In Vitro Studies Investigating the Effect of Subcutaneous Phosphatidylcholine Injections in the 3T3-L1 Adipocyte Model: Lipolysis or Lipid Dissolution?

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Background: The demand for lipolytic injection therapies for aesthetic indications increases continuously. The substance most frequently discussed in this context is phosphatidylcholine solubilized in sodium deoxycholate, a composition known in Europe as Lipostabil (Artesan Pharma, Lüchow, Germany). The evidence for its lipolytic effect is based mainly on clinical studies that suggest a decrease in adipose tissue volume after subcutaneous injections. However, neither the actual effect nor the effective supplemental effect of Lipostabil has been clearly identified so far. The purpose of this study was to investigate the effects caused by lipolytic formulations on adipocytes using an in vitro model.
Methods: 3T3-L1 adipocytes in 12-well plates were exposed to varying doses of isolated phosphatidylcholine, deoxycholate, and the combination of both (Lipostabil). Subsequently, changes in the cell membrane integrity were evaluated microscopically, and assays measuring the amount of glycerol as a biochemical lipolysis substrate (lipolysis assay) together with dimethyl thiazolyl diphenyl tetrazolium assays were performed to quantify the lipolytic effect and the cell viability.
Results: Deoxycholate reduced cell viability significantly ($p < 0.05$), even at low concentrations. Neither phosphatidylcholine nor deoxycholate led to a significant ($p < 0.05$) induction of a lipolytic pathway. Lipostabil, the combination of deoxycholate and phosphatidylcholine, led to a significant ($p < 0.05$) decrease in cell viability at low doses and to a highly significant ($p < 0.01$) reduction at high doses. The loss in cell viability is attributable to changes in the cell membrane integrity.
Conclusions: These results suggest that no enzymatic lipolytic pathway is induced. The decrease in volume after Lipostabil injections is likely attributable to the detergent effect of deoxycholate. (*Plast. Reconstr. Surg.* 124: 419, 2009.)

Originally, Lipostabil (Artesan Pharma, Lüchow, Germany) was introduced in the 1960s as an intravenous medication to prevent or treat fat embolism. This substance consists mainly of lipid phosphatidylcholine dissolved in the bile salt deoxycholate.[1,2] Considering that this medication was developed for another indication (fat embolism) and for intravenous application only, it is hardly surprising that there is a paucity of knowledge regarding the effect(s) caused by its subcutaneous injection. Because of publications regarding its fat-reducing effect, the subcutaneous injection of Lipostabil has become a popular alternative to liposuction over the past few years.[3–8]

The term "lipolysis" necessitates differentiation between the biochemical mechanism behind lipolysis and the simple lysis of cell membranes. The biochemical mechanism behind lipolysis is

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mediated by adrenergic β_1 and α_2 receptors; together with hormones such as glucagon and insulin, the mediator cyclic adenosine monophosphate is induced. This nucleotide then activates lipases that hydrolyze the triglycerides into glycerol and free fatty acids.[9] In contrast, the detergent-induced lyses of cell membranes are not mediated by receptors. More accurately, this type of lipolysis is the dissolution of the lipid bilayer into micelles, caused by molecules with hydrophilic and lipophilic features.[10,11] The overall clinical "lipolytic" effect is likely to be similar, and has been reported numerous times as a decrease in adipose tissue volume.[3–7,9] Both definitions of lipolysis have been suggested to be the reason for the decrease in volume caused by injecting Lipostabil in the subcutaneous fatty tissue.[7,12]

In this study, our aim was to determine in an in vitro setting how phosphatidylcholine, deoxycholate, or the synergism of both can cause a reduction in volume of adipose tissue. Furthermore, we aimed to investigate whether the treated adipocytes react to the treatment with simple cell death or by inducing a pathway of lipolytic enzymes that split intracellular lipids.

