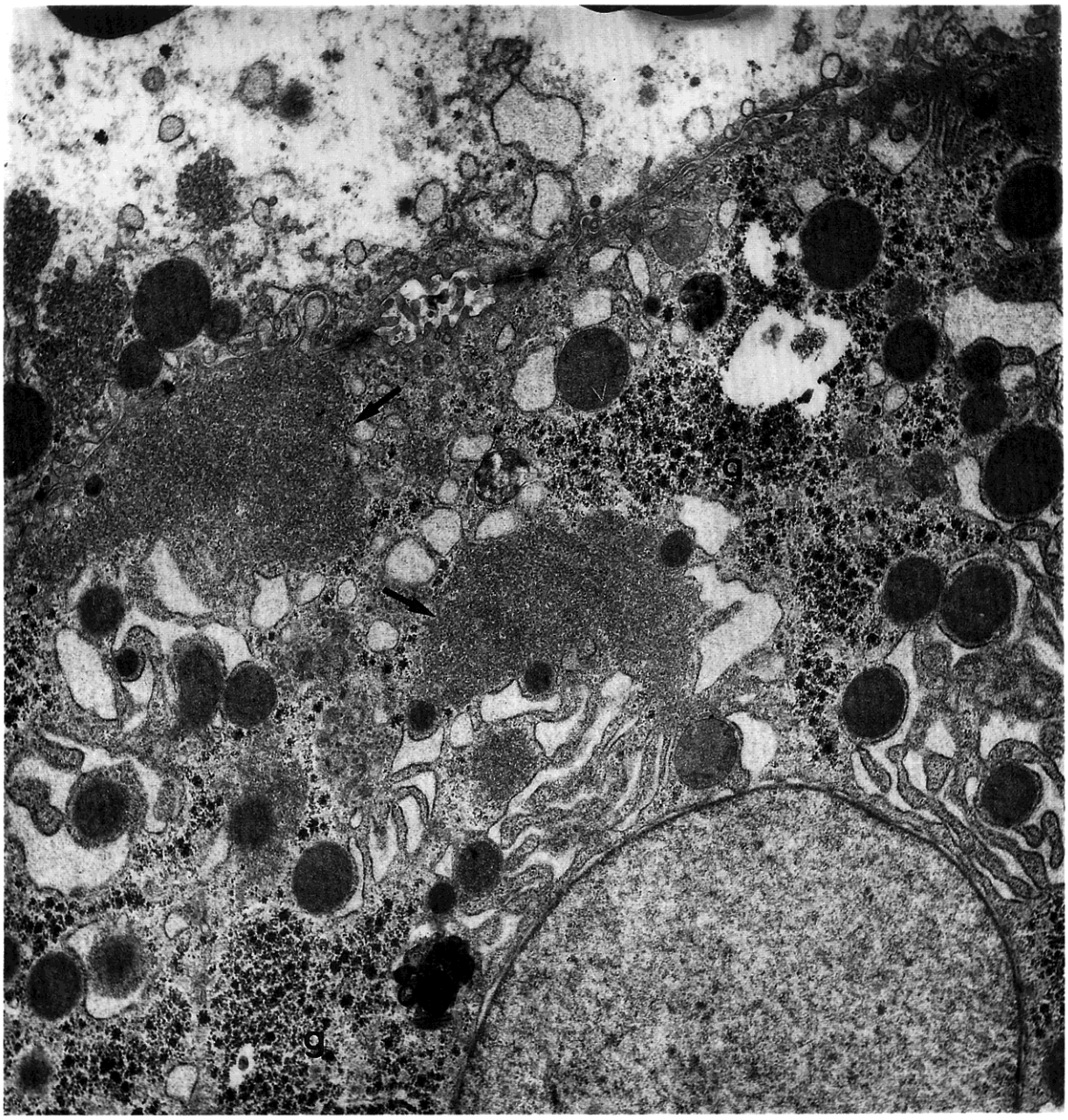



Fig. 3. The dilated cisternae of rough-endoplasmic reticulum containing a moderately electron-dense substance (arrow) are observed at the electronmicrograph of the hyperlipiemic. group. Note the intracytoplasmic gycogen particles (g). Uranyl acetate lead citrate ×13500

also been reported that probucol increases the catabolism of high-density lipoprotein 3 (HDL3) to HDL2[15], and reduces the size of HDL[2]. Probucol was evaluated in hypercholesterolemic Japanese quail, in order to assess its antioxidant effect in preventing the development of atherosclerosis. Probucol reduced the incidence of lesions containing esterase positive cells and the macrophage content of the lesion[8].

