Infusions of large synthetic HDL containing trimeric apoA-I stabilize atherosclerotic plaques in hypercholesterolemic rabbits

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BRIEF SUMMARY

The present study aimed at evaluating the effect on atherosclerosis of infusion of synthetic HDL containing trimeric apoA-I (TN-sHDL). Moreover, the impact of TN-sHDL on key biomarkers of reverse cholesterol transport was investigated.
Our results showed that TN-sHDL promotes plaque stabilization, reduces plaque macrophage content and increases both plasma cholesterol efflux capacity and free cholesterol concentration. Besides recent failures in proving its efficacy, sHDL treatment remains a fascinating therapeutic option for reduction of cardiovascular risk.

ABSTRACT

Background: Among strategies to reduce the remaining risk of cardiovascular disease, interest has focused on using infusions of synthetic HDL (sHDL).

Methods: New Zealand rabbits underwent a perivascular injury at both carotids and were randomly allocated into two protocols: 1) a single dose study, where rabbits were treated with a single infusion of sHDL containing a trimeric form of human apoA-I (TN-sHDL, 200 mg/kg) or with Placebo; 2) a multiple dose study, where four groups of rabbits were treated five times with Placebo or TN-sHDL at different doses (8, 40, 100 mg/kg). Plaque changes were analysed *in vivo* by IntraVascular UltraSound (IVUS). Blood was drawn from rabbits for biochemical analyses and cholesterol efflux capacity (CEC) evaluation.

Results: In both protocols, atheroma volume in the Placebo groups increased between the first and the second IVUS evaluation. A stabilization or a slight regression was instead observed vs baseline in the TN-sHDL treated groups ($p<0.005$ vs Placebo post-infusion). TN-sHDL treatment caused a sharp rise of plasma free cholesterol levels and a significant increase of total CEC. Histological analysis of carotid plaques showed a reduced macrophage accumulation in TN-sHDL treated rabbits compared to Placebo ($p<0.05$).

Conclusions: Our results demonstrate that acute and sub-acute treatments with TN-sHDL are effective in stabilizing atherosclerotic plaques in a rabbit model. This effect appears to be related to a reduced intra-plaque accumulation of inflammatory cells. Besides recent failures in proving its efficacy, sHDL treatment remains a fascinating therapeutic option for reduction of cardiovascular risk.

Introduction

Drugs affecting lipid metabolism have revolutionized the treatment of atherosclerosis reducing the risk of cardiovascular disease (CVD) by 30-40%. There is, however, an urgent need for further reduction of the unacceptably high remaining risk [1]. Therefore, the development of drugs targeting the atherosclerotic process still represents an important area of contemporary clinical research.[1,2]

It is a well-known fact that the concentration of high-density lipoprotein cholesterol (HDL-C) is inversely related to the risk of cardiovascular events.[3,4] The most popular mechanistic hypothesis underlying this association is the stimulation of the reverse cholesterol transport (RCT).[5] However, recent studies have suggested that the plasma HDL-C concentration may not always reflect HDL function or, more significantly from a clinical perspective, explain the reduced CVD risk.[4,6-10] Indeed, carriers of inherited HDL disorders accumulate small cholesterol-poor HDL particles, which are very efficient in cholesterol efflux capacity as a first step of RCT and are not at increased risk for CVD.[11-13] Additionally, HDL has been shown to protect the endothelium, inhibit low-density lipoprotein oxidation, play an important role in host defence and exert anti-inflammatory and antithrombotic effects.[5,14-16]

Taken together, these data justify the rationale of testing infusion therapies with cholesterol-poor HDL (i.e. synthetic HDL, sHDL), as a treatment for patients with atherosclerotic disease. This therapeutic approach showed very promising results in pre-clinical studies and first clinical trials, where atherosclerosis regression was demonstrated.[17,18] However, in more recent investigations, infusion of sHDL did not show a convincing clinical benefit.[19]

In the context of this therapeutic approach, a recombinant high-molecular mass variant of human apoA-I, named Tetranectin-apoA-I, has been engineered by fusing three apoA-I molecules with the trimerization domain of human tetranectin.[20] This trimeric apoA-I is not filtered by glomeruli and hence shows a prolonged half-life as compared to normal apoA-I,[20] thus potentially improving its efficacy. Indeed, sHDL containing the dimeric form of a human apoA-I molecular variant, apoA-I_{Milano}, characterized by a longer half-life, have been formulated and proved effective in both pre-clinical and clinical studies.[17,23,32] Moreover, sHDL containing trimeric apoA-I (TN-sHDL) maintains the biological functions of monomeric apoA-I by promoting cell cholesterol efflux, stimulating LCAT-mediated cholesterol esterification, and exerting anti-inflammatory effects.[21,22]

