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Lipolytic effects of nano particle sized polyenylphosphatidylcholine on adipose tissue: First promising *in vitro* and *in vivo* results

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Abstract.
BACKGROUND: Lipolytic injectables for body contouring procedures have been reported for necrotic effects on adipose tissue causing side effects as swelling, pain and hematoma. Deoxycholic acid is widely used as a solvent in lipolytic injectables and is associated with necrosis when applied to cells. A new lipolytic preparation (NWL-10) containing only polyenylphosphatidylcholine in nano particle size, glycerrhizinate and maltose has been reported for its lipolytic action on adipose tissue. However, no data exist whether the NWL-10 mixture is responsible for apoptosis or necrosis in adipose tissue which can be associated with severer side effects as reported for deoxycholic acid preparation.
METHODS: 3T3-L1 mouse cells and human adipose tissue derived stem cells were exposed to the NWL-10 mixture and to each ingredient of the mix in order to investigate cytotoxic, lipolytic, necrotic or apoptotic effects. Furthermore, a Balb/C mouse animal model was used to investigate inflammatory responses to NWL-10 by bioluminescence monitoring and histological examination.
RESULTS: A high extent of lipolysis was detected for the NWL-10 mixture when applied to both cell types with no cytotoxic effect. Interestingly, low concentration of NWL-10 resulted in necrosis whereas high concentration of NWL-10 showed a certain amount of apoptosis. Application of single ingredients of NWL-10 or various combinations of two component mixtures did not result in any apoptosis or necrosis. In addition, no inflammatory effects of NWL-10 were observed in the mouse model.
CONCLUSIONS: The NWL-10 mixture provided promising results regarding lipolysis on adipose tissue with limited apoptosis and necrosis when compared to currently available injectables. These first promising results require further fundamental and more detailed research on essentials for drug approval. NWL-10 has the potential to become a second generation product for future lipolytic injectables.

1. Introduction

Lipostabil® N is approved for the treatment of fat embolism by intravenous administration for decades [1, 2]. In addition, the drug is off-label used for subcutaneous injection lipolysis (IL), especially for smaller fat pads [3–6].

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Lipostabil® N contains a combination of different phosphatidylcholines (PPC, a molecule combination of different phosphatidylcholines) from soybean and deoxycholic acid (DOC) as a solvent. The first *in vitro* study on 3T3-L1 mouse cell lines revealed that no enzymatic lipolytic pathway is induced. The decrease in volume after Lipostabil® N injections is likely attributable to the detergent effect of deoxycholate [7]. The literature of the last decade provides evidence that DOC is responsible for the destruction of adipocyte cell walls and initiating necrosis - an undesired effect of lipolytic injectables [8–10]. The role of PPC for the fat metabolism of destructed adipocytes - as second part of the underlying mechanism of action (MOA) - is still in a state of discourse [11].

NWL-10, a new substance combination for IL was developed by Profs. Alexander Archakov and Olga Ipatova from the Research Institute of Biomedical Chemistry in Moscow/Russia and reported for its lipolytic effects without any side effects. These first data are not published yet. NWL-10 is a lyophylisate containing PPC 50 mg/mL in nano particle size (<40 nm), glycerrhizinate (GLY) 20 mg/mL and maltose (MAL) 180 mg/mL. Interestingly, DOC is not required as a solvent as reported for other lipolytic injectables. To date, no data exists regarding the lipolytic effect on adipose tissue or adipocytes. Moreover, no study exists regarding apoptosis, necrosis or cytotoxicity of the NWL-10 drug composition.

2. Methods

2.1. Cell culture

Human adipose tissue derived stem cells (ASCs), were isolated from adipose tissue generated from liposuctions by collagenase digestion, centrifugation and plastic adherence. These cells were expanded in a 2D cell culture and stored in liquid nitrogen for further experiments. Cells of passages 2–4 were used for all experiments. Murine 3T3-L1 preadipocytes were obtained from ATCC (Manassas, VA). ASCs were allowed to differentiate into adipocytes by adding an adipogenic cocktail containing dexamethason (1 μM), 3-isobotyl-1-methylxanthine (500 μM), indomethacin (200 μM) and insulin (1,7 μM).

2.2. Cytotoxicity assay

3T3-L1 mouse cells were stimulated and differentiated into adipocytes for 8 days.

Differentiated 3T3-L1 adipocytes were incubated with NWL-10 in ascending concentrations (PPC 5 mg/mL /GLY 2 mg/mL/ MAL 18 mg/mL, 10/4/36 mg/mL, 20/8/72 mg/mL, 30/12/108 mg/mL and 50/20/180 mg/mL) for 4 hours. Propidium iodide was added to the cell dish and investigated by confocal laser-scan microscopy (63x). 3T3-L1 cells supplemented with normal growth media served as control.