Lipid-regulating drugs including probucol should be evaluated with special attention for their ability to cause reproducible morphological degenerative

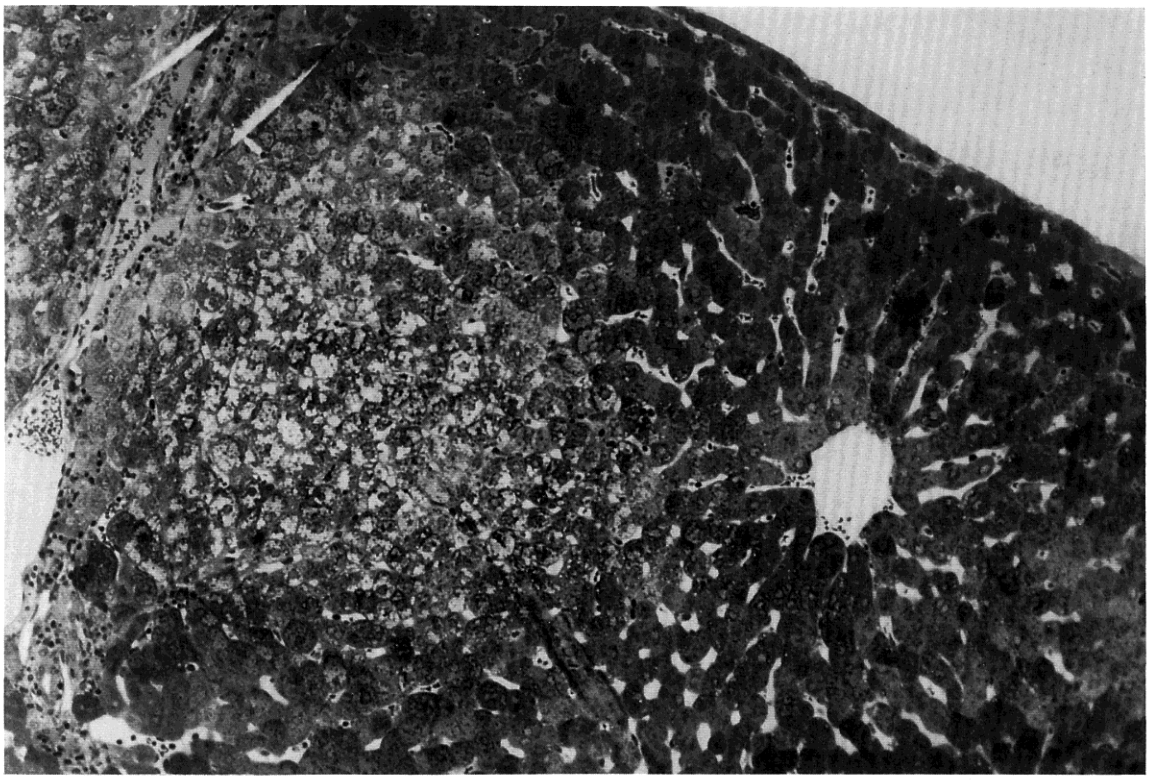


Fig. 4. Micrograph of the probucol-treated hyperlipidemic group presenting the differing localisation and amount of degeneration between lobules. Methylene blue-Azur II ×10.

changes in means of physicians apply lipid-lowering medications in high-risk patients to reduce cardiovascular mortality and morbidity. Adverse effects of probucol are generally reported to be mild and gastrointestinal side effects such as diarrhea are infrequently predominating[15–18]. Increased frequency of ventricular arrhythmia and sudden death in association with QT interval prolongation were reported in some studies[19,20].

Studies on the effects of probucol and other hypolipidemic agents on the liver are of limited number. Svoboda *et al.* compared the effects of several hypolipidemic agents excluding probucol morphologically and reported the increase of peroxisomes at the subcellular level[5]. The effects of probucol on rat liver cell ultrastructure were comparatively studied with two other hypolipidemic drugs in a unique study of Barnard *et al.*[6]. In this study, probucol is reported not to cause any significant changes at the subcellular level with the absence of hepatocytomegaly, cytoplasmic eosinophilia and granularity comparing to other agents. The peroxisomes were also reported to preserve their normal appearance. The only change at subcellular level due to probucol treatment was an occasional increase in smooth-surfaced endoplasmic reticulum when compared to the control group[6].