The aim of the present study was to evaluate the effect on atherosclerosis of TN-sHDL infusion in rabbits.[17,23] This experimental approach takes advantage of the *in vivo* assessment of plaque volume through IntraVascular UltraSound (IVUS). Additionally, the impact of TN-sHDL on key biomarkers of RCT was evaluated. The results obtained in the present study may guide future developments towards clinical success.

Materials and methods

Male New Zealand white rabbits, weighing 2.0-2.2 kg, were used for the study. Procedures involving animals and their care were conducted in accordance with institutional guidelines that are in compliance with national (D.L. No. 26, March 4, 2014, G.U. No. 61 March 14, 2014) and international laws and policies (EEC Council Directive 2010/63, September 22, 2010: Guide for the Care and Use of Laboratory Animals, United States National Research Council, 2011). The study was approved by the Italian Ministry of Health (Progetto di Ricerca Protocollo 2012/4).

Preparation of TN-sHDL

Synthetic HDL containing trimeric apoA-I (TN-sHDL) was prepared by Roche Diagnostics, Penzberg, Germany. Briefly, recombinant TN was expressed in Escherichia coli (StrataGene) and a protein extract was made using the phenol extraction protocol.[20] The crude protein was purified using Zn-chelate, followed by SP-Sepharose chromatography and lyophilized. To remove endotoxins and E. coli lipids, the TN was washed with chloroform:methanol, re-dissolved in a guanidinium-HCl buffer, gel-filtrated using Sephadex G-25 into 25 mM (NH4)2CO3 (pH 8.8) and lyophilized. TN was finally bound to 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) and dipalmitoyl phosphatidylcholine (DPPC) in a 1:45:15 molar ratio, as described.[24]

Experimental protocols

Lipid-rich plaque formation was induced as previously described.[25] Rabbits were anesthetized, common carotid arteries perivascularly injured by electric current using a bipolar microcoagulator and all animals fed a 1.5% cholesterol diet throughout the study. Ninety days after surgery, a complete scan of right carotids was recorded by IVUS. Cross-sectional area at the point of maximal stenosis was measured, and only animals with stenosis between 25-50% were enrolled for the study.[24] The right jugular vein was cannulated for treatment/blood drawing. Rabbits were then treated following two protocols, named single dose study and multiple dose study, respectively. For the single dose study, rabbits were divided into 2 groups of 8 animals each and treated with a single intra-jugular infusion, at a constant rate of 1.0 mL/min, of 200 mg/Kg of TN-sHDL or with Placebo (5mM Sodium phosphate and 240mM sucrose, pH 7.3). For the multiple dose study, selected rabbits were divided into 4 groups of 8 animals each and treated five times (once every 3 days) with Placebo or 8, 40, 100 mg/Kg body weight of TN-sHDL.

Three days after the single or after the fifth (last) dose, rabbits were anesthetized and subjected to a second IVUS analysis. Carotids were then excised, embedded in OCT compound and stored at –80^{o}C. Operators responsible for treatment, animal handling, IVUS analyses, and histological quantifications were totally blinded with respect to the treatment.

IVUS imaging

IVUS evaluations were performed before the start of the treatments and at sacrifice using a mechanical IVUS system (Galaxy^{TM} 2, Boston Scientific), as previously described.[17] Atheroma area was calculated as external elastic membrane area minus luminal area. The sum of areas was multiplied by the slice thickness

value (0.5 mm) to obtain plaque volume. The cross-section with the maximal plaque area was referred as the point of maximal plaque formation (maximal stenosis).

Biochemical evaluations

Biochemical evaluations were performed on rabbits enrolled in the single dose study. Blood was collected before and 2', 30', 60', 4h, 24h, 48h and 72h after the end of the single infusion, in EDTA-containing tubes. Plasma was separated by centrifugation and stored at -20°C.[26,27] Total and free cholesterol concentrations were measured by using a Roche Diagnostics Cobas autoanalyser.[28,29] Trimeric apoA-I plasma levels were measured using an anti-human apoA-I antibody (F. Hoffmann-La Roche, Basel, Switzerland), which does not recognize rabbit apoA-I.