2.3. Lipolysis assay

3T3-L1 mouse cells were differentiated into adipocytes and incubated with NWL-10 in ascending concentrations (10/4/36 mg/mL, 25/10/90 mg/mL and 50/20/180 mg/mL) for 4 hours. The lipolytic activity was identified by the level of glycerol release. Basal lipolytic activity was measured by cells supplemented with bovine serum albumin (BSA)/phosphate-buffered saline (PBS). 10 μM isoproterenol incubated cells were used as positive control.

2.4. Necrosis/apoptosis assay

Human adipose tissue derived stem cells (hASCs) were differentiated into adipocytes and subsequently incubated with ascending concentrations of NWL-10 (10/4/36 mg/mL, 25/10/90 mg/mL and

50/20/180 mg/mL) for 4 hours. Apoptosis and necrosis were detected by Cell Death ELISA Kit (Roche) at various time point (i.e. 0.5, 2 and 4 hours).

In addition lipolytic effects of NWL-10 on differentiated hASCs were analyzed. HASCs were differentiated into adipocytes and incubated with ascending concentrations of NWL-10 (10/4/36 mg/mL, 25/10/90 mg/mL and 50/20/180 mg/mL) for 4 hours. Lipolytic activity was quantified by glycerol release after 0.5 and 3 hours. Basal lipolytic activity was obtained by culturing cells in BSA/PBS. 50 μM isoproterenol was used in cells to provide a positive control group.

Furthermore, cytotoxic effects of NWL-10 as well each single ingredient were investigated. Adipocytes were incubated with ascending concentrations of NWL-10 (2,5/1/9 mg/mL; 10/4/36 mg/mL; 25/10/90 mg/mL) as well with various concentration of its ingredients (PPC 10 mg/mL, PPC 25 mg/ mL, GLY 4 mg/mL, GLY 10 mg/mL, MAL 36 mg/mL, MAL 90 mg/mL) and combinations of its individual substances (PPC 25 mg/mL/GLY 10 mg/mL, GLY 10/MAL 90 mg/mL, PPC 25/MAL 90 mg/mL, and PPC 25/GLY 10/MAL 90 mg/mL) for 4 hours. Triton X treatment served as a positive control and cells without treatment serves as a negative control. Propidium iodide staining was performed and confocal laser-scan microscopy were used for analysis [12].

Then, lipolytic effects were investigated by using ascending concentration of NWL-10 and various concentrations of single substances of the NWL-10 and different combinations. Subsequently the adipocytes were incubated with NWL-10 in ascending concentrations (1/0,4/3,6 mg/mL, 2,5/1/9 mg/mL, 10/4/36 mg/mL and 25/10/90 mg/mL). Furthermore, single substances of GLY and MAL were also used in different concentrations (GLY 0,4 mg/mL, GLY 1 mg/mL, GLY 4 mg/mL, GLY 10 mg/mL, MAL 36 mg/mL, MAL 90 mg/mL) and substance combinations for 4 hours. Afterwards, lipolytic activity was identified by measuring glycerol release. For all experiments positive controls were treated with 10 μM or 50 μM isoproterenol whereas basal lipolytic activity was identified by cells cultured in BSA/PBS.

2.5. *In vivo experiments*

The inflammatory effect of NWL-10 was investigated using BALB/C mice. First, murine ASCs (mASCs) were isolated from subcutaneous fat tissue of BALB/c mice. (Fat tissue was rinsed in PBS and minced into pieces of <2 mm^3. Serum-free minimum essential medium (αMEM, 1 mL/g tissue) and Liberase Blendzyme 3 (Roche) (2 U/g tissue) were added and incubated at 37°C under continuous shaking for 45 min. The digested tissue was filtrated with 100 and 40 μm filters (Fisher Scientific™) sequentially, and afterwards centrifuged for 10 min. The supernatant containing adipocytes and debris was discarded. The pelleted cells were washed with Hanks' Balanced Salt Solution (Cellgro™) twice and finally resuspended in growth medium containing αMEM, fetal bovine serum (FBS, 20%), l-glutamine 2 mM and penicillin 100 U/mL with streptomycin 100 μg/mL. Afterwards, plastic-adherent cells were grown in culture vials (Nunc™) in a humidified atmosphere containing 5% CO_2 at 37°C and washed daily to remove red blood cells and not attached cells.

Murine ASCs were labeled with GFP/luciferase vector controlled by a longterminal-repeat promoter. Cells were transduced using polybrene (8 μg/mL) (Chemicon) and a lentiviral vector ($2 \bullet 10^5$ TU/mL virus) encoding green fluorescent protein (GFP) and luciferase reporter. Lentivirus production were accomplished by three plasmid co-transfections with 40 μg shRNA DNA, 30 μg pCMV-ΔR8.91 and 10 μg pMD2.G into human embryonic kidney derived cells (HEK 293T) using Calcium Phosphate Transfection Kit (Invitrogen). 72 hours after transduction GFP-positive mASCs were sorted by fluorescence-activated cell sorting.

Contemporaneously, E. coli bacteria (ATCC 15746) were incubated with 5 ml brain-heart infusion (BHI; Difco Laboratories) in an atmosphere containing 95% air and 5% CO_2 at 37°C overnight.

