

EFFECT OF PROGESTERONE AND LOVASTATIN ON THE SECRETION OF VERY LOW DENSITY LIPOPROTEIN 1 AND 2 IN THE NORMAL GUINEA PIG MODEL

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Abstract- Liver secretes a large range of very low density lipoprotein (VLDL) particles of different sizes and Svedberg flotation rates (Sf). In plasma VLDL is converted to low density lipoprotein (LDL) which in turn plays an important role in the development of atherosclerotic diseases; however, triglyceride (TG) rich VLDL1 has been shown to be more deleterious than cholesteryl ester (CE) rich VLDL2. There is evidence that the liver intracellular pool of CE and TG can regulate secretion of VLDL. To study the effect of CE pool on VLDL1 and VLDL2 secretion, guinea pig liver was perfused with Krebs-Henseleit buffers containing lovastatin, progesterone and lovastatin plus progesterone. Perfusate pools of VLDL1 and VLDL2 were separated by cumulative flotation ultracentrifugation, confirmed by electron microscopy, and in each pool TG, CE and total lipid were measured. Progesterone had no significant effect on total lipid analysis in either VLDL1 or VLDL2 pools while lovastatin lowered the total lipid by 20% in VLDL1 and 41% in VLDL2 based on percent 90 minute point. Lovastatin decreased TG by 21% and CE by 20% in VLDL1 while it decreased TG by 39% and CE by 56% in VLDL2, based on percent 90 minute point slope change. On the other hand, while lovastatin plus progesterone lowered TG by 22% and CE by 23% in VLDL1, it lowers TG and CE by 39 and 64% in VLDL2, respectively. CE has an important role in the lipitation process and secretion of VLDL2 particles in normal guinea pig liver perfusion system.

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INTRODUCTION

Very low density lipoprotein (VLDL) particles are synthesized in the liver and are secreted into the plasma (1). In plasma, VLDL is converted to low density lipoprotein (LDL) through the delipidation

process by lipolytic step (2). LDL particle size and composition have been demonstrated to be important risk factors for coronary artery disease (3-5). However, oxidized VLDL has also been proposed to play a role in the development of atherosclerotic lesions (2).

VLDL is comprised of heterogeneous population of particles varying in Svedberg flotation rate (Sf), origin, structure and receptor interaction. Various subclasses of VLDL will, therefore, differ regarding their metabolism, immunoreactivity, response to lipid

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lowering therapy and possibly their role in the development of atherosclerosis (6). So, the metabolic fate of a lipoprotein has been shown to be determined by its composition (7).

There are two major subclasses of VLDL particles produced by the liver: 1) large triglyceride rich VLDL1 with 450-900 Å diameter (Sf 60-400) and 2) the smaller and more dense VLDL2 with 300-450 Å diameter (Sf 20-60) (8). Demant and Packard have demonstrated that VLDL1 is the precursor of a slowly catabolized LDL particle, which is the atherogenic lipoprotein subtraction (9).

Dixon and Ginsberg believe that the rate of synthesis of VLDL depends on the amount of available lipid substrate rather than on the production of apolipoprotein B (apo B) (10). Moreover, there is accumulating evidence from both *in vitro* and *in vivo* studies that cholesterol, cholesteryl ester (CE) and the mass of neutral lipid availability can regulate secretion of hepatic apo B containing particles (11). In this regard, the mechanism by which the liver is able to vary the amount of secretion of large versus small VLDL is unknown. Packard and Shepherd believe that the release of VLDL2 is possible by addition of a relatively small quantity of triglyceride (TG) or CE to the nascent particle while VLDL1 is formed by the addition of a substantial amount of TG to it (12).

Clinical trials data have demonstrated the efficacy of the statins drugs in reducing plasma cholesterol and decreasing risk of cardiovascular diseases (13). However, some studies have shown that reduced rates of CE production induced by statins do not result in reduced rate of VLDL secretion (14,15). On the other hand a number of studies have provided evidence that the inhibition of cholesterol synthesis by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors decreases the hepatic assembly and secretion of apolipoprotein containing lipoproteins (16,17). Interestingly progesterone, female sex steroid hormone, has been shown to increase VLDL apo B secretion rate in the baboons (18), although some studies are in contrast (19).

This study was undertaken to see if perfusion of progesterone and lovastatin can affect the composition and the rate of secretion of different subclasses of VLDL particles.