Histological evaluation

Cryosections were stained with hematoxylin (Mayer's Haemalum, Bio-Optica, Milano, Italy) and eosin (Bio-Optica, Milano, Italy) for plaque area measurement. In the single dose study, neutral lipid accumulation was identified staining selected sections with Oil Red O (Sigma-Aldrich, St. Louis, MO, USA),[30] whereas macrophages were detected incubating the selected sections with the specific antibody RAM-11 (Dako Italia S.p.A, Cernusco sul Naviglio, Italy).[24]

The Aperio ScanScope GL Slide Scanner (Aperio Technologies, Vista, CA, USA) was used to acquire digital images.[31,32] Plaque volume was evaluated by measuring cross-sectional areas of the intima every 0.5 mm within the area of plaque accumulation. The lipid or macrophage content was measured as plaque area occupied by lipids or macrophages/total plaque area X 100.[33,34]

Efflux experiments

Cholesterol efflux capacity (CEC) of rabbit plasma samples was quantified in J774 murine macrophages.[35] Cells were labelled with [1,2-^{3}H]-cholesterol (PerkinElmer, Milano, Italy) for 24 h in the presence of an ACAT inhibitor (Sandoz S.p.A., Origgio, Italy) used at 2 µg/ml and. After 18 h in BSA-containing medium, cholesterol efflux was promoted for 4 h using 0.5% (v/v) of rabbit plasma samples collected before infusion, and at 4 h and 72 h after the end of the single infusion. Aqueous diffusion (AD)-mediated cholesterol efflux was evaluated in J774 murine macrophage under basal conditions, i.e. in the absence of cAMP (Sigma Aldrich, Milano, Italy). In this condition, specific lipid transporters are expressed at undetectable levels.[36] Total release of cholesterol, i.e. AD-dependent + ATP binding cassette transporter A1 (ABCA1)-mediated, was measured by adding cAMP to the J774 cells. ABCA1-mediated-CEC was then calculated as the difference between total and AD-dependent-CEC.[36]

Statistical analysis

Group differences in IVUS absolute plaque volume, free cholesterol levels and CEC were tested for statistical significance by ANOVA for repeated measurements. IVUS percentage variations and histology data were evaluated by paired two sample t-test or by one-way ANOVA. All ANOVA analyses were followed by Tukey post hoc test. The Pearson correlation coefficient was calculated for inter-

and intra-observer variability of IVUS measurements and for the association between plasma FC changes and total-CEC changes. A value of $p<0.05$ was considered statistically significant. The statistical analyses were performed using the SYSTAT software (Version 13; Systat Software, Inc., Chicago, IL) or Prism (version 6.0) (GraphPad Inc., San Diego, CA).

Results

IVUS scans were analysed for plaque volume measurements. The inter-observer and the intra-observer variability in plaque volume measurements were in line with those calculated in previous studies performed by our group (0.853, $p<0.001$ and 0.915, $p<0.005$, respectively).[17,25]

Single dose study - In three out of sixteen animals the quality of IVUS images did not allow reliable measurements of the plaque area, therefore the results described below refer to thirteen rabbits (6 Placebo and 7 TN-sHDL treated rabbits). Figure 1 shows absolute plaque volumes and percentage variations that occurred during the treatment period. Pre-treatment plaque volumes were not different between the two groups ($p>0.05$). By looking at plaque changes vs. baseline, atheroma volume in the Placebo group increased in the time between the first and the second IVUS evaluation (+0.94 ± 0.33 mm^3, Fig. 1A). In contrast, a stabilization was observed vs. baseline in TN-sHDL treated rabbits (-0.05 ± 0.26 mm^3). Comparison between post-treatment plaque volumes of the two groups showed a difference that was very close to statistical significance ($p=0.06$). Nevertheless, absolute and percentage changes of atheroma volume in the Placebo group were significantly different from those found in TN-sHDL treated rabbits (+0.94 ± 0.33mm^3 vs. -0.05 ± 0.26mm^3, respectively; $p<0.0001$, and Fig. 1B). Examples of IVUS images of atherosclerotic plaques recorded before and after treatment with Placebo or TN-sHDL are shown in Supplemental Figure S1.