Cultures were aliquoted into 0.7 ml BHI, supplemented with 15% glycerol and frozen at –80°C. For subsequent experiments stock cultures were thawed and incubated with 20 ml BHI overnight. Afterwards, bacterial culture was centrifuged for 15 min and supernatant was discarded. The E. coli bacteria pellet was resuspended in PBS and used for experiments.

NWL-10, E. coli bacteria (2×10^7 colony-forming units), or PBS were injected subcutaneously into the 4th right fat pad of Balb/C mice. GFP/luciferase labelled mASCs were administered 48 hours later intraperitoneally. Mice injected with PBS served as control.

The migration of the applied GFP/luciferase labelled mASCs was monitored by bioluminescence. For this reason, BALB/C mice received an intraperitoneal injection with D-Luciferin (GoldBio) resuspended in PBS 400 mg/mL. BALB/C mice were positioned in a dark imaging chamber and photographed 20 minutes after D-Luciferin injection. First, a surface image under dim lighting was obtained. The surface image was superimposed by the pseudo-color image generated by bioluminescence emerging because of the catalytic activity of luciferase. The exposure time was 0.15 seconds. Bioluminescence intensity was quantified in units of photons per image using Living Image Software 3.0 (Caliper Life Sciences).

The BALB/C mice were sacrificed 7 days after injection of NWL-10, PBS or E. coli bacteria. Tissue of their 4th right fat pads was harvested and placed into formalin 4 % for fixation and subsequent paraffin processing. Paraffin embedded tissue was sliced in 4 μm sections and stained with hematoxylin and eosin (H & E).

The study was approved and given informed consent by the local ethics committee.

3. Results

3.1. Cytotoxic effects

No cytotoxic effect on adipocytes was apparent when incubated with different concentrations of NWL-10. The analysis by confocal laser scan microscopy showed that all adipocytes were vital and the cell membrane was intact (Fig. 1).

3.2. Lipolysis, necrosis and apoptosis for NWL-10

NWL-10 showed a significant lipolytic effect for all three different concentration when compared to control group ($p < 0.001$). Moreover, lipolytic effects on adipocytes incubated with NWL-10 increased significantly for ascending concentrations of NWL-10. In contrast to isoproterenol incubated adipocytes glycerol release did not increase significantly for adipocytes incubated with a certain concentration NWL-10 depending on time of incubation (instead they reached steady state after 0.5 hours) (Fig. 5). Noteworthy, NWL-10 showed highest glycerol release already after 0.5 hours without further release for all investigated concentrations. (Fig. 2).

Interestingly, necrotic effects on adipocytes were only observed for the low and medium concentration of NWL-10 for incubation of more than 2 hours. All other concentrations of NWL-10 did not show a necrotic effect on adipocytes (Fig. 3). Apoptotic effects on adipocytes increased significantly for all NWL-10 concentrations depending on time of incubation (Fig. 4).

Furthermore, there were cytotoxic effects on few isolated adipocytes after incubation with the highest concentration of NWL-10 detected by propidium iodide staining. Adipocytes incubated with PPC 10 mg/mL and PPC 25 mg/mL showed no cytotoxic effects. The same results were observed for adipocytes incubated with GLY 4 mg/mL and GLY 10 mg/mL. In contrast, there was obvious cell membrane damage of adipocytes incubated with MAL 90 mg/mL detected by propidium iodide staining.