MATERIALS AND METHODS

Lovastatin and progesterone were provided by Razak and Iran Hormone Co. (Tehran, Iran), respectively. Bovine serum albumin (BSA), polyethylene glycol (PEG), ethanol, heparin, glucose, NaCl, KCl, KH₂PO₄, CaCl₂ and NaHCO₃ were purchased from Merck Co. Aspartate transaminase (AST) and lactate dehydrogenase (LDH) assay kits were purchased from Zist-Chimi Co. (Tehran, Iran).

Albino male guinea pigs (350-400 gr) were obtained from Iran Razi institute and all experiments on animals were performed in accordance with UK legal requirements.

Guinea pigs were randomly assigned to one of the four different experimental groups: control, lovastatin, progesterone and lovastatin plus progesterone.

Animals were fasted for 12 hr prior to experiments. Water was given *ad libitum*. Liver perfusion was done based on the procedure of Fitzharris (20). Briefly, animals were anesthetized with chloroform through a mouth cap. Heparin (1250 units/kg) was injected i.p. to prevent blood clotting. Perfusion of isolated livers was performed in the completely closed system such that a cannula was inserted into portal vein and the outflow of the perfusate was recirculated via a cannula into the vena cava (2.5 ml/min/gr liver) using peristaltic pump (Minipuls 3 peristaltic pump; Gilson, Middleton). Initially, non-recirculating perfusion was performed for 30 min (flush period) to allow the release of plasma lipoproteins remaining in the liver prior to experimental manipulation.

Recirculating perfusion (100 ml) was performed for 90 min (drug period) and perfusate aliquots (10 ml) were taken every 15 min.

Liver was kept moist and warm throughout the perfusion and pH was adjusted to 7.3 - 7.4 with the gas pressure (95% O₂ /5% CO₂). The temperature of perfusate was maintained at 37 °C. Liver viability was monitored by measuring perfusate AST and LDH levels.

After perfusion, liver samples were fixed in 10% formalin, stained with hematoxylin and eosin and examined under light microscope to ensure structural integrity of the liver.

Experimental conditions

Doses of lovastatin and progesterone were determined by doses-response tests on the basis of liver viability markers (AST and LDH). For this reason each dose was examined on 4 guinea pig livers. Each drug was dissolved in PEG 300: ethanol 95% (95:5; v/v), conjugated with BSA and added to Krebs-Henseleit buffer [containing 118.9 mM NaCl, 4.79 mM KCl, 1.19 mM KH_2PO_4 , 2.55 mM CaCl_2 , 24.8 mM NaHCO_3 (gassed separately in 95% O_2 / 5% CO_2 for 1 hr and added last), 2.5% glucose and 3% BSA fraction V]. In all experiments the concentration of vehicle (PEG + ethanol) was 0.02%. Treatments were achieved in three different groups (lovastatin, progesterone and lovastatin plus progesterone), so that each group included 4 guinea pig livers.

Isolation of VLDL1 and VLDL2 particles

Isolation was performed by cumulative flotation ultracentrifugation (21). In this procedure, aliquot of perfusates was adjusted to density 1.10 g/ml with solid NaCl. Four ml of perfusate and 3 ml of each of gradients (1.065, 1.020 and 1.006 g/ml) were added into the ULTRA clear tubes. Ultracentrifugation was performed in SW Ti 40.3 swinging bucket rotor (Beckman x-100 ultracentrifuge) at +15 °C. Two consecutive runs were performed to separate VLDL1 (37800 rpm, 4 hr) and VLDL2 (40000 rpm, 15 hr). After each centrifugation, the top 0.5 ml of the gradient containing the respective lipoprotein subclass was collected and 0.5 ml of 1.006 g/mL density salt solution was used to refill the tube before the next run. All salt solutions were adjusted to pH 7.4. Lipoproteins in the perfusate were concentrated 5 fold by centrifugation at a density of 1.065 g/ml (83150 g for 18 h).

Electron microscopy analysis

VLDL1 and VLDL2 particle sizes were confirmed by electron microscopy. A drop (20 μL) of VLDL1 or VLDL2 was applied to formvar-coated copper grid. After 60 seconds excess fluid was removed by absorption onto filter paper and a drop of 2% potassium phosphotungstate (pH 6.3-6.4) was added. Following evaporation the grids were examined using Siemens 101 electron microscope (Siemens Corp, Iselin, NJ).