To answer these queries, an in vitro adipocyte model was exposed to either phosphatidylcholine, deoxycholate, or phosphatidylcholine that was dissolved in deoxycholate (Lipostabil) in varying doses. Subsequently, assays were performed to measure whether a lipolytic enzymatic pathway was induced and to quantify what proportion of the cells underwent cell death. In addition the cells were stained to evaluate changes in the membrane structure under the microscope.

MATERIALS AND METHODS

Lipostabil N 5 ml (50 mg/ml) intravenously consists of its active substance being 250 mg of soy-derived phospholipids (93% phosphatidylcholine), solubilized in 126.50 mg of deoxycholate, supplemented by 4539 mg of water, 45 mg of benzyl alcohol (for preservation), 18 mg of sodium chloride, 15 mg of ethanol, 12.65 mg of sodium hydroxide, and 0.75 mg of DL-α-tocopherol (vitamin E). It was purchased from Artesan Pharma (Luechow, Germany). Pure phosphatidylcholine was acquired from Lipoid GmbH (Ludwigshafen, Germany). Ethanol (99%) for the solubilization solution was delivered by Mallinckrodt Baker (Griesheim, Germany). Triton X and deoxycholate were obtained from Fluka Analytical (Seelze, Germany).

Murine 3T3-L1 preadipocytes were obtained from American Type Culture Collection (Manassas, Va.). Dulbecco's Modified Eagle Medium, fetal bovine serum, and trypsin (1:250) were purchased from Seromed Biochrom KG (Berlin, Germany); phosphate-buffered saline and penicillin-streptomycin were obtained from Life Technologies (Karlsruhe, Germany). Alpha minimal essential medium (alpha modification), corticosterone, indomethacin, and propidium iodide were produced by Sigma-Aldrich (Deisenhofen, Germany); 3-isobotuyl-1-methylxanthine was purchased from Serva Electrophoreses GmbH (Heidelberg, Germany); and insulin was provided by Hoechst (Frankfurt, Germany). Cell culture materials were obtained from Sarstedt AG and Co. (Nuembrecht, Germany). The lipolysis assay kit was purchased from Zen-Bio, Inc. (Research Triangle Park, N.C.). The dimethyl thiazolyl diphenyl tetrazolium (MTT) assay was produced by AppliChem (Darmstadt, Germany).

Cell Culture

3T3-L1 preadipocytes were plated in 12-well plates at a density of 5000 cells/cm^2. The preadipocytes were cultivated in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin (100 U/ml) together with streptomycin (0.10 mg/ml). Every 2 days, the preadipocytes were washed in phosphate-buffered saline; the medium was always changed at the same time.

Four days after plating, adipogenesis was induced by applying induction medium (0.1 μM corticosterone, 0.50 mM 3-isobotuyl-1-methylxanthine, 60 μM indomethacin, 5% fetal bovine serum, 1 μM insulin, penicillin at 100 U/ml, and streptomycin at 0.10 mg/ml in alpha minimal essential medium). After 2 days, the induction medium was replaced by the differentiation medium (1 μM insulin, 5% fetal bovine serum, penicillin at 100 U/ml, and streptomycin at 0.10 mg/ml in alpha minimal essential medium). All experiments were performed on day 10 of the differentiation period.

Measurement of Lipolysis

3T3-L1 adipocytes were cultivated in 12-well plates. In preparation for the experiment, the cells were washed in phosphate-buffered saline, and the differentiation medium was replaced by phosphate-buffered saline 2 hours in advance. The β-agonist isoprenaline was used to induce lipolysis to have a positive control in this assay. Pure phosphate-buffered saline/bovine serum albumin served as the negative control for this experiment. Ethanol,