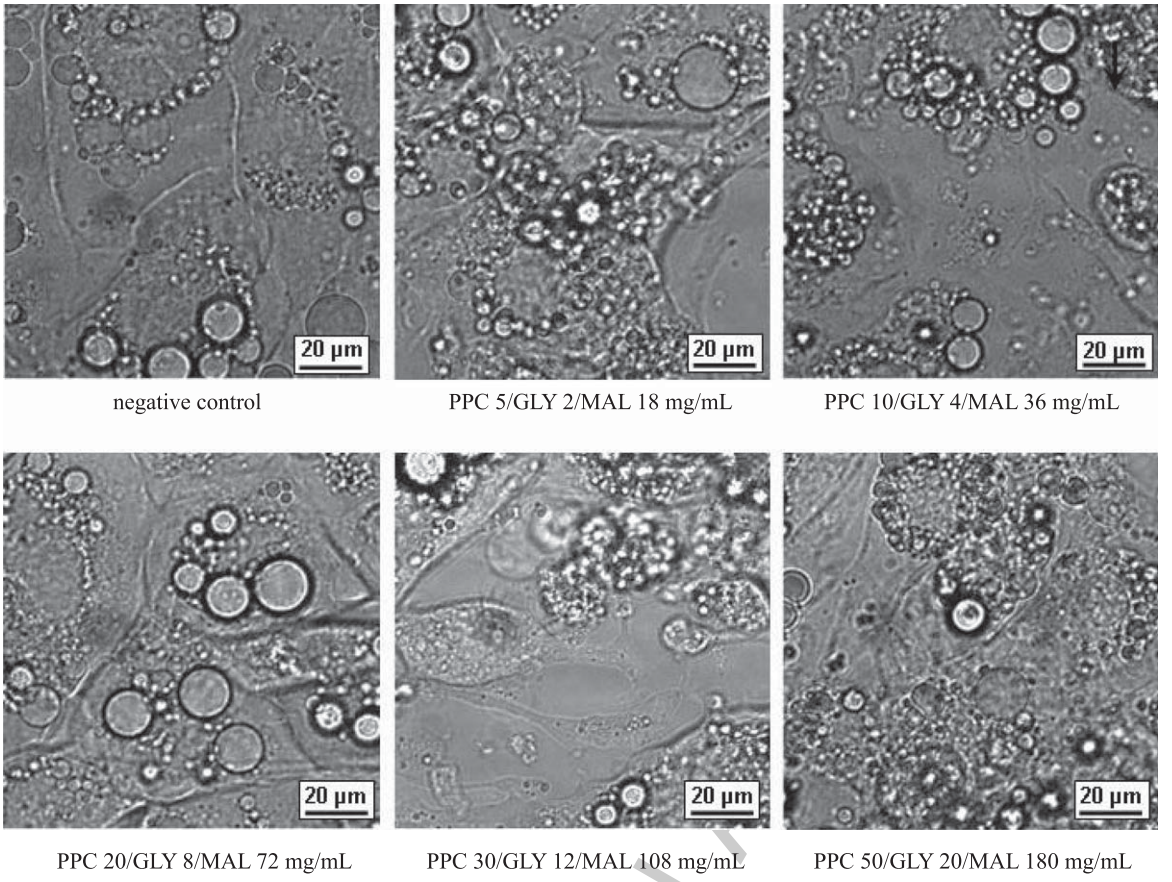

Fig. 1. Confocal laser-scan microscopy of differentiated 3T3-L1 mouse cells after incubation with different concentrations of NWL-10 to investigate cytotoxic effects. 3T3-L1 mouse cells were stimulated and afterwards differentiated into adipocytes. The mature adipocytes were incubated with NWL-10 in ascending concentrations (PPC 5/GLY 2/MAL 18 mg/mL, 10/4/36 mg/mL, 20/8/72 mg/mL, 30/12/108 mg/mL, 50/20/180 mg/mL) for 4 hours. Not incubated cells were used as control. The analysis was made by confocal laser-scan microscopy. There were no cytotoxic effects on adipocytes incubated with NWL-10.

This effect was observed also for adipocytes incubated with substance combinations containing MAL 90 mg/ml (Fig. 6).

Very high lipolytic effects on adipocytes were discovered after incubation with NWL-10. Even after incubation with very low concentrations of NWL-10 (1/0,4/3,6 mg/mL) distinct lipolytic effects could be shown. The highest lipolytic effects were measured after incubation with medium concentration of NWL-10 (10/4/36 mg/mL). It was equivalent to the effects on adipocytes incubated in 50 µM isoproterenol (positive control). No lipolytic effects were observed neither for adipocytes incubated with the individual substances nor for adipocytes incubated with combinations of them. In preceding investigations the individual substance phosphatidylcholine likewise did not show any lipolytic effects.

Furthermore, there was no significant difference between mASCs migration in BALB/C mice injected with NWL-10 and mice treated with PBS (accumulation in liver and lien, not in the injected area). Whereas mASCs migrated toward E. coli bacteria injected areas meaning ongoing inflammatory processes (Fig. 7). Histology confirmed significantly higher inflammatory processes in E. coli