Lipid and protein analysis

Lipids were extracted from VLDL1 and VLDL2 in ether-ethanol 1:3 (v/v) and TG was measured by spectrophotometric method (22). Total protein was measured by modified Lowry method (23). Phospholipid and cholesterol were measured by chemical methods. Total lipid was measured by total lipid method (24).

Statistical analysis

Percent 90 minute point change of total lipid, TG and CE among different groups were compared by ANOVA through sigma stat package. In each VLDL fraction slope linear regression change of each of these parameters was analyzed by *t* test.

RESULTS

At First, based on dose response tests and using cytotoxic markers (AST and LDH), 1.5 and 25 μM doses were selected for lovastatin (Fig. 1) and progesterone (Fig. 2) to be optimal.

On the other hand, hepatocellular integrity was determined by light microscopy study. Figure 3 (A and B) shows a cross section of liver; the liver was perfused as described earlier (in experimental procedure). Perfusion of the liver over a period of 120 min did not cause any morphological changes (Fig. 3B) in comparison to the intact liver (Fig. 3A).

Isolated VLDL1 and VLDL2 were confirmed by counting 60 particles in each micrograph of EM and particle sizes were expressed as mean \pm SEM. Particle sizes were determined as $839.9 \pm 0.5 \text{ \AA}$ (Fig. 4A) and $409.7 \pm 0.36 \text{ \AA}$ (Fig. 4B) for VLDL1 and VLDL2, respectively.

Based on division of VLDL into two different sizes (300 - 450 \AA for VLDL1 and 450 - 900 \AA for VLDL2) there were no significant difference in total lipid accumulation in perfusate VLDL1 and VLDL2 pools by progesterone in comparison to control group (Table 1).

We also examined the effects of lovastatin on the secretion rate of VLDL2 particles. Based on the change percentage of 90 minute point, progesterone has no significant effect on total lipid analysis in either VLDL1 or VLDL2 while lovastatin lowered total lipid by 20% in VLDL1 and 41% in VLDL2 pools.

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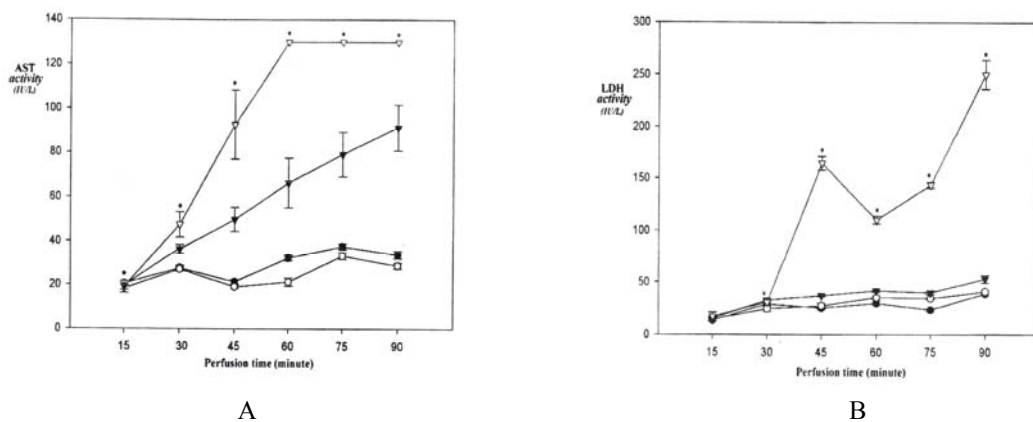


Fig. 1. Dose-response tests for lovastatin in different concentrations, 1 μM (-○-), 1.5 μM (-▼-) and 2 μM (-▽-) in comparison to control (-●-). (*) shows significant difference. Cytotoxicity was determined by cytotoxic markers, AST (A) and LDH (B), in perfusate during 90 min recirculating perfusion.

