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UPTAKE AND SUBCELLULAR DISTRIBUTION OF LIPOSOMES IN RAT LIVER IN NORMAL CONDITIONS AND IN CHRONIC HEPATIC INSUFFICIENCY

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Abtract

Liposomal absorption and subcellular distribution in the rat liver in normal conditions and in chronic hepatic insufficiency (CHI) due to venous congestion were compared. The rats were intravenously administered with empty liposomes (EL) and α-tocopherol containing liposomes (TL). Both liposomal types contained 3H-lecithin as a radioactive label. Liposomal absorption and distribution were assessed by the tritium activity in the liver sample and its subcellular fractions. It was found that either type of liposomes is absorbed both by the normal and diseased liver. Intact rats showed around 70% of the label in the liver 1h after EL or TL administration. CHI rats had around 75% and 60% of the label within 1h after TL and EL administration, respectively, which demonstrated the preserved ability of the affected liver to absorb liposomes. During the first 6 hours the slow-down of the liposomal label activity was slower in CHI rats.

Liposomal membrane modification by incorporating α-tocopherol had an impact both on liposomal absorption and cellular distribution. In particular, both groups showed higher affinity of TL to mitochondria as compared to EL. Thus, the results suggest that liposomal compositions can be used for targeted delivery of biologically active substances both in normal and diseased liver and can be the basis for the development of highly effective pharmaceuticals for intracellular therapy.

Key words*: liposomes, mitochondria, hepatoprotection.*

1. INTRODUCTION

Nowadays, liposome-based pharmaceuticals are used as means of drug delivery to the damaged organs. Inclusion of biologically active substances into liposomes allows to carrying out their targeted transport, reducing toxicity and protecting them from breakdown by blood enzymes [11, 13].

On the one hand, liposomes absorption depends on the structure of liposomes themselves (size, surface charge, structural rigidity of the bio layer, membrane modification by blood factors, presence of polysaccharides and proteins on the surface *etc*.) [4, 10,13]. On the other hand, it depends on the structure of the organ (the structure of the vascular bed, presence and size of fenestrae in capillaries, availability of macrophages) [4, 5, 13].

It has been shown that most of the intravenously administered unmodified liposomes are absorbed by different liver cells [4, 10]. This feature of the liposomes allows using them for targeted drug delivery to a particular organ. In spite of the high interest to this problem, the process of liposomes absorption by the liver has been studied only in the experiments on healthy animals. The characteristics of absorption and distribution of liposomes in the diseased liver are still have to be investigated.

Intracellular distribution of liposomes plays an important role in the liposome-mediated delivery of biologically active substances to the tissues. Cell damage caused by pathological processes is accompanied by dysfunction of subcellular organelles [8, 9]. That is why targeted delivery of the molecules directly to the damaged subcellular structures has become a very important task in creating modern pharmaceuticals.

In the present study, α-tocopherol was chosen as a model pharmaceutical for evaluation of the liposomal transport of lipophilic agents to the liver since α-tocopherol is water insoluble and cannot be introduced directly into the bloodstream. Furthermore, α-tocopherol is an important natural antioxidant.

The aim of the present work was to carry out a comparative study of the absorption and subcellular distribution of empty liposomes and liposomes with α-tocopherol in the liver of rats in normal and chronic liver failure caused by venous congestion.

2. MATERIALS AND METHODS

The model of chronic liver failure

All procedures conform to the Directive 2010/63/EU of the European Parliament and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The experiments were carried out on 300-330 g male Wistar rats. The abdominal cavity of the animals was dissected under the isoflurane anaesthesia. Chronic liver failure was modeled by ligation of the right and the central hepatic veins distal to the confluence of the inferior vena cava. Ligation of the vein was performed gradually over 2-3 minutes. Chronic liver failure (CLF) was developed in 8 weeks as a result of venous congestion. The presence of hepatic failure was confirmed by assessing morphological integrity of the organ and activity of the marker enzymes in the blood (lactate dehydrogenase, sorbitol dehydrogenase, arginase, and γ-glutamyl). Two types of liposomes were used in the experiments, empty liposomes (EL) and liposomes with α-tocopherol (TFL).