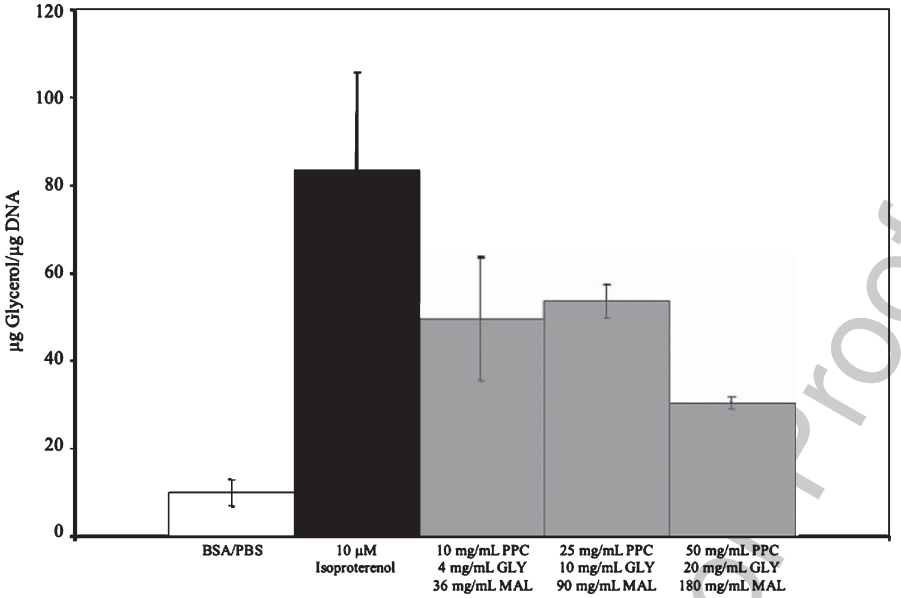

Fig. 2. Lipolysis assay with differentiated 3T3-L1 mouse cells after incubation with different concentrations of NWL-10 to investigate lipolytic effects. 3T3-L1 mouse cells were stimulated and afterwards differentiated into adipocytes. The mature adipocytes were incubated with ascending concentrations of NWL-10 (PPC 10/GLY 4/MAL 36 mg/mL, 25/10/90 mg/mL, 50/20/180 mg/mL) for 4 hours. Lipolytic activity was identified by measuring glycerol release. Basal lipolytic activity was measured by cells incubated with BSA/PBS. 10 µM isoproterenol incubated cells were used as positive control. There were very high lipolytic effects on adipocytes incubated in NWL-10.

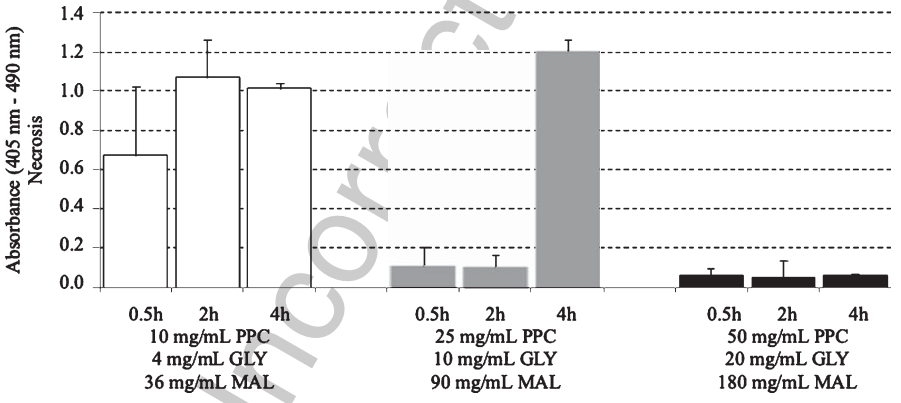

Fig. 3. Cell death analysis of differentiated hASCs with different concentrations of NWL-10 to investigate necrotic effects. HASCs were stimulated and differentiated into adipocytes. The mature adipocytes were incubated with ascending concentrations of NWL-10 (PPC 10/GLY 4/MAL 36 mg/mL, 25/10/90 mg/mL, 50/20/180 mg/mL). Necrosis was detected by Cell Death ELISA Kit (Roche) at different points of time. Necrotic effects on adipocytes were only observed for the low concentration of NWL-10 and for the medium concentration after an incubation of more than 2 hours. For higher concentrations of NWL-10 almost no necrotic effects on adipocytes were detected.

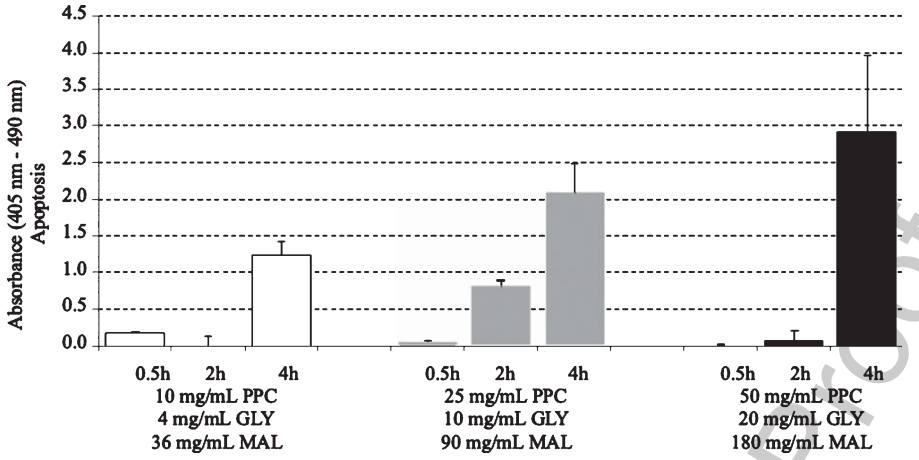

Fig. 4. Cell death analysis (Apoptosis) of differentiated hASCs with different concentrations of NWL-10 to investigate apoptotic effects. HASCs were stimulated and differentiated into adipocytes. Afterwards, these cells were incubated with ascending concentrations of NWL-10 (PPC 10/GLY 4/MAL 36 mg/mL, 25/10/90 mg/mL, 50/20/180 mg/mL). Apoptosis was detected by Cell Death ELISA Kit (Roche) at different points of time. Apoptotic effects on adipocytes increased significantly for all NWL-10 concentrations depending on time of incubation.