phosphatidylcholine, and Lipostabil were tested separately for their ability to cause lipolysis. To determine the effect after exposure to each of the substances, the cells were incubated (37°C, 5% carbon dioxide) for 4 hours, with varying concentrations of the substances (Figs. 1 and 2) and phosphate-buffered saline, which is free of fatty acids. Subsequently, the cells were separated from the phosphate-buffered saline and the investigated substance. Phosphatidylcholine itself showed a drastic influence on absorbance. Therefore, the samples had to be filtered with 0.20-μm filters to extract the phosphatidylcholine before photometric measurements. The amount of DNA in each well was measured to obtain a reference for the number of cells included in each well, as recommended by the manufacturer. The samples (phosphate-buffered saline together with the investigated substance) were mixed with GPO-Trinder A (Sigma-Aldrich), an enzyme that produces quinoneimine dye out of glycerol in several steps. At 540 nm, quinoneimine dye increases the absorbance in the photometer directly proportional to the glycerol concentration in the sample.

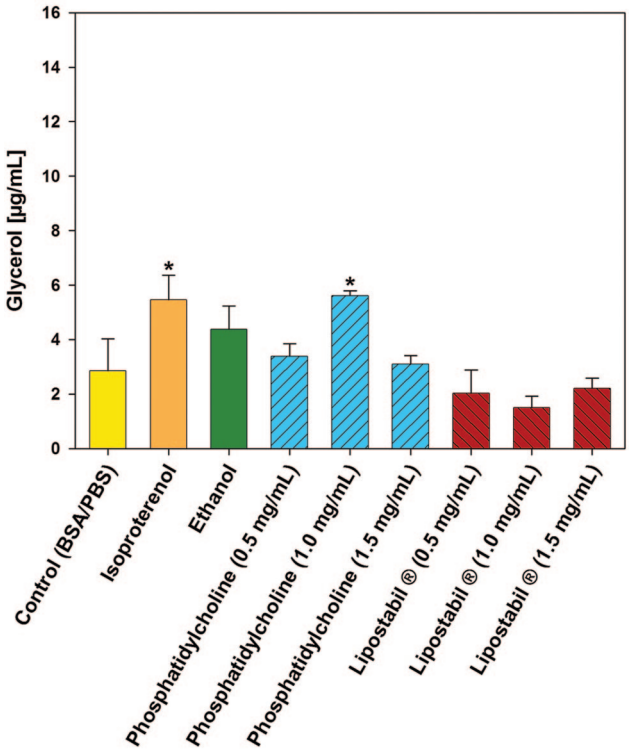

Fig. 1. Lipolysis assay with 3T3-L1 adipocytes ($n = 3$). The rise of glycerol indicates the induction of a lipolytic catabolism inside the adipocytes. The lipolysis assay demonstrates a significant rise of glycerol for the positive control, but neither phosphatidylcholine, deoxycholate, nor the combination of both (Lipostabil) was able to induce significant lipolysis. The negative control was bovine serum albumin (*BSA*)/phosphate-buffered saline (*PBS*); the positive control was isoproterenol. The *vertical bars* and *T bars* represent means ± SD; *statistically significant ($p < 0.05$).