Multiple dose study - A total of 32 rabbits was treated, but only 27 animals (7 Placebo, 5 TN-sHDL 8 mg/Kg, 7 TN-sHDL 40 mg/Kg and 8 TN-sHDL 100 mg/Kg) allowed reliable plaque measurements. Pre-treatment plaque volumes were not statistically different among the four experimental groups ($p>0.05$, Fig. 2A). Atheroma volume in the Placebo group increased during the time between the first and the second IVUS evaluation (+1.55 ± 0.58 mm^3). A slight progression or regression was instead observed in TN-sHDL treated rabbits vs baseline (+0.18 ± 0.25 mm^3, -0.01 ± 0.63 mm^3 and -0.21 ± 0.57 mm^3 in the TN-sHDL 8, 40 and 100 mg/Kg groups, respectively, Fig. 2A). As a consequence, absolute and percentage changes of total atheroma volume vs. baseline in each TN-sHDL treated group were significantly different from those measured in the Placebo group ($p<0.005$, Fig. 2B). No statistical differences were observed among the three TN-sHDL treated groups.

Effect of TN-sHDL infusion on plaque macrophage content and plasma free cholesterol

Plaque volume, evaluated by histology, did not differ between Placebo and TN-sHDL treated rabbits ($p>0.05$). Moreover, TN-sHDL treatment did not affect plaque lipid accumulation, measured by Oil Red O staining (65.7 ± 18.0% vs 70.3 ± 13.0% in Placebo, $p>0.05$). On the contrary, TN-sHDL treated rabbits displayed a significantly lower plaque macrophage content compared to that measured in Placebo (69.5 ± 13.4% vs 84.3 ± 9.3%, $p< 0.05$, Fig. 3).

Total cholesterol concentration did not change throughout the treatment period, and between the two groups of animals (Fig. 4). However, as shown in Fig. 4, starting from 2 min after the end of the infusion and up to 24 h, a significant increase in plasma free cholesterol levels was observed in rabbits treated with TN-sHDL ($p<0.05$ vs Placebo).

Plasma concentration of apoA-I was also measured at each time point. Based on these data, a half-life of 22 hours was calculated (Fig. 5).

Effect of TN-sHDL infusion on cholesterol efflux capacity

TN-sHDL infusion elicited a marked increase of total, AD-dependent and ABCA1-mediated CEC (Fig. 6). As shown in Figure 6A, total CEC of rabbit plasma, collected 4h after the end of TN-sHDL infusion, was significantly increased compared to total CEC measured in the Placebo group ($p<0.0001$). No differences between the two treatments were observed instead in plasma samples collected before infusion and after 72 h. Moreover, for TN-sHDL treated rabbits, plasma total CEC at 4h was significantly different from that measured before infusion or 72h after the end of the infusion ($p<0.0001$).

Four hours after TN-sHDL infusion, AD-dependent CEC was increased compared with Placebo ($p<0.005$), and it was significantly higher than that measured before and 72h after infusion ($p<0.05$; Fig. 6B). The ABCA1-mediated CEC was significantly increased at 4h after TN-sHDL treatment compared to pre-treatment values and returned to baseline at 72h after the end of the single infusion (Fig. 6C). No significant differences were observed vs. Placebo at each time point analysed.

In the Placebo group, no variations were detected in total, AD-dependent as well as ABCA1-mediated CEC at each time point (Fig. 6A, 6B and 6C).

Delta plasma FC at 4 hours after infusion correlated strongly and positively with the increase of total efflux capacity at the same time point (R^2 = 0,868, $p<0.0001$).

Discussion

The main result of the present study is that a single intravenous infusion of TN-sHDL promoted plaque stabilization in a rabbit model of atherosclerosis.[25] This stabilization occurred without plaque lipid removal, but was paralleled by a significant reduction in plaque macrophage content. Similar effects were achieved by treating the rabbits with five infusions of TN-sHDL at different doses (8, 40 and 100 mg/Kg), where stabilization or a moderate regression of atherosclerotic lesions was observed. These results were obtained in a rabbit model, already used to test the efficacy of sHDL infusion on atherosclerotic lesions[37,17], that allows the *in vivo* assessment of plaque volume through IVUS, one of the clinical imaging modalities to evaluate the impact of therapies on plaque progression/regression.[38-40] The efficacy of TN-sHDL treatment must be evaluated considering that these results were obtained within a short time and after one or few administrations. Of note, first choice pharmacological treatments, i.e. statins, minimally affect plaque size and this effect occurs only when these drugs are administered at high doses and for 18-24 months[41].