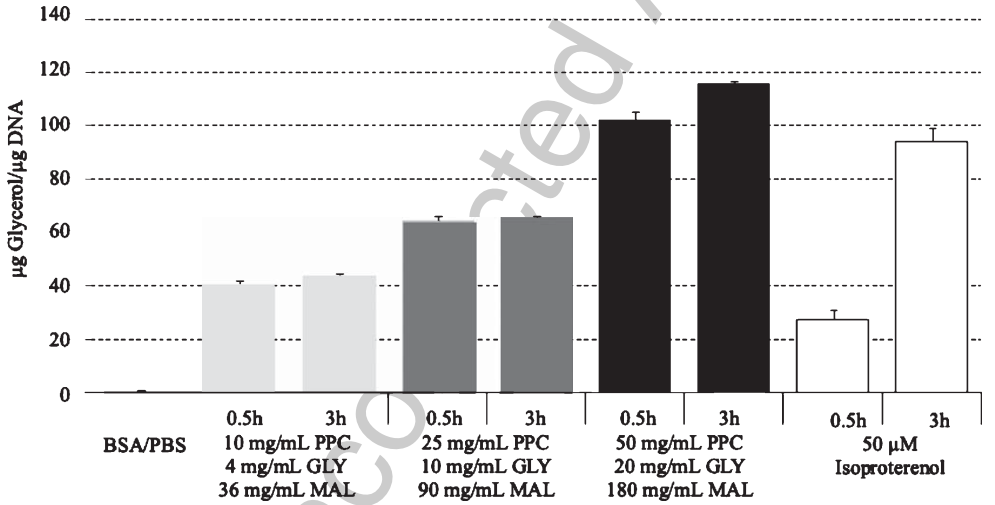

Fig. 5. Lipolysis assay with differentiated hASCs after incubation with different concentrations of NWL-10 to investigate lipolytic effects. HASCs were stimulated and differentiated into adipocytes. The mature adipocytes were incubated with ascending concentrations of NWL-10 (PPC 10/GLY 4/MAL 36 mg/mL, 2,5/1/9 mg/mL, 25/10/90 mg/mL, 50/20/180 mg/mL) for 4 hours. Lipolytic activity was identified by measuring glycerol release after 0.5 and 3 hours. Basal lipolytic activity was measured by cells incubated in BSA/PBS. 50 µM isoproterenol incubated cells were used as positive control. Lipolytic effects on adipocytes incubated with NWL-10 increased significantly for ascending concentrations of NWL-10. In contrast to isoproterenol incubated adipocytes glycerol release did not increase significantly for adipocytes incubated with a certain concentration of NWL-10 depending on time of incubation (steady state after 0.5 hours).

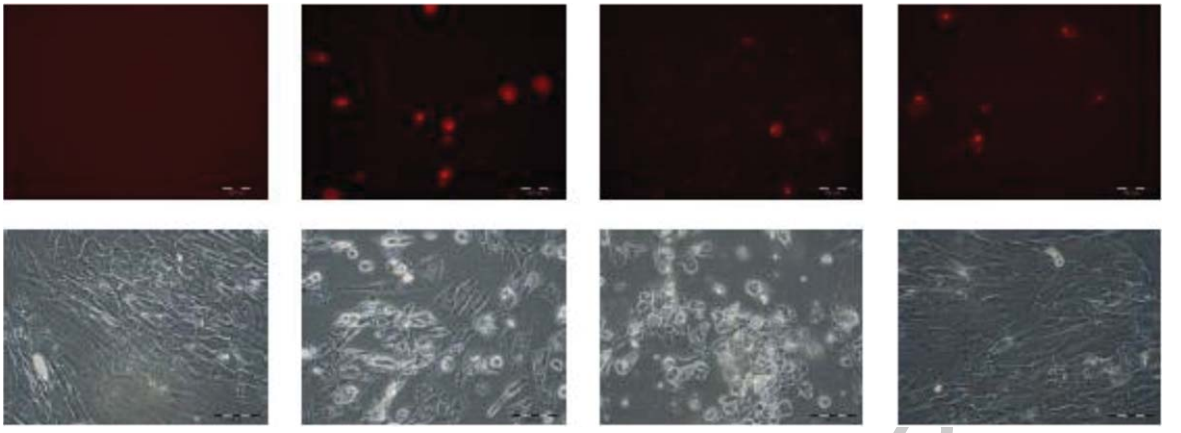

Fig. 6. Confocal laser-scan microscopy and propidium iodide staining of differentiated hASCs after incubation with different substance combinations of PPC, GLY and MAL to investigate cytotoxic effects. HASCs were stimulated and differentiated into adipocytes. The mature adipocytes were incubated with the following combinations: PPC 25/GLY 10 mg/mL, GLY 10/MAL 90 mg/mL, PPC 25/MAL 90 mg/mL, and PPC 25/GLY 10/MAL 90 mg/mL for 4 hours. Not incubated cells were used as negative control, as positive control Triton X was used. The analysis was made by confocal laser-scan microscopy after propidium iodide staining. Size bar = 100 μm. Cytotoxic effects could be observed for all combinations containing MAL 90 mg/mL.

bacteria injected areas by measuring the number of granulocytes compared to PBS or NWL-10 injected areas. The difference regarding the granulocyte numbers of PBS and NWL-10 injected areas was not statistically significant (Fig. 8).