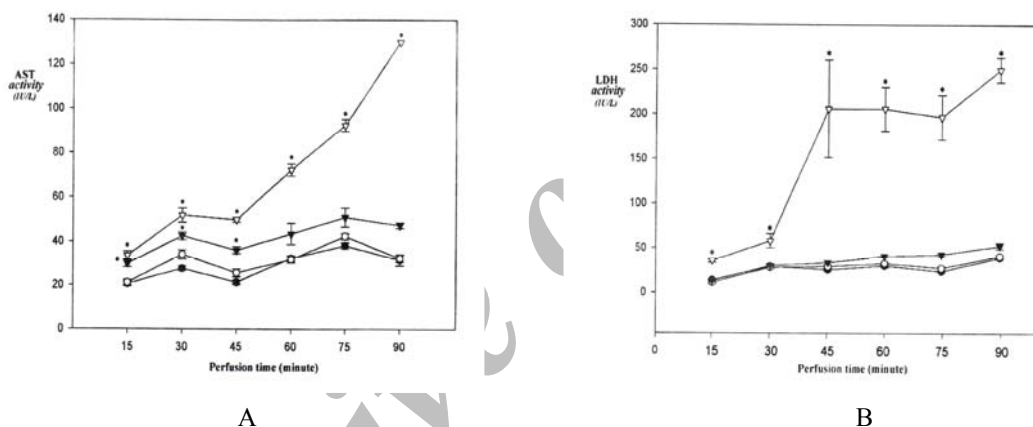
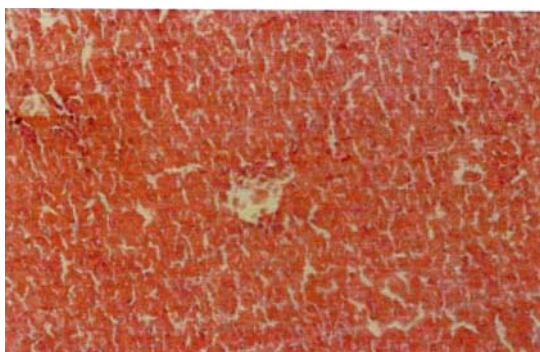
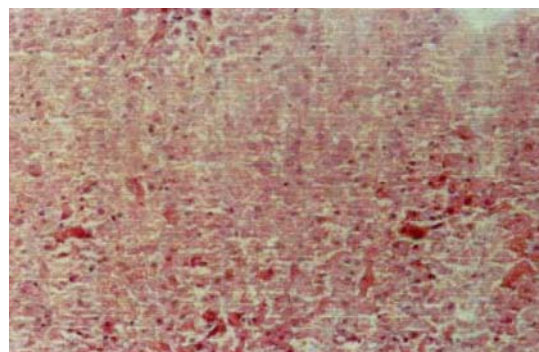


Fig. 2. Dose-response tests for progesterone in different concentrations, 20 μM (-○-), 25 μM (-▼-) and 30 μM (-▽-) in comparison to control (-●-). (*) shows significant difference. Cytotoxicity was determined by cytotoxic markers, AST (A) and LDH (B), in perfusate during 90 min recirculating perfusion.



A



B

Fig. 3. Light microscopic appearance of guinea pig liver in staining with hematoxylin and eosin in intact liver before perfusion (A) and after perfusion (B). Structural integrity of the latter was compared with the former.

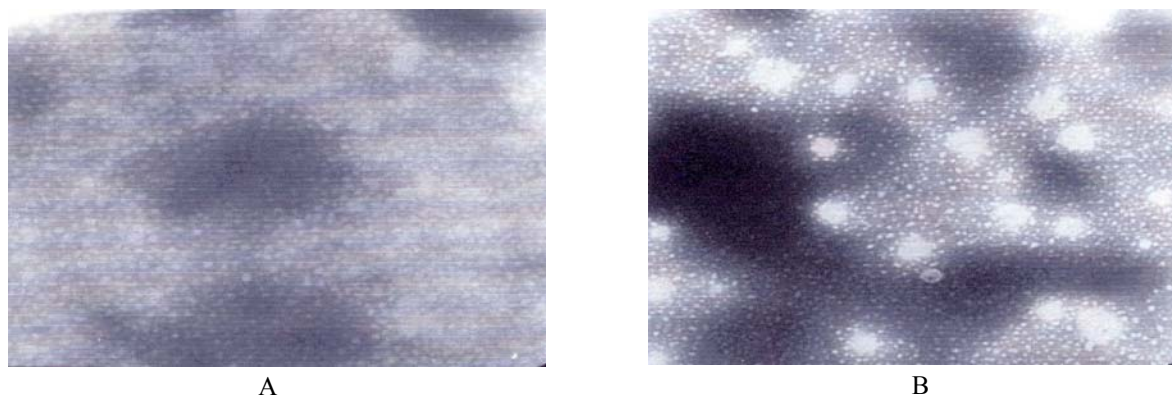


Fig. 4. Electron microscopic photograph of VLDL1 (A) and VLDL2 (B), 50000 magnification, after negatively staining. VLDL size was calculated by counting 60 particles and is expressed as mean \pm SEM.