Preparation of radiolabeled liposomes.

Liposomes were prepared from multilamellar vesicles by extrusion through polycarbonate filters with an extruder (Lipex Biomembranes Inc., Canada). Before extrusion, lipid film consisting of egg lecithin (Lipoid, Germany) and cholesterol (Sigma) in a molar ratio of 7:5 was prepared on a 0.5 l glass flask using a rotary evaporator (Heidolph, Germany). When producing TFL, α-tocopherol was added to the flask of the evaporator at the stage of production of the lipid film. The molar ratio of lecithin/cholesterol/ α-tocopherol for TFL was 7:3:1 respectively. α-tocopherol was labelled with ^{3}H-phosphatidylcholine at the stage of production of the lipid film at 1333.3 μCi /g lipid. The resulting lipid film was hydrated with PBS pH-7.4 (Gibco) and shaken until formation of the multilamellar vesicles. The suspension of the vesicles was passed 10 times through the extruder using polycarbonate filters (Costar, Canada) with a pore size of 100 nm. The lipid content in the resulting product was 25 mg/ml.

Infusion of the labelled liposomes and determination of radioactivity level.

The labeled liposomes (0.3 ml per animal) were administered via the rat tail vein. Total radioactivity of the administered liposomes was 10 μCi.

Rats were divided into four groups: 1 – intact rats with EL (Control EL); 2 – intact rats with TFL (Control TFL); 3 – CLF rats with EL (CLF-EL); and 4 – CLF rats with TFL (CLF-TFL). After 1, 2, 4, 12, 24 and 48 h following the infusion of the liposomes, rats were killed by stunning and cervical dislocation, liver was excised and weighed. Eight rats of each group at each time point were used. Liver samples were homogenized in a glass homogenizer with PBS (4 ml/g of liver tissue). Part of the homogenate was used for determination of the overall radioactivity of the label tissue. For this purpose, chloroform/methanol mixture was added to 0.2 ml of the homogenate and the radioactivity of the sample was determined by the method described below. These data were used to calculate the percentage of the lipid radiolabel normalized for the total weight of the liver.

Another part of the homogenate was used to prepare subcellular fractions. For this purpose, 1 ml of the homogenate was centrifuged sequentially at 3000 g for 30 min, at 10,000 g for 30 min and at 100,000 g for 40 min. Thus the crude, mitochondrial and microsomal fractions were separated [3]. The crude fraction containing nuclei and cell debris was not used in the study. Fractions obtained during the second and third centrifugation were resuspended in 1 ml of PBS. After the third centrifugation, 0.4 ml of methanol and 0.2 ml of chloroform were added to 0.2 ml of the suspension of resultant pellet and supernatant. The mixture was stirred vigorously for 30 min at room temperature and 0.2 ml of PBS and 0.4 ml of chloroform were added. After separation of the chloroform and water fractions by centrifugation at 10000 g for 1 min, 0.1ml of the chloroform, fraction was used for determination of radioactivity by the liquid scintillation counter RackBeta (LKB, Sweden) in 5 ml of the standard toluene scintillator. In order to evaluate quenching of scintillation in the solution, after the initial counting, an aliquot (10 μl) of the chloroform solution containing ^{3}H-lecithin at radioactivity of about 100,000 cpm was added to each vial and the vials were counted again.

Activity of tritium in each sample was calculated using the following formula:

$$I = I_1 / (I_0/I_2-I_1),$$

I_1 - the rate of counting of the sample at the first reading (cpm), I_2 – the rate of counting of the sample at the second reading (cpm), I_0 - the rate of counting of the additional aliquots of lecithin (cpm).