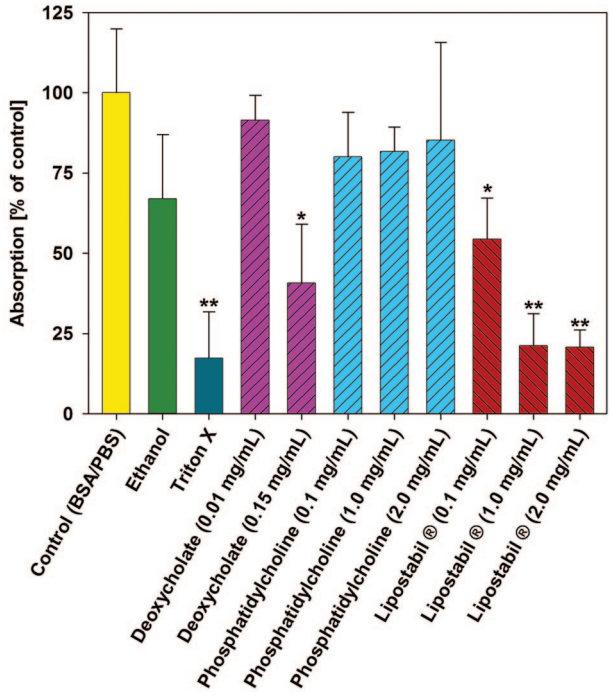

Fig. 2. MTT assay with 3T3-L1 adipocytes ($n = 3$). The loss of absorption reflects the amount of dead cells. MTT assay showed a significant loss in cell viability for neither phosphatidylcholine nor ethanol used as a solubilizer. Deoxycholate and Lipostabil led to a significant loss in cell viability of adipocytes, even at concentrations of 0.15 mg/ml. A highly significant reduction in cell viability was detected in bovine serum albumin concentrations of Lipostabil starting from 1.0 mg/ml. The negative control was bovine serum albumin (*BSA*)/phosphate-buffered saline (*PBS*); the positive control was Triton X. The *vertical bars* and *T bars* represent means ± SD; *statistically significant ($p < 0.05$); **highly significant ($p < 0.01$).

Measurement of Cell Viability

Cell viability was determined using the MTT assay, which consists of a tetrazolium-based change in the photometric extinction resulting from a bioreduction by mitochondrial enzymes of metabolically active (living) cells. The change in viability can be quantified by the extinction in a spectrophotometer at 490-nm wavelength.

3T3-L1 adipocytes were cultivated in 12-well plates (for details, see earlier under Cell Culture). In preparation of the experiment, the differentiation medium was replaced by phosphate-buffered saline 2 hours in advance. After 2 hours, the cells were incubated with phosphatidylcholine, de-

oxycholate, or Triton X (1%), which is a laboratory detergent used as the negative control for cell viability experiments. The incubation was performed for 4 hours (37°C, 5% carbon dioxide). Subsequently, the MTT agent was added and the assay was performed according to the manufacturer's instructions.

Propidium Iodide Staining

Propidium iodide stains DNA but is not able to penetrate the intact bilayers of cell membranes. Therefore, only those cells without an intact cell membrane are stained. In our experiments, the staining was used to evaluate the cell membrane integrity of the exposed cells. The cells were incubated with varying concentrations of the isolated substances (Figs. 3 and 4) for 4 hours (37°C, 5% carbon dioxide). Subsequently, the cells were exposed to propidium iodide (5 μg/ml), which was documented under a confocal laser scanning microscope with 63× magnification.

Statistical Analysis

All analyses were performed using SigmaPlot and SigmaStat (SPSS, Inc., Chicago, Ill.). Data are given as mean ± SD of three cultures. For each culture, three samples were measured. A *t* test was used to verify differences between glycerol levels under different lipolytic stimuli and respective controls. No correction was made for multiple testing. Values of $p < 0.05$ were considered significant, values of $p < 0.01$ were considered highly significant, and corresponding results were marked using one (significant) or two asterisks (highly significant) within the graphs, respectively. One adipocyte culture intended for the measurement of glycerol levels under 1.50 mg/ml Lipostabil did not grow; thus, $n = 2$ for that particular measurement.

RESULTS

Lipolysis Assay

The lipolysis assay with 3T3-L1 adipocytes showed glycerol amounts of 2.86 ± 1.17 μg/ml in the bovine serum albumin/phosphate-buffered saline control (negative control) (Fig. 1). The isoprenaline samples showed a significant rise to 5.46 ± 0.91 μg/ml ($p < 0.05$) of glycerol, which was the positive control in this experiment (for details, see earlier under Measurement of Lipolysis). For all other tested substances, no significant rise of glycerol could be measured.