4. Discussion

The almost complete absence of necrotic, cytotoxic, and inflammatory effects indicates that NWL-10 might have less side effects like pain, swellings and bruises [4, 9, 10, 13] compared to current lipolytic injectables containing DOC. The high lipolytic activity reaching steady state after 0.5 hours and the apoptotic cell death of adipocytes implicates two different, consecutive modes of action. Firstly, a glycerol release empties the adipocytes directly after incubation [14, 15] and, secondly, apoptotic cell death follows this first step leading to longer lasting fat reduction by decreasing the number of adipocytes [16].

At the current status of investigation we can only show the potential of NWL-10. Further investigations need to be initiated to validate these first results. Additional investigations should focus on the molecular mechanism of apoptosis [17, 18] caused by NWL-10. Firstly, apoptosis should be analyzed for its dependency of cytochrome c release or Apaf-1 activation [19]. Cytochrome c plays a key role in the regulation of apoptosis and is released into the cytosol from the mitochondria triggered by the intrinsic apoptotic pathway [20, 21]. However, procaspases can also be activated by Apaf-1 cytochrome c independently [22]. Secondly, using western blot analysis it has to be investigated whether NWL-10 induced apoptosis is dependent of caspase 3 activity [23, 24]. In addition, the caspase inhibitor Q-VD-OPh should be used to check for a decrease of apoptotic cell death [25]. Thirdly, after injection of NWL-10 into muscle tissue possibly inflammatory reactions should be analyzed by using an *in vivo* model [26–28]. Furthermore, it has to be confirmed that NWL-10 does not induce inflammatory processes or uncontrolled muscle cell death, if administered into muscle tissue as an event of incorrect

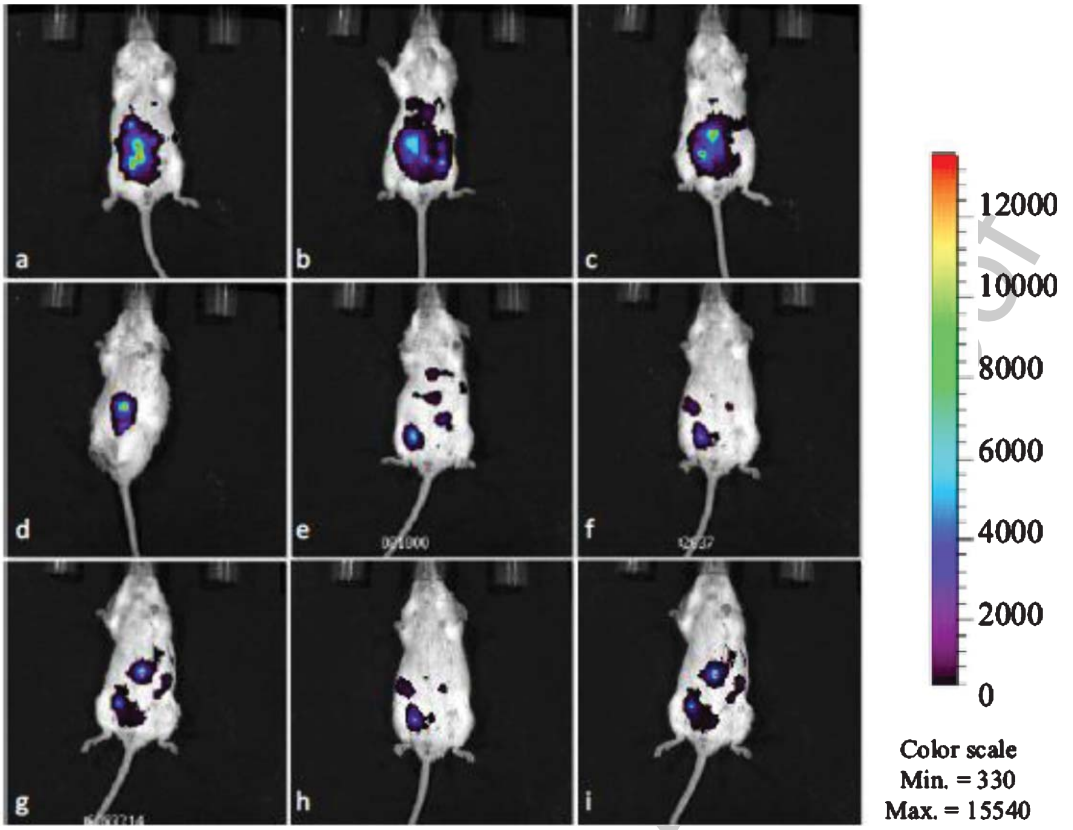

Fig. 7. Bioluminescence monitoring of mASC migration after injection of PBS, NWL-10, or E. coli bacteria to investigate inflammatory effects. BALB/C mice were treated with subcutaneous injection of E. coli bacteria (7a-c), NWL-10 (7d-f), or PBS(7g-i) into the 4th right mammary fat pad. Luciferase labeled mASCs were injected intraperitoneally 48 hours afterwards and monitored using bioluminescence. There was no significant difference between mASC migration in BALB/C mice injected with NWL-10 and mice treated with PBS (accumulation in liver and lien, not in the injected area). Whereas mASCs migrated toward E. coli bacteria injected areas.

injection. Finally, a phase 1 study with human volunteers should be initiated. For these investigations different concentrations of NWL-10 should be used.