In each sample, the tritium activity in the fractions of mitochondria, microsomes and the supernatant were summed and defined as 100%. Then the percentage of the radiolabeled content of lecithin was calculated in each fraction.

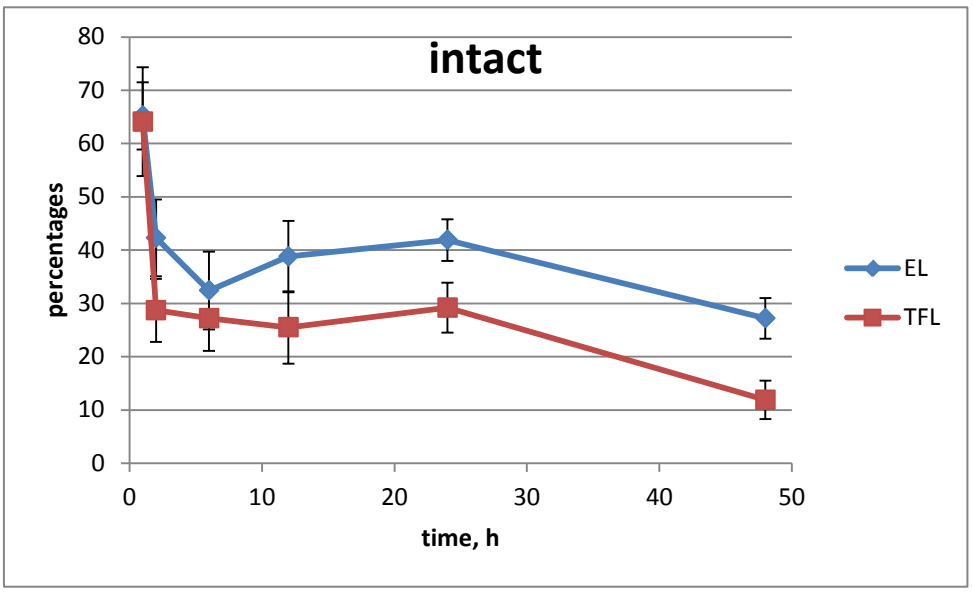
Radioactivity of the samples was measured at the Institute of Chemical Biology and Fundamental medicine SB RAS, Novosibirsk, Russia.

Statistical Analysis was carried out using the software Statistica, version 7.0. Data are presented as mean ± SEM. Statistical significances of the differences between groups were evaluated using one-way ANOVA followed Tukey's multiple comparison post hoc test. Differences were considered significant where $P<0.05$.