MTT Assay

Under controlled conditions, the MTT reaction of 3T3-L1 adipocytes amounted to 100 ± 19.90 percent (Fig. 2). Triton X (positive control) led to a highly significant reduction of the extinction to 17.28 ± 14.50 percent ($p < 0.01$). Of the investigated drugs, 0.15 mg/ml deoxycholate significantly reduced the extinction to 40.75 ± 18.37 percent ($p < 0.05$). Lipostabil 0.10 mg/ml reduced the extinction significantly to 54.36 ± 12.85 percent ($p < 0.05$), and at a concentration of 1 mg/ml, Lipostabil led to a highly significant reduction to 21.16 ± 9.97 percent ($p < 0.01$). The ethanol control did not show any significant reduction of the extinction in the cell viability assay (78.15 ± 6.76 percent).

DISCUSSION

Phosphatidylcholine has been in use for several indications ranging from the prevention of fat embolism to the reduction of serum triglycerides and even for the prevention of fibrosis in damaged livers.[13,14] Multiple clinical trials support the idea that subcutaneously injected phosphatidylcholine leads to a reduction in volume of adipose tissue.[3–8]

These facts have led to controversial theories concerning the role of the molecule phosphatidylcholine in the formulations used for subcutaneous injections. One theory states that—because of its bipolar character—phosphatidylcholine dissolves the fatty tissue by breaking the cell membrane lipid bilayer into micelles.[15–17] Another published theory suggests that the induction of lipases by phosphatidylcholine causes a catabolic pathway ending in a liver metabolism.[7] Recently, Rotunda et al. concluded that phosphatidylcholine is not the active substance in injectable formulations for subcutaneous lipolysis.[12] The authors found a similar volume-reducing effect on keratinocyte cells and porcine skin after injection of plain sodium deoxycholate—a bile salt that acts as a solubilizer for phosphatidylcholine in water—isolated from phosphatidylcholine.

Our first objective was to investigate whether phosphatidylcholine—when isolated from deoxycholate—has a lipolytic effect on adipocytes as previously postulated.[7] Thus, it was necessary to identify a solubilizer for phosphatidylcholine other than deoxycholate. Phosphatidylcholine can be solubilized in low concentrations of ethanol. To ensure that ethanol as the solvent agent does not have any relevant effect on the lipolytic activity or viability of adipocytes, one group was

Fig. 3. Propidium iodide staining for the analysis of necrotic cell death by confocal microscopy ($n = 3$). (*Above*) The stained cells are shown in *red*; (*below*) the corresponding phase-contrast images (*scale bar* = 20 μm; original magnification, × 63). (*First column*) Negative control (ethanol); (*second column*) positive control (Triton X); (*third column*) 1 mg/ml phosphatidylcholine; (*fourth column*) 5 mg/ml phosphatidylcholine; (*fifth column*) 10 mg/ml phosphatidylcholine. It can be seen that throughout the entire range of concentrations hardly any cells are stained by propidium iodide. Even at a concentration of 10 mg/ml phosphatidylcholine, no cells are stained, indicating the intactness of the cellular membrane bilayer.