The trimeric human apoA-I has been synthesized with the aim of increasing the half-life as compared to normal apoA-I,[20] based on the hypothesis of a size-dependent rate of catabolism for apoA-I.[42] Indeed, in the present experimental conditions a half-life of 22 h was found, a much higher value than that measured for normal apoA-I.[43] This observation seems to exclude the formation of immune complexes with trimeric apoA-I that were shown to cause an accelerated catabolism of TN-sHDL in a previous study in monkeys.[44]

When TN-sHDL was infused at three different doses, only a trend towards a dose-related effect on plaque volume was detected, since no significant differences were observed among treatments. This result is in line with those obtained by infusion of other sHDL preparations, i.e. ETC-216 and CER-001. Specifically, ETC-216 administered at 45 mg/kg dose did not show significantly higher efficacy than the 15 mg/kg dose and CER-001 showed its best efficacy at the lowest dose tested.[38,45] These results may be explained by recent epidemiological observations indicating that HDL-C levels do not correlate linearly with CVD risk, but they follow a U-shaped association, thus suggesting that HDL functionality is not reflected by HDL concentration.[46] As a consequence, the highest sHDL dose tested may not necessarily determine a greater effect on plaque burden when compared with a lower dose used. Although speculative, 8, 40, and 100 mg/Kg of TN-sHDL may be close to the bottom of the U-curve, thus showing a comparable efficacy in their atheroprotection.

Animal[47] and human[48] studies have shown that the cholesterol efflux potential of HDL is a better inverse predictor of CVD than plasma HDL-C levels per se. For this reason, CEC was evaluated in rabbit plasma after Placebo or TN-sHDL infusion. TN-sHDL caused a marked increase of total-CEC in rabbit plasma collected 4h after the end of the infusion. In agreement with these data, a rapid increase of plasma free cholesterol was detected after TN-sHDL infusion that positively correlated with the delta total-CEC at 4 hours. A similar sharp rise in free cholesterol concentration was also observed after the infusion of MDCO-216, CSL112 and CER-001, both in animal and human studies.[49,50] Efflux data revealed that TN-sHDL infusion increased the ABCA1-mediated route, but also importantly affected AD-dependent CEC. A physical explanation for the use of the AD pathway by TN-sHDL may be related to its size. It has been shown that reconstituted HDL with diameter greater than 9 nm is a good acceptor in the AD pathway, whereas smaller particles are efficient acceptors

of cholesterol via ABCA1.[51] Coherently, TN-sHDL particles, which have a mean diameter of about 9.5 nm (unpublished results), efficiently use the AD pathway. The relevant use of this efflux modality differentiates TN-sHDL from other sHDL tested for atherosclerosis regression, such as MDCO-216, CSL-112, that, being constituted by smaller particles, mainly promote cholesterol efflux via the ABCA1 transporter.[49,50] In addition, the observed increase in AD-dependent CEC could be also partially related to the phospholipid component of the sHDL particles, since phospholipids promote AD efflux.[52]

Although it is clear that the ABCA1 pathway plays a major role in cholesterol removal from cell components of atherosclerotic plaque, pharmacological interventions increasing non-ABCA1-mediated cholesterol efflux[53,54] did not provide cardiovascular benefit in patients with atherosclerotic CVD.[55] It is thus interesting to note that no significant removal of neutral lipids from plaques was observed in TN-sHDL infused rabbits vs Placebo. This result differs from that obtained after infusion of A-I_{Milano}-containing sHDL where a dose-dependent lipid removal from plaques was detected.[23] Interestingly, TN-sHDL treated rabbits displayed a significantly lower macrophage content in carotid plaques compared to the Placebo group, implying that the stabilization of the stenosis process mediated by this sHDL treatment may be a consequence of reduced monocyte migration into the atherosclerotic lesions. Indeed, in previous studies, sHDL infusion was shown to significantly lower endothelial expression of VCAM-1, ICAM-1 and MCP-1[56,57]. Alternatively, the lower macrophage content of plaques in TN-sHDL treated rabbits could be the result of macrophage cholesterol removal occurring through the ABCA1 pathway. Cholesterol unloading has in fact been shown to restore the migration ability of macrophages[58], possibly inducing their transmigration out from plaques.[59]