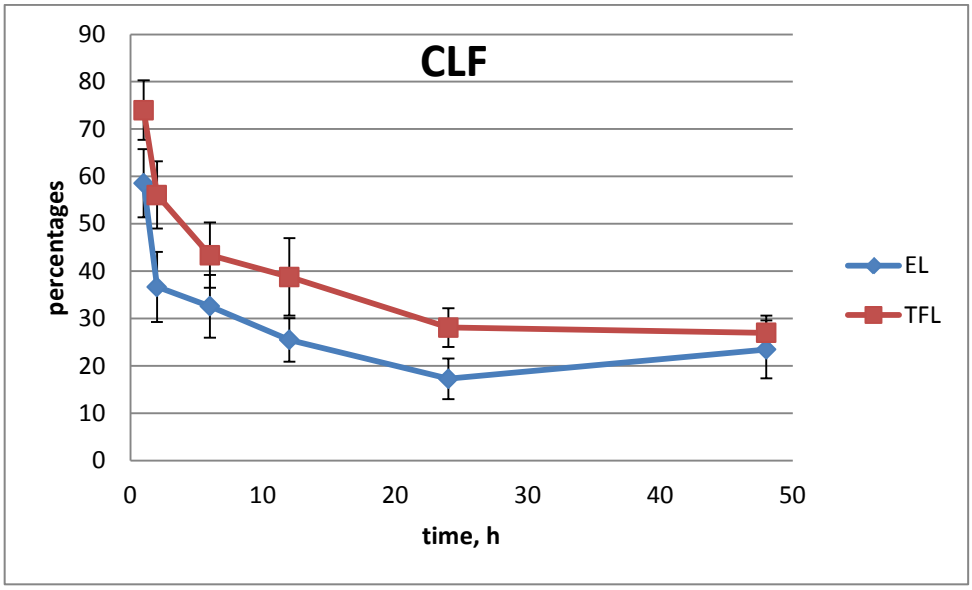
3. RESULTS AND DISCUSSION

Study of the inclusion of liposomes in the liver of intact animals and redistribution of the radiolabel included in EL and TFL has shown in general similar dynamics of changes in the activity of the radiolabel but with some quantitative differences. These differences might be associated with changes in the properties of liposomal membrane when α-tocopherol was included in composition of the liposomes.

Thus, at 1 h after administration of both EL and TFL, up to 70% of the activity of radiolabel was recorded in the liver. After 2 h, 33% of the absorbed radiolabel of EL and 54% of the label of TFL left the organ (Fig. 1). This could be associated with the metabolism and utilization of liposomes by liver tissue. Then, in the case of TFL, current activity of radiolabel remained approximately unchanged during the first day. In contrast, in the case of EL, increase in activity of the label was observed by the end of the first day. The latter may indicate a re-capture of the excreted label from the bloodstream by the liver cells. Since the capture and transport of lipids with blood lipoproteins play an important role in metabolism, we can assume that after the release of ^{3}H-lecithin in the bloodstream, part of the label became bound to lipoproteins. Then, the label included into lipoproteins was again accumulated in the liver cells. Such migration cycle of liposomal lipid was confirmed in experiments of Scherphof *et. al*. [10]. Another possible mechanism of the reuptake of radiolabel by liver cells can be realized by their biliary excretion followed by reabsorption by the gastrointestinal tract. By the end of the second day in both groups, the concentration of radiolabel in the liver decreased significantly, which can be explained by excretion of the products of catabolism of the liposomes. It should be noted that the concentration of the label after the administration of TFL was somewhat lower throughout the whole period of observation.



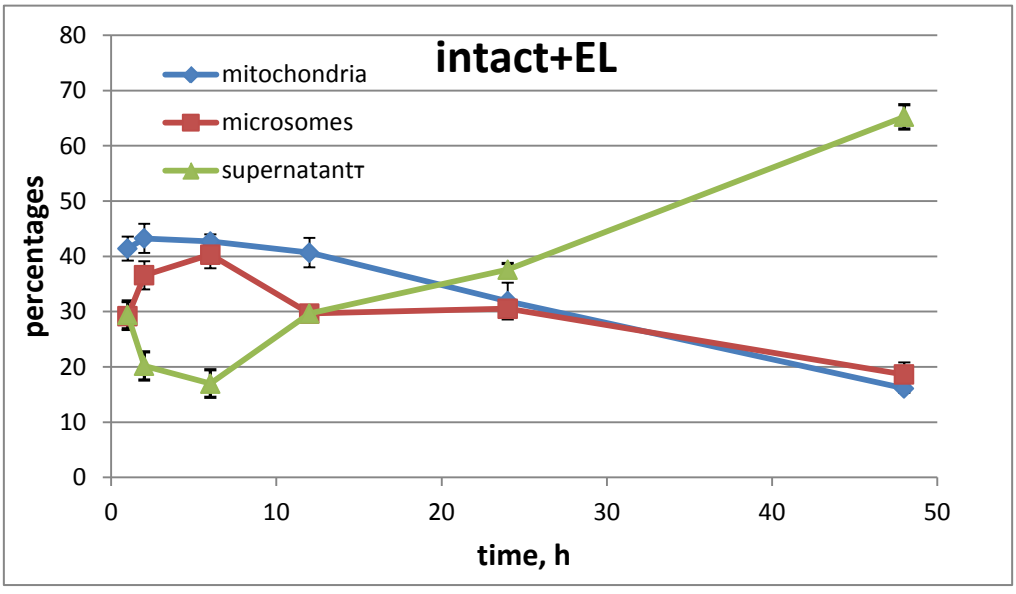
A



B

Fig.1. Kinetics of activity changes of ^{3}H-lecithin incorporated in the composition of the EL and TFL in the liver of intact (A) and CLF rats (B).