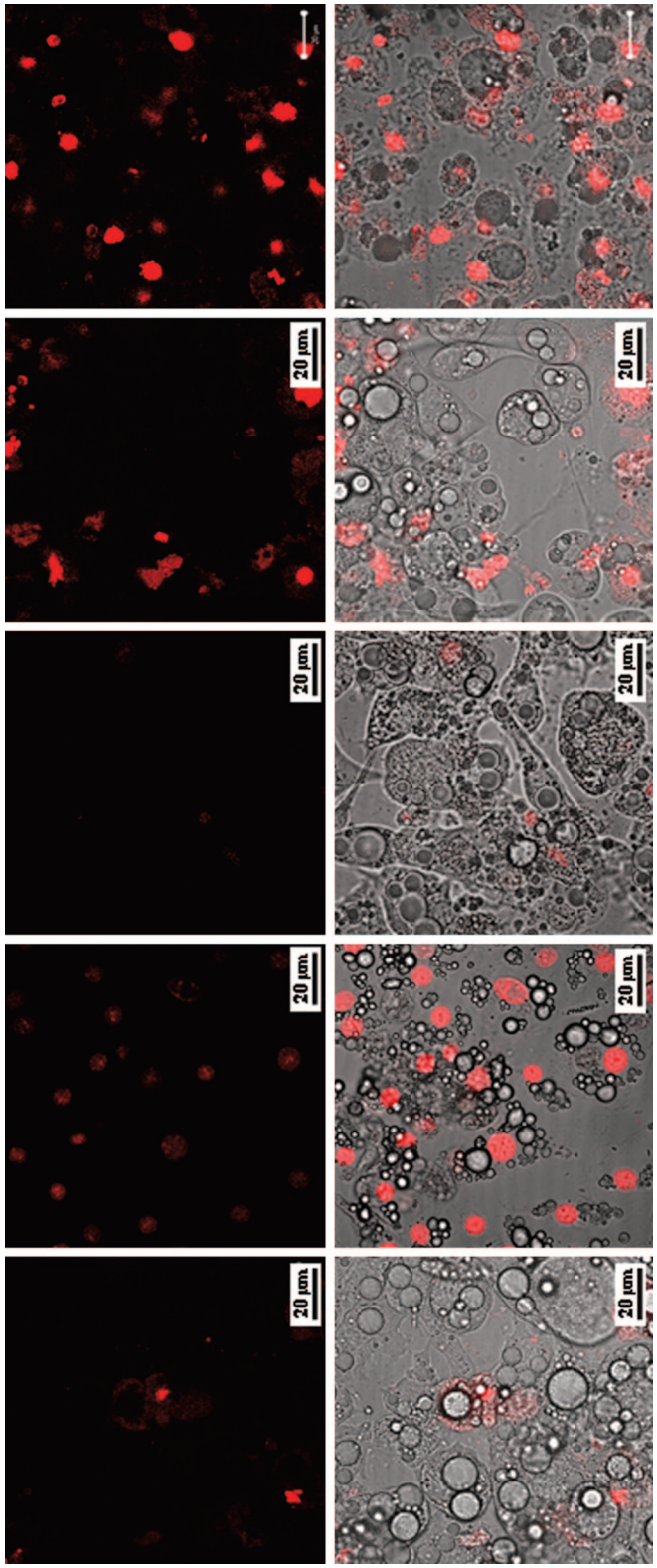

Fig. 4. Propidium iodide staining for the analysis of necrotic cell death by confocal microscopy ($n = 3$). (*Above*) The stained cells are shown in *red*; (*below*) the corresponding phase contrast images (*scale bar* = 20 μm; original magnification, ×63). (*First column*) Negative control (ethanol); (*second column*) positive control (Triton X); (*third column*) 0.01 mg/ml deoxycholate; (*fourth column*) 0.05 mg/ml deoxycholate; (*fifth column*) 0.10 mg/ml deoxycholate. It can be seen that the number of cells stained by propidium iodide increases with the increasing dose of deoxycholate. At a concentration of 0.10 mg/ml deoxycholate, almost all cells are stained because of the damage to the cellular membrane bilayer.

treated with pure ethanol in each assay (Figs. 1 through 3, *first column*). The ethanol concentration was chosen equal to the highest concentration needed for the phosphatidylcholine solubilization in our experiments. The ethanol control showed neither a significant lipolytic effect nor a significant reduction of the extinction in the cell viability assay (Figs. 1 and 2). Furthermore, no effect could be observed with pure ethanol in the group stained with propidium iodide (Fig. 3, *first column*). The lipolysis assay proved a significant rise of glycerol for the positive control (Fig. 1), but neither phosphatidylcholine, deoxycholate, nor the combination of both (Lipostabil) induced lipolytic pathways. This effect was reproduced throughout the entire range of concentrations. These results suggest that phosphatidylcholine does not induce any endogenous lipases. Thus, we cannot support the theory of phosphatidylcholine acting as an inductor of an enzymatic lipolytic pathway.