In this study, the samples of the hyperlipidemic group revealed the presence of several basophilic bodies at the light microscopical level which were proved to be groups of dilated rough surfaced endoplasmic reticulum complexed with mitochondria by electron microscopy. The overworking appearance of the rough surfaced endoplasmic reticulum can be the consequence of increased protein synthesis needed for the production of more lipoproteins to carry the excess amount of lipids in blood. The close relation of mitochondria with the rough surfaced endoplasmic reticulum can be explained as the increased need of energy for increased synthesis of proteins. Formation of lysosomal bodies must be the consequence of the trial to metabolise the excess lipid loaded.

In the probucol treated hyperlipidemic group the rough surfaced endoplasmic reticulum gained back its normal structure probably due to the fact that

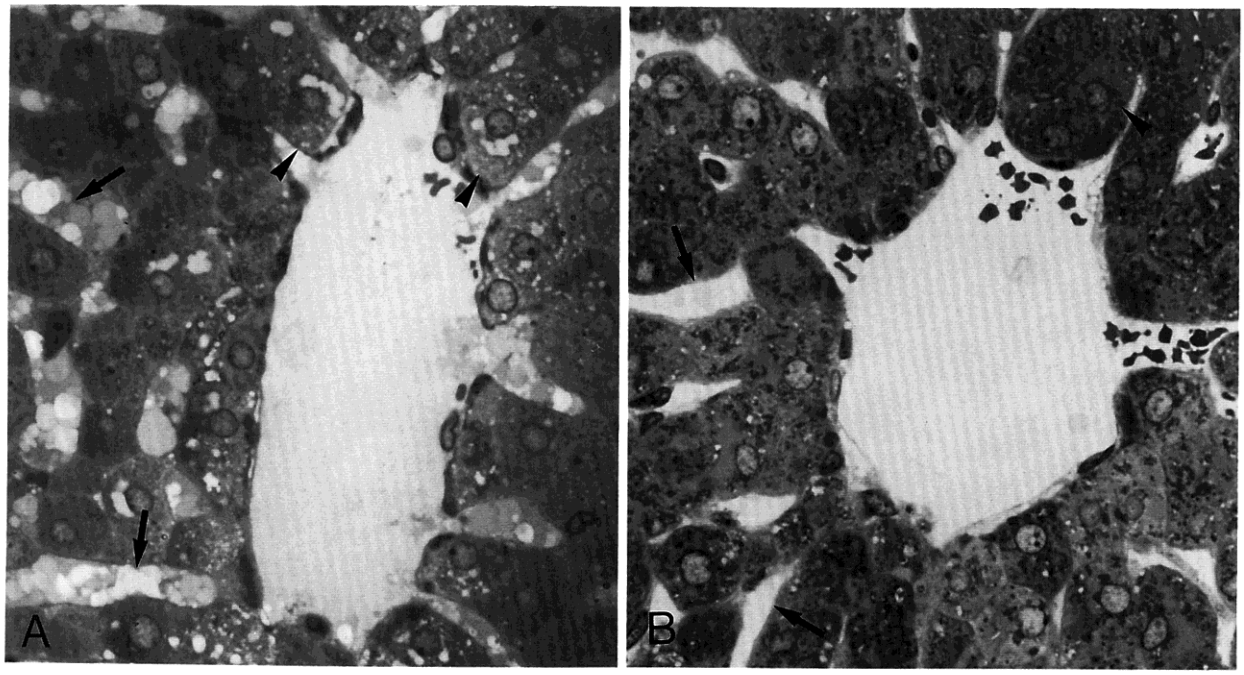



Fig. 5A, B. (A): Note the dilated and lipid-filled sinusoids (arrows) in the vicinity of the central vein and the vacuolated appearence of the parenchymal cells (arrow heads), and in another lobule, at the semi-thin section from the probucol-treated hyperlipidemic group. (B). Except the dilatation, in the vicinity of the central vein., parenchymal cells (arrow heads) and sinusoids appear normal (arrows) in the same group. Methylene blue-Azur II ×40.

probucol decreased the blood lipid levels and there was no need for carrier lipoproteins any more. In this group, the smooth surfaced endoplasmic reticulum increased in amount in accordance with Bernard *et al.* finding[6]. Furthermore, the smooth-surfaced endoplasmic reticulum had a degenerative appearance and was in close relation with the mitochondria at the subcellular level. The metabolic fate of probucol is not clear yet. There are few studies on the pharmakokinetics and tissue distribution of probucol showing that the drug concentration is higher in plasma than in adipose tissue, adrenal glands and muscle, heart and liver during long periods of administration[21]. The cause of the disseminated and degenerated appearance of smooth surfaced endoplasmic reticulum may be the trial of metabolisation in this organelle and forcing it beyond physiological borders having toxic effects on it. The close relation of smooth surfaced endoplasmic reticulum with the mitochondria must be because of excess energy needs. The enforcing and toxic effects of the drug may cause mitochondrial degeneration.