With the administration of the EL and TFL to animals with CLF, absorption of the radiolabeled liposomes by the liver tissues was also observed. This confirms that in the congestive type of CLF liver retains the capacity to absorb liposomes. However, dynamic characteristics of the absorption process in these rats differed from those in intact animals. In CLF animals, within 1 h after administration of TFL, there was accumulation of up to 80% of the radiolabel in the liver whilst only 60% of the label was accumulated after administration of EL (Figure 1B). The concentration of the label in the hepatic tissue in both groups of liver failure was levelled off only 2 days after the administration of liposomes. During this period, there was a gradual decrease in activity of the label. There was no reuptake of the radiolabel observed in the CLF animals. It is worth noting that during the first 6 hours, decrease in activity of the labels in animals with CLF was slower as compared with the intact rats. Apparently, this was due to the general decrease in the activity of metabolic processes in the diseased liver. During the 24 h period, a trend of increased absorption of TFL relative to PL could be noticed in the "congested" liver. It can be assumed that the more active capture of TFL by the failing liver was caused by participation of α-tocopherol in the processes of inhibition of lipid peroxidation in hepatocyte membranes.



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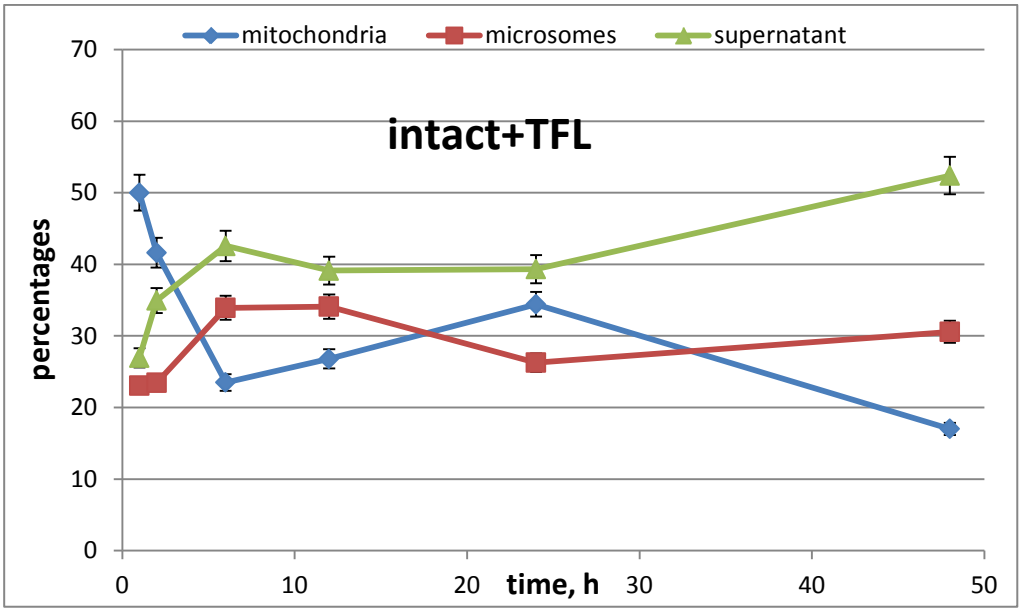
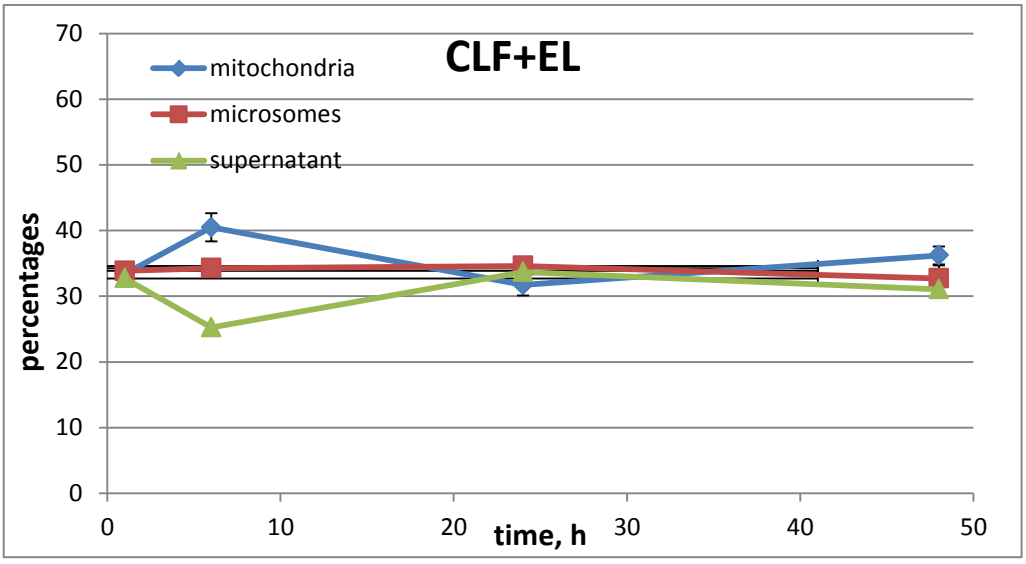