5. Conclusions

The first investigations have shown that NWL-10, a new substance combination for injection lipolysis containing PPC 50 mg/mL (in nano particle size of less than 40 nm), GLY 20 mg/mL and MAL 180 mg/mL has the potential to become a second generation product for future lipolytic injectables. In our *in vitro* studies on 3T3-L1 mouse cells and human adipose tissue derived stem cells NWL-10 showed almost no cytotoxic, but high lipolytic effects. Only very low concentrations of NWL-10 caused necrotic effects; higher concentrations led to apoptotic effects. Furthermore, neither the individual substances nor combinations of the individual substances different from NWL-10 showed these effects. Besides, no significant inflammatory response of NWL-10 could be detected in our *in vivo* study on Balb/C mice.

The first promising results need to be confirmed and added by further fundamental and more detailed research on essentials for drug approval.

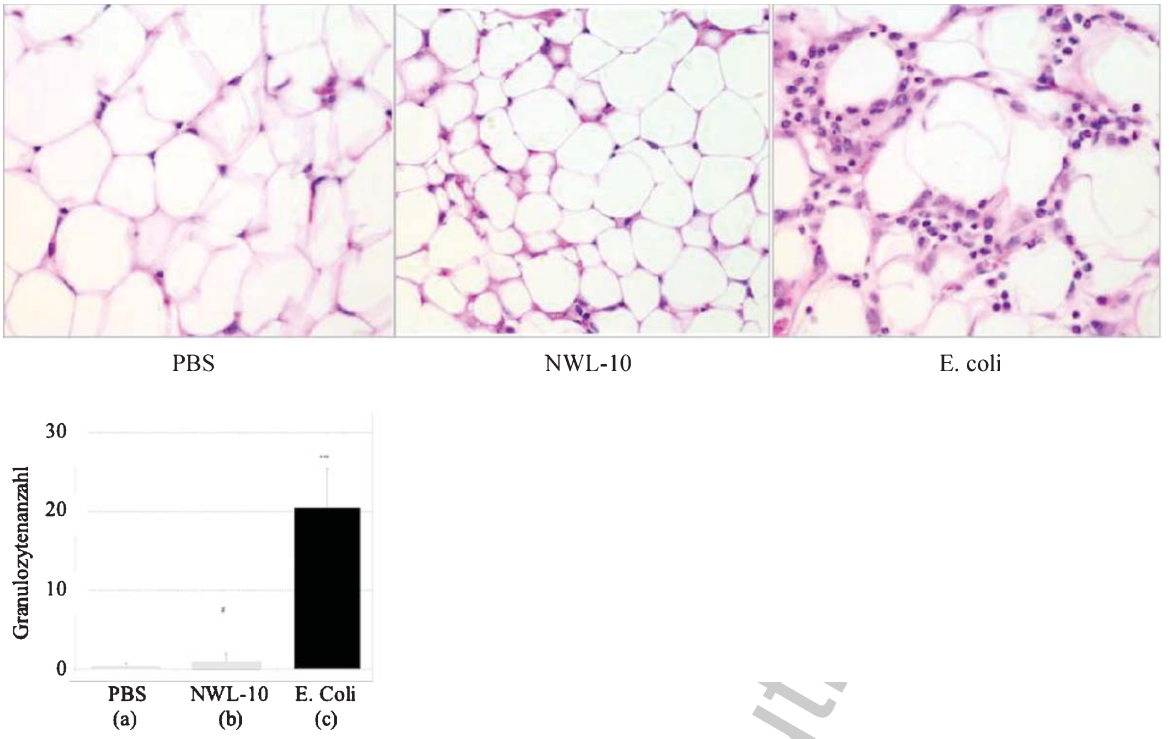

Fig. 8. Haematoxylin and eosin staining of prepared fat pad tissue after injection of PBS, NWL-10 or, E. coli bacteria to investigate inflammatory effects measured by granulocyte migration. BALB/C mice were treated by subcutaneous injection of PBS, NWL-10 = PMC or E. coli bacteria into the 4th right mammary fat pad. The BALB/C mice were sacrificed 7 days afterwards, fat tissue of the injected areas was harvested and after preparation stained with haematoxylin and eosin. Granulocyte migration and consequently inflammatory processes were significantly higher in E. coli bacteria injected tissue compared to PBS or NWL-10 injected tissue. There was no significant difference of granulocyte migration between PBS and NWL-10 injected tissue.

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