These proposed effects of probucol may be reversible at this dosage and duration of application, as we observed lobules of differing amounts and localisation of degeneration. In conclusion, probucol does not appear to be a completely safe drug for the liver parenchymal cell. It may have a time and dose-dependent toxic effect. Further studies investigating these concepts and the reversibility of them should be performed. The metabolic pathway of the drug may be searched by biochemically labelled markers.

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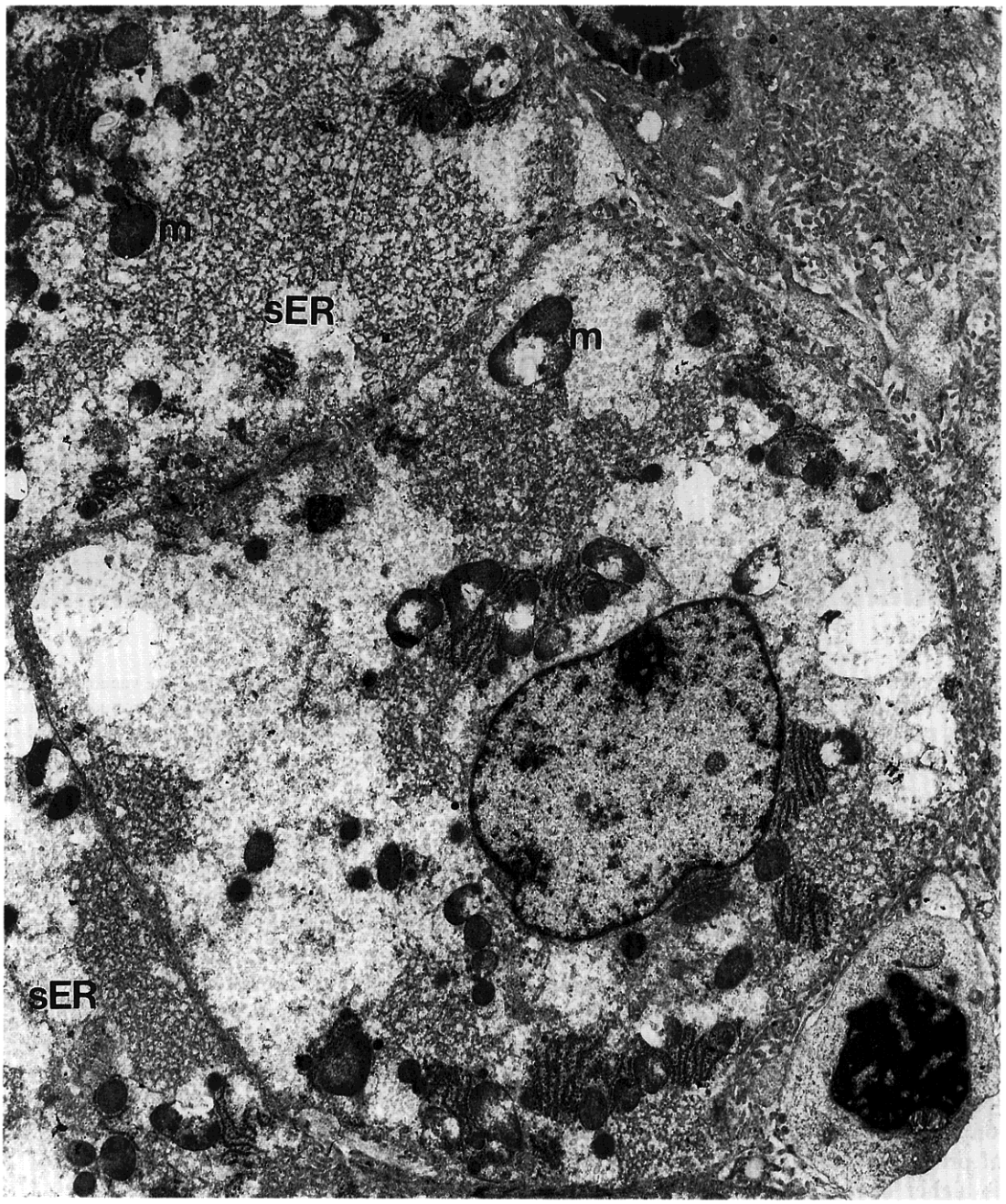