As mentioned above, Rotunda et al. demonstrated a detergent effect of phosphatidylcholine solubilized in deoxycholate for keratinocyte cells.[12] Considering these data, the purpose of this study was to determine whether this detergent effect can also be detected in the adipocyte in vitro model used in our studies. Moreover, our objective was to investigate whether phosphatidylcholine has the same detergent effect caused by its bipolar structure when being applied isolated from deoxycholate. To demonstrate whether the cell viability of adipocytes is influenced by isolated phosphatidylcholine, the MTT assay and propidium iodide stainings together with native microscope image analysis were performed. The MTT assay showed no significant loss in cell viability for phosphatidylcholine or for the ethanol

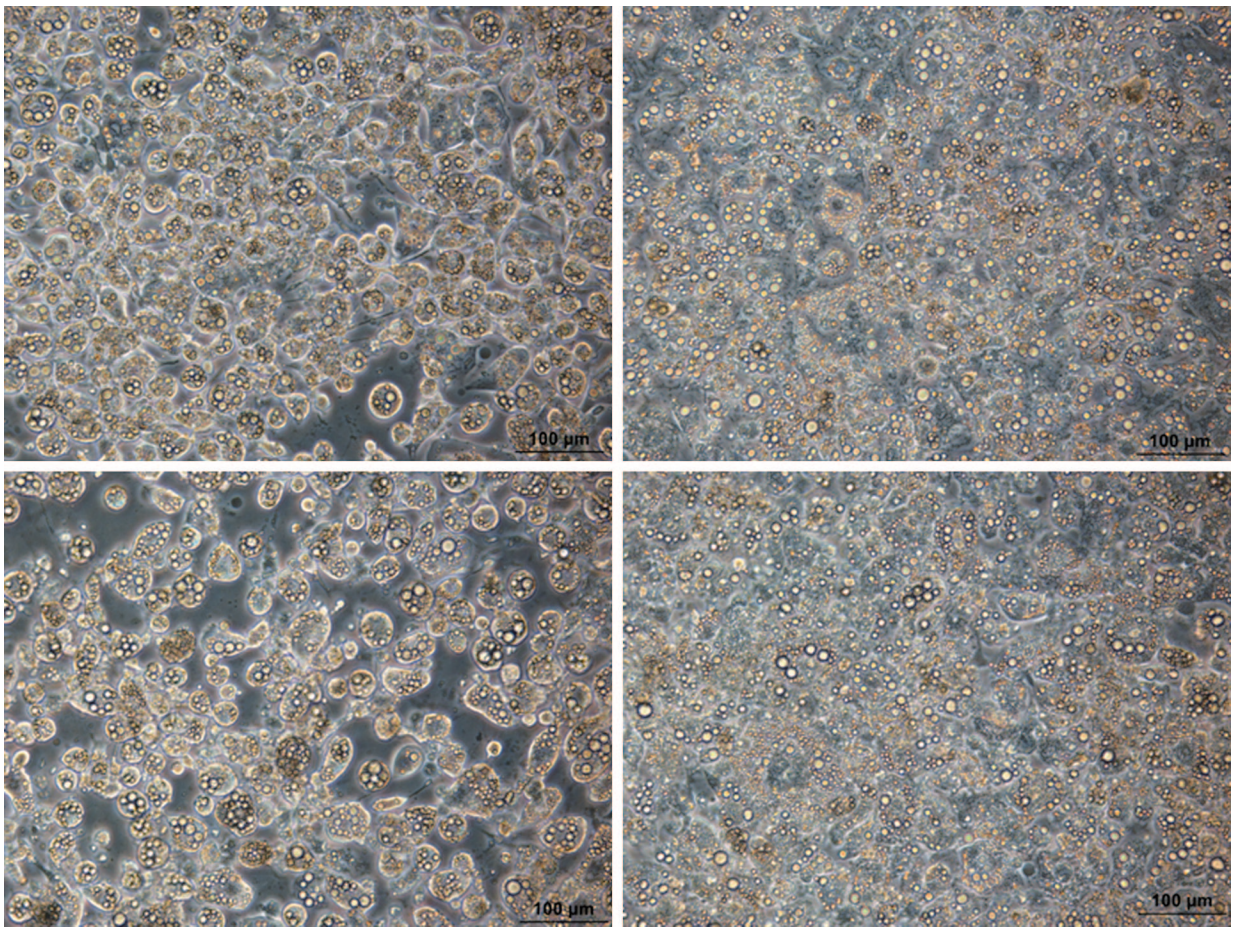

Fig. 5. Native microscopic images of adipocytes after 6 hours of incubation (original magnification, ×60). (*Above*, *left*) Negative control (ethanol); (*above*, *right*) positive control (Triton X); (*below*, *left*) 10 mg/ml phosphatidylcholine; (*below*, *right*) 0.10 mg/ml deoxycholate. (*Left*) The cell membranes seem to be intact. (*Right*) A strong loss of cell barriers can be seen. These findings confirm the results of the propidium iodide stainings in Figures 3 and 4.

formulation, which served to solubilize phosphatidylcholine in this study (Fig. 2). In contrast, deoxycholate and Lipostabil had already begun to result in a significant loss in adipocyte cell viability at concentrations of 0.15 mg/ml or lower (Fig. 2). This loss was highly significant for concentrations of Lipostabil at or above 1 mg/ml (Fig. 2). The decrease in viability with Lipostabil is likely attributable to the detergent effect of deoxycholate described by Rotunda et al., because phosphatidylcholine exhibited no such effect at equivalent doses in the MTT assay (Fig. 2).[12] Although the MTT assay does not enable us to conclude by what specific mechanism cell death has been induced (apoptosis or necrosis), it is likely—together with the propidium iodide staining and the native microscope images—that the cell death is a result of the detergent effect of deoxycholate (Figs. 4, *fourth* and *fifth columns* and 5, *below, right*).