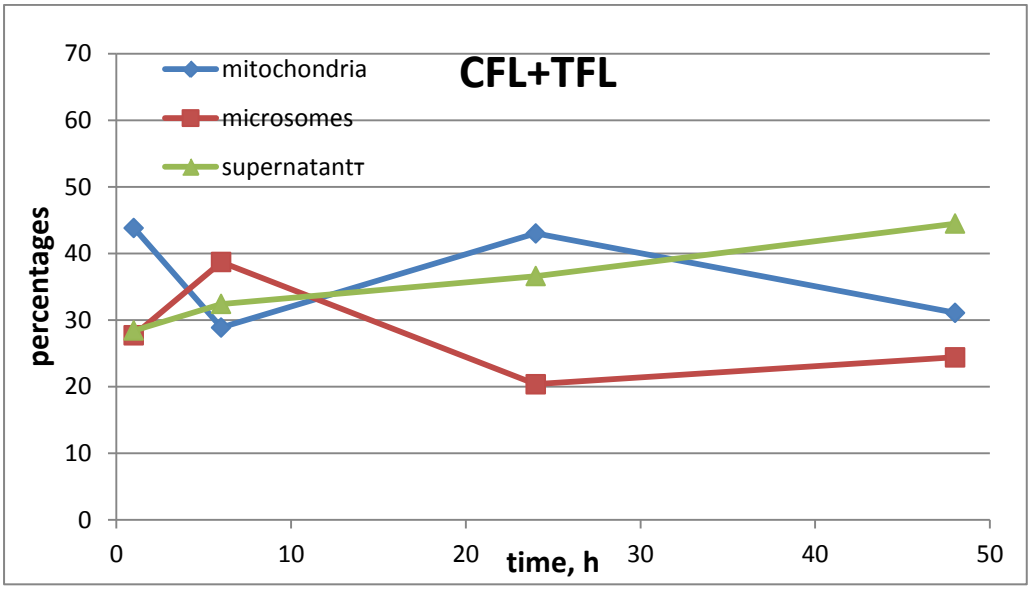
B

Fig.2. Kinetics of the subcellular distribution of ^{3}H-lecithin administered with EL (A) and TFL (B) in the intact rat hepatocytes.