Fig. 6. The extensive amount of degenerated smooth-surfaced endoplasmic reticulum (sER), the condensed and degenerated mitochondria (m) and their close apposition are observed at the electronmicrograph of the probucol treated hyperlipidemic group. Uranyl acetate lead citrate ×5700

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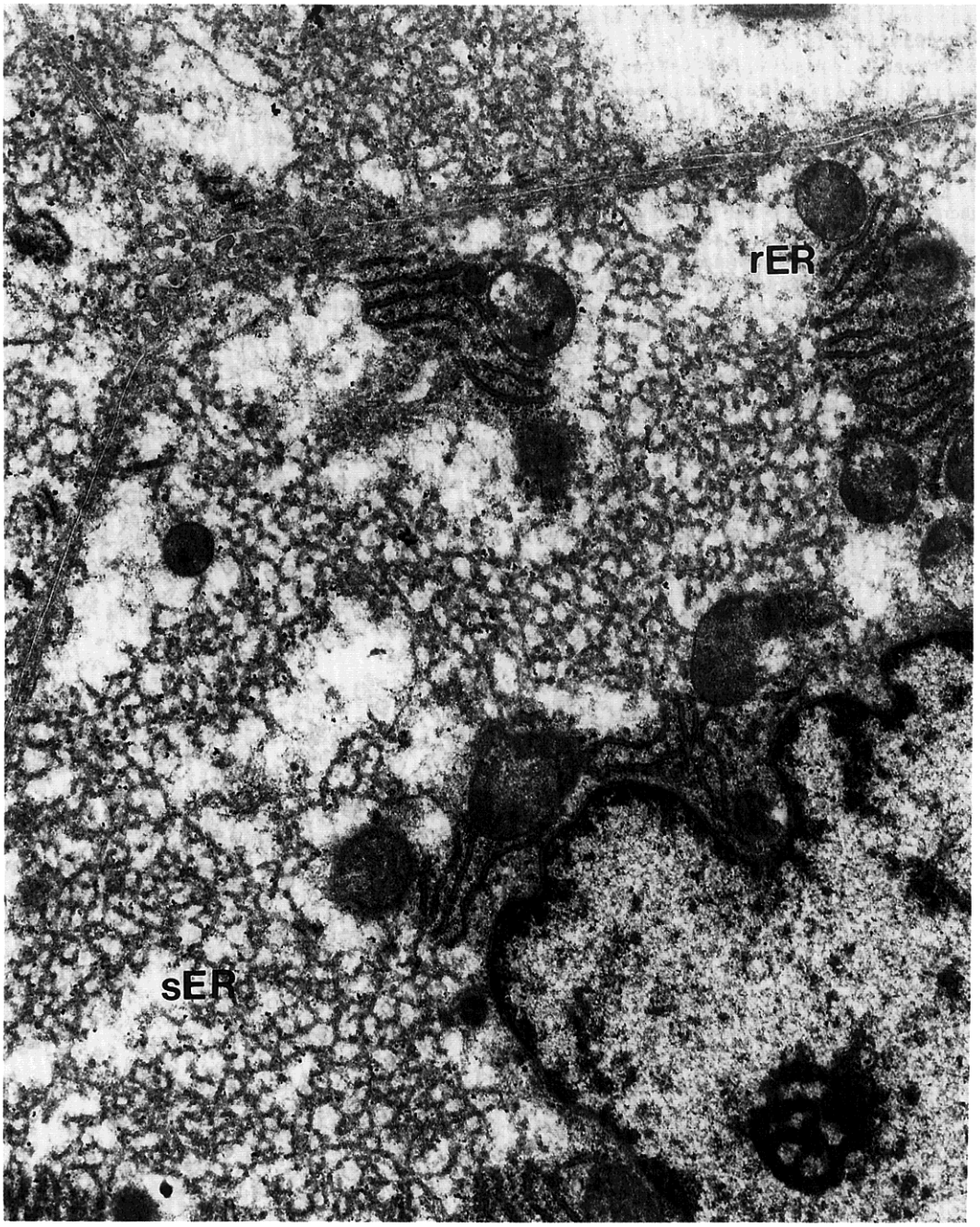



Fig. 7. Electronmicrograph of the probucol-treated hyperlipidemic group presents normal appearence of the rough-surfaced endoplasmic reticulum (rER) and the reticular pattern of the smooth-surfaced endoplasmic reticulum (sER). Uranyl acetate lead citrate ×13500

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