Moreover, total lipid in treatment with progesterone plus lovastatin decreased by 21 and 44% in VLDL1 and VLDL2, respectively (Table 1). Figure 5A shows the effects as measured by TG content and figure 5B as CE content of VLDL2 as linear regression of accumulation rate.

While in VLDL1 there was no significant decrease (Fig. 5C and 5D), there was statistical

decrease in VLDL2 as analyzed by *t* test ($P < 0.05$). Further study showed lovastatin lowered TG by 21% and CE by 20% in VLDL1 and TG by 39% and CE by 56% in VLDL2.

In this respect, while progesterone plus lovastatin lowered TG by 22% and CE by 23% in VLDL1 it decreased TG and CE by 39 and 64% in VLDL2, respectively.

Table 1. Total lipid accumulation of perfusate VLDL1 and VLDL2 pools in control, lovastatin, progesterone and progesterone plus lovastatin treatment groups during 90 min recirculating perfusion*

Perfusion time(min)	VLDL1- total lipid accumulation (mg/dl)				VLDL2- total lipid accumulation (mg/dl)			
	C	P	L	P+L	C	P	L	P+L
15	0.61 \pm 0.12	0.57 \pm 0.03	0.46 \pm 0.08	0.47 \pm 0.02	0.23 \pm 0.09	0.24 \pm 0.46	0.14 \pm 0.04	0.13 \pm 0.05
30	1.14 \pm 0.29	1.19 \pm 0.05	0.86 \pm 0.22	0.85 \pm 0.07	0.98 \pm 1.9	1.01 \pm 0.4	0.67 \pm 0.09	0.61 \pm 0.11
45	1.82 \pm 0.33	1.87 \pm 0.07	1.48 \pm 0.33	1.34 \pm 0.1	1.75 \pm 0.29	1.8 \pm 0.53	1.13 \pm 0.14	1.07 \pm 0.12
60	2.59 \pm 0.49	2.68 \pm 0.12	2.2 \pm 0.45	2.13 \pm 0.08	2.42 \pm 0.39	2.45 \pm 0.34	1.56 \pm 0.19	1.48 \pm 0.17
75	3.17 \pm 0.6	3.28 \pm 0.13	2.59 \pm 0.51	2.48 \pm 0.14	3.01 \pm 0.46	3.08 \pm 0.42	1.9 \pm 0.23	1.84 \pm 0.18
90	3.83 \pm 0.64	3.98 \pm 0.16	3.05 \pm 0.61	3 \pm 0.12	3.59 \pm 0.57	3.66 \pm 0.3	2.31 \pm 0.27	2.17 \pm 0.24
			(-20%)			(-14%)		

Abbreviations: VLDL, very low density lipoprotein; C, control; L, lovastatin; P, progesterone; P+L, progesterone plus lovastatin
* Data are presented as mean \pm SEM

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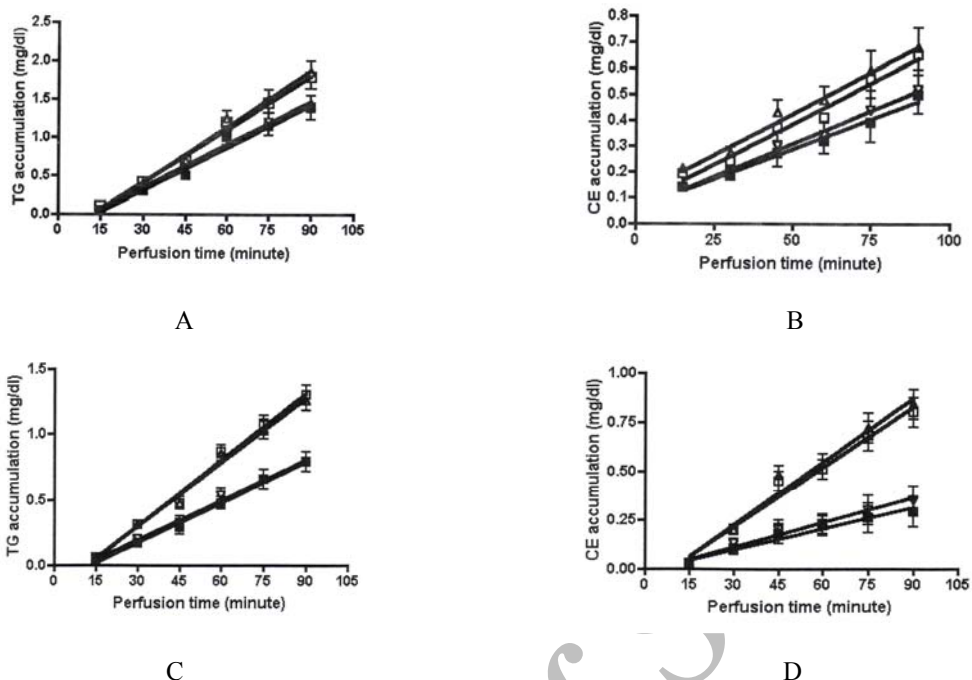