Comparative study of the kinetics of the subcellular distribution of ^{3}H-phosphatidylcholine in hepatocytes of intact and CLF rats treated with EL and TFL (Fig. 2) showed that 1 hour after administration of EL, over 40% of the administered label was found in the mitochondria of the control animals whilst in the microsomes and the supernatant, there was 30 % of the label. Between 2 and 6 hours, the relative content of the labels in mitochondria remained constant while it was increased in microsomes due to translocation from the supernatant. Between 12 and 48 hours, the label content was gradually increasing in the supernatant and decreasing in the mitochondria and microsomes.



A



B

Fig. 3. Kinetics of the subcellular distribution of ^{3}H-lecithin administered with EL (A) and TFL (B) in the hepatocytes with CLF.

In animals with congestive CLF (Fig. 3), even distribution of the liposomal label between the factions was observed during 1 h after administration of EL. After 6 hours, the radiolabel appeared to be accumulated in the mitochondrial fraction and reduced in the supernatant. After 24 and 48 h, the label content was equilibrated between the fractions and fluctuated in the range of 30-36%.

Meanwhile, when TFL were used, the concentration of the label in the mitochondrial fraction was close to 50% in 1 hour after the administration. The label content in microsomes and the supernatant was about 25%. Then, in the period up to 6 h, almost linear decrease in the activity of the label in mitochondria was observed while the activity in the microsomal fraction and the supernatant was increasing. In the period between 6 and 48 h, concentration of the label in the microsomal fraction was maintained at about 30% whilst the changes of this parameter in the mitochondria and the supernatant were multidirectional.

Thus, in intact animals, empty liposomes are more evenly distributed over the subcellular fractions and faster move to the supernatant than the antioxidant ones. At the same time, microsomal structures, which are more stable than mitochondria, retain the TFL

We can assume that an even distribution of the radiolabel between factions was associated with reduced activity of biochemical processes in chronic liver failure. In 1 h after administration of TFL, in animals with CLF, more than 40% of the radiolabel appeared in the mitochondrial fraction and about 30% in each of microsomes and supernatant fractions. By 6 h, the label content in mitochondria decreased. However by 24 h, it was increased again. The characteristic feature of this group was the increase of the label content in the supernatant fraction with multidirectional changes of this parameter in the fractions of mitochondria and microsomes. Preferential accumulation of the radiolabel in mitochondria similar to that observed in the group of intact animals could be caused by reparative effect of α-tocopherol in the ischemic cells.

It should be noted that the inclusion of α-tocopherol into the membrane influences both absorption of the liposomes by the cells and their intracellular distribution. Since the composition of the liposomal membrane largely determines the nature of the interactions of liposomes with cells [4, 5], one can assume that the presence of α-tocopherol in the lipid phase of the membrane modifies its physicochemical characteristics and, consequently, parameters of absorption and distribution of the liposomes.

It is noteworthy the increase in tropism of TFL towards mitochondria found in this study. It is known that various liver lesions are accompanied by dysfunction of mitochondria [1, 4, 8, 9]. Mitochondrial dysfunction leads to the production of reactive oxygen species and uncontrolled activation of free radical oxidation, resulting in cell destruction. Apparently, previously obtained data demonstrating high therapeutic efficacy of treatment of the congestive liver failure with liposomal formulations containing antioxidants [6, 7] are associated with the inactivation of reactive species production by the mitochondria.

4. CONCLUSION

In conclusion, it can be suggested that liposomal formulations can be used to deliver biologically active substances into cells both in normal conditions and in liver disease. In spite of the various quantitative and qualitative characteristics of the absorption, the fact of inclusion and intracellular utilization of liposomes by the liver cells is in no doubt. In general, the ability of liposomes to penetrate hepatocytes may allow in the near future to create compositions targeted to certain cell organelles, and can serve as a basis for the development of highly effective pharmaceutical products for cellular therapy.

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