The Lipostabil formula contains low amounts of benzyl alcohol for antimicrobial reasons. Although an effect of benzyl alcohol on the fluidity of cell membranes has been reported, we did not isolate this benzyl alcohol because of its small quantity in the formula.[18,19] The described lipolysis assay supports the theory that detergents are the major feature of injectable phosphatidylcholine formulations, because no parameters for lipolysis occurred with phosphatidylcholine, deoxycholate, or Lipostabil (which is the combination of both mentioned substances) (Fig. 1).[12] The performed MTT assays proved a significant loss in viability for deoxycholate and Lipostabil at doses where phosphatidylcholine alone did not show any effect (Fig. 2). The results of the MTT assay together with the propidium iodide stainings confirm observations published in clinical trials that revealed local inflammatory reactions such as erythema, pain, and edema, most likely caused by cell membrane destruction.[20]

CONCLUSIONS

Our findings suggest that neither phosphatidylcholine nor sodium deoxycholate induces an enzymatic lipolytic pathway (Fig. 1). Deoxycholate reduces the cell viability already at low doses (0.15 mg/ml) (Fig. 2). Phosphatidylcholine neither significantly influences the cell viability nor starts to cause membrane destruction at the tested doses (Figs. 2 and 4), whereas Lipostabil demonstrated a highly significant effect on cell viability at a concentration of only 1 mg/ml (Fig. 2). This comparably strong effect could be related to a synergistic effect of phosphatidylcholine or the benzyl alcohol in the Lipostabil formula. The propidium iodide stainings and native microscope images prove that the detergent bile salt deoxycholate induces membrane destruction (Figs. 4, *fourth* and *fifth columns* and 5, *below, right*), thereby leading to cell death of exposed adipocytes (Fig. 2).

According to published clinical trials, the subcutaneous injection of phosphatidylcholine formulations appears to be an effective tool with which to reduce subcutaneous fatty tissue, although it has to be noted that the membrane destruction is most likely not restricted to adipocytes. Therefore, physicians should be well trained and cautious when injecting these substances. Further studies investigating the effect under in vivo conditions should be conducted to ensure the safety and efficacy of lipolytic formulations.

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