Fig. 5. Perfusate accumulation of VLDL2-TG (A), VLDL2-CE (B), VLDL1-TG (C) and VLDL1-CE (D) in progesterone (Δ), lovastatin (∇) and lovastatin + progesterone (\blacksquare) treatment groups compared to control group (\square). Slope linear regression of each group was compared with control group by *t* test analysis.

DISCUSSION

Burnett, Wilcox and Huff, in line with this study, believe that CE availability can regulate hepatic VLDL secretion (11). Present study shows such a decrease in total lipid level of VLDL1 and VLDL2 pools during the treatment with lovastatin (Table 1). In spite of about 21% decrease in VLDL1-TG and 20% in VLDL1-CE, a significant decrease was observed only in perfusate VLDL2 CE and TG accumulation with lovastatin in *t* test analysis of slope linear regression. Since levels of TG and CE in VLDL1 pool was persistently lower with administration of lovastatin compared to control (Fig. 5), it can be concluded that lovastatin also lowers TG and CE in VLDL1, but only half as much as in VLDL2 pool. These changes in VLDL particles may be due to the changes in the rate of cholesterol synthesis, CE availability (20) or microsomal transfer protein activity (25-27).

Lovastatin decreases the rate of cholesterol

production and reduces cardiovascular risks (11, 13, 17), but we have not been able to show this effect in our study. This can be due to short term effect of lovastatin and the animal modelling. Krause and Newton believe that LDL in guinea pig is directly produced from the liver and is the major cholesterol transporting lipoprotein (28). On the other hand, although LDL is produced directly from the liver, it should be considered that like human subjects, the fraction of cholesteryl ester of VLDLs that converts to LDL is considerably larger than the other animal modellings for studying of cardiovascular disorders (16).

Krause and Newton believe that cholesterol-lowering effect of HMG-CoA reductase inhibitors correlate with triglyceride lowering in LDL animal models such as guinea pig (28). Present study shows this correlation more in VLDL2 than VLDL1, in which whole TG and CE have a significant decrease. Absence of such a decrease in VLDL1 pool is probably due to the effect of short perfusion time (90

minute) on VLDL secretion, as Isusi and coworkers believe that short term effect of statins is different from the long term effect of them on the secretion of CE and TG from the liver (29). Greater effect of lovastatin on VLDL2 may be due to presence of higher levels of CE in VLDL2 compared to VLDL1.

As Avramoglu, Cianflone and Sniderman have mentioned, the secretion rate of apo B100 lipoprotein particles by the liver can differ as much as their lipid composition (30). On the other hand, a close relationship between hepatic CE concentration and apo B containing lipoprotein has been reported by Tanaka *et al.* (31). Although guinea pig has a big reservoir of free cholesterol in liver, it seems that newly synthesized free cholesterol is required for biosynthesis of VLDL. Availability of cholesterol in a putative hepatic metabolic pool, required for secretion and transport of triglyceride in VLDL, is a factor contributing to decreased secretion of the VLDL.

Although Carr, Parks and Rudel showed that lower acyl CoA cholesterol acyl transferase (ACAT) activity in perfused green monkey liver lead to less CE and reduction in VLDL secretion (32), in the present study treatment with progesterone had no significant decreasing effect on VLDL1 and VLDL2 cholesterol and TG accumulation in perfusate. It seems that these differences are due to different animal modelling and use of more specific ACAT inhibitors rather than progesterone. Field and Mathur used oxysterol as an activator of ACAT (19) and Dashti used progesterone as an inhibitor of ACAT (33) and both of them believe that these sterols have several other effects rather than solely activating and inhibiting effect on ACAT.

In conclusion, it seems that lovastatin has a decreasing effect on secretion of VLDL2 particles in perfused guinea pig liver in short term.

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