Conclusions

We showed that infusion of sHDL containing trimeric human apoA-I led to a stabilization of atherosclerotic lesions. This result might be the consequence of different HDL-mediated mechanisms, including anti-inflammatory effects related to the increased cholesterol efflux. The long-term efficacy of this kind of therapeutic strategies has still to be proven in the clinic, but an experimental study clearly demonstrated that acute regression of atherosclerotic plaques by sHDL infusion was maintained up to six months.[57]

In conclusion, our results may provide the scientific rationale to further develop lipidated TN-ApoA-I and support the evidence of health benefits by sHDL infusion in the treatment of atherosclerosis.

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Disclosures

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Figure captions

Figure 1. A single infusion of TN-sHDL promotes plaque stabilization. (**A**) Absolute values and (**B**) percentage change of atheroma volume evaluated by IVUS at the right carotids in rabbits treated with Placebo or 200 mg/Kg of TN-sHDL. In A, open bars represent pre-treatment and solid bars represent post-treatment. Data are expressed as mean ± SD; with n=6 for Placebo and n=7 for TN-sHDL treated rabbits. *p<0.0001 vs Placebo by paired two-sample t-test.

Figure 2. Five infusions of 8, 40 and 100 mg/Kg of TN-sHDL are effective in stabilizing or moderately regressing rabbit carotid plaques. (**A**) Absolute values and (**B**) percentage change of atheroma volume evaluated by IVUS at the right carotids in rabbits treated with Placebo or different doses of TN-sHDL. In **A**, open bars represent pre-treatment and solid bars represent post-treatment. Data are expressed as mean ± SD; with n=7 for Placebo, n=5 for TN-sHDL 8 mg/Kg, n=7 for TN-sHDL 40 mg/Kg and n=8 for TN-sHDL 100 mg/Kg treated rabbits. *p<0.005 vs Placebo by one-way ANOVA.

Figure 3. TN-sHDL infusion reduces plaque macrophage content. Representative photomicrographs of immunostaining for macrophages in rabbit carotid plaques infused with Placebo or 200 mg/Kg of TN-sHDL. A decreased macrophage content is visible in the TN-sHDL-treated rabbit. Scale bar = 100 μm.

Figure 4. Top: Total cholesterol plasma levels measured before (0) and 2', 30', 60', 4h, 24h, 48h and 72h after the end of the infusion in Placebo and TN-sHDL treated rabbits. **Bottom:** A single TN-sHDL infusion causes a rapid increase of free cholesterol levels. Percentage change of free cholesterol levels measured in plasma of rabbits treated with Placebo (squares) or 200 mg/Kg of TN-sHDL (diamonds). Blood was collected before (0') and 2', 30', 60', 4h, 24h, 48h and 72h after the end of the infusion. Data are expressed as mean ± SD; with n=6 for Placebo and n=7 for TN-sHDL treated rabbits. *p<0.05 vs Placebo by ANOVA for repeated measurements.

Figure 5. Plasma clearance of trimeric human apoA-I in hypercholesterolemic rabbits infused with 200 mg/kg of TN-sHDL. Blood was collected before and 2', 30', 60', 4h, 24h, 48h and 72h after the end of the infusion. Data are expressed as mean ± SD; with n=7.

Figure 6. TN-sHDL infusion increases cholesterol efflux capacity (CEC). CEC was measured on rabbit plasma collected before infusion (0h), at 4h and 72 h after the end of infusion with Placebo (open bars) or 200 mg/kg of TN-sHDL (solid bars). (**A**) Total, (**B**) aqueous diffusion (AD)-dependent and (**C**) ABCA1-mediated CEC of rabbit plasma are shown. All efflux values are reported as the average of 3 determinations in different wells. Data are expressed as mean ± SD; with n=5. In **A**, *p<0.0001 vs 4h Placebo; † p<0.0001 vs 0h and 72h TN-sHDL. In **B**, $ p<0.005 vs 4h Placebo; § p<0.05 vs 0h and 72h TN-sHDL. In **C**, † p<0.0001 vs 0h and 72h TN-sHDL. Data were analysed by ANOVA for repeated measurements.