Anticancer Agents

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A Liposomal System Capable of Generating CO_2 Bubbles to Induce Transient Cavitation, Lysosomal Rupturing, and Cell Necrosis**

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Drug delivery using nanoscale liposomal vehicles is a promising strategy for cancer treatment owing to their merits in terms of safety and ease of surface modification.[1] Liposomes can also hold a large payload of anticancer drugs such as doxorubicin. After killing the cancer cells, however, the remaining anticancer agent may harm normal cells and tissues as well. Additionally, certain drugs may undergo transformation to generate chemically reactive metabolites that can cause significant adverse side effects. For example, it is well documented that doxorubicinol (i.e., the metabolite of doxorubicin) can induce acute and chronic cardiac toxicities.[2]

To solve the aforementioned problem associated with conventional chemotherapy, herein we describe a thermally responsive liposomal system that contains no anticancer drug but is still capable of killing cancer cells. Figure 1 shows a schematic of the new liposomal system and how it works. The key component of this new system is ammonium bicarbonate (NH_4HCO_3), which is widely used as a rising agent in the food industry for the generation of gas bubbles in baked goods.[3] Upon heating to a temperature of 40 °C or above, it can quickly decompose to generate CO_2 bubbles.[4] This compound can also be readily incorporated into the aqueous compartment of a liposome. After the internalized liposomes have gone through endocytosis and intracellular trafficking to lysosomes, they can be thermally triggered at 42 °C to generate CO_2 bubbles. As a result of the transient formation, growth, and collapse of CO_2 bubbles, a disruptive force will be produced similar to the cavitation effect induced by ultrasound.[5] The cavitation force acting on lysosomes can mechanically disrupt the membranes to release their proteolytic enzymes into the cytosol, resulting in cell necrosis without leaving behind any toxic agents. In this way, only the cells that internalize the liposomes containing NH_4HCO_3 will be destructed when heated up to 42 °C, whereas the neighboring cells can still remain alive and unharmed. It is worth pointing out that most normal tissues remain undamaged following treatment for 1 h at temperatures up to 44 °C.[6] This feature makes the new liposomal system potentially safe for clinical use.

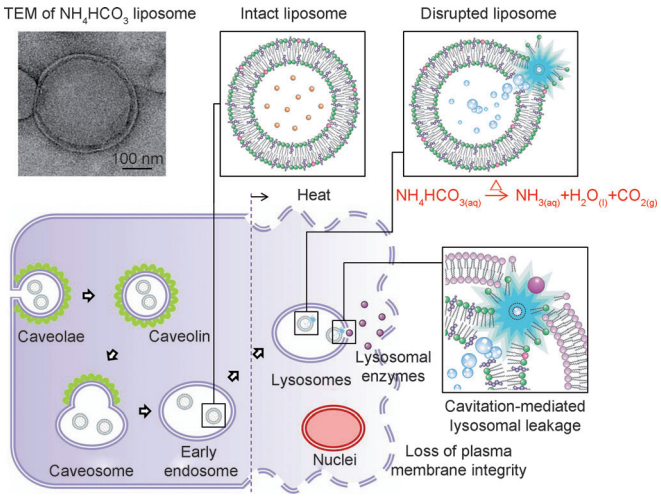

Figure 1. A schematic illustration showing the composition/structure of the liposomes that generate bubbles upon heating, and how they can be used to rupture cancer cells by transient cavitation, thus resulting in cell death.

The liposomes were prepared in a phosphate buffered saline (PBS) containing NH_4HCO_3 with hydrogenated soy phosphatidylcholine (HSPC), cholesterol, and 1,2-di-*O*-octadecenyl-3-trimethylammonium propane (DOTMA) at a molar ratio of 6.0:4.0:0.5. As a control, liposomes were also prepared in PBS in the absence of NH_4HCO_3 (PBS liposomes). From the transmission electron microscopy (TEM) image (Figure 1), it is clear that the as-prepared NH_4HCO_3 liposome had a unilamellar vesicle structure. Dynamic light scattering (DLS) measurements indicate that the sizes and surface potentials were 295.0 ± 13.7 nm and 39.5 ± 2.5 mV ($n = 6$ batches),respectively, for the NH_4HCO_3 liposomes, and 300.0 ± 14.5 nm and 41.4 ± 1.3 mV, respectively, for PBS liposomes.

The temperature sensitivity of both PBS and NH_4HCO_3 liposomes was evaluated by their ability to generate CO_2 bubbles when they were suspended in aqueous media and

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heated to 37 °C and 42 °C. Test tubes containing either PBS or NH_4HCO_3 liposomes were immersed in a water-filled tank, and the formation of bubbles was examined at a macroscopic level by using an ultrasound imaging system.[7] For the PBS liposomes, no bubbles were observed at both temperatures (Figure 2a). While no bubbles appeared in the sample of NH_4HCO_3 liposomes at 37 °C, a large number of bubbles were observed to form when the sample was heated to 42 °C. The bubbles quickly grew and then collapsed violently, similar to cavitation, which has been demonstrated to play a major role in a wide range of novel therapeutic applications involving ultrasound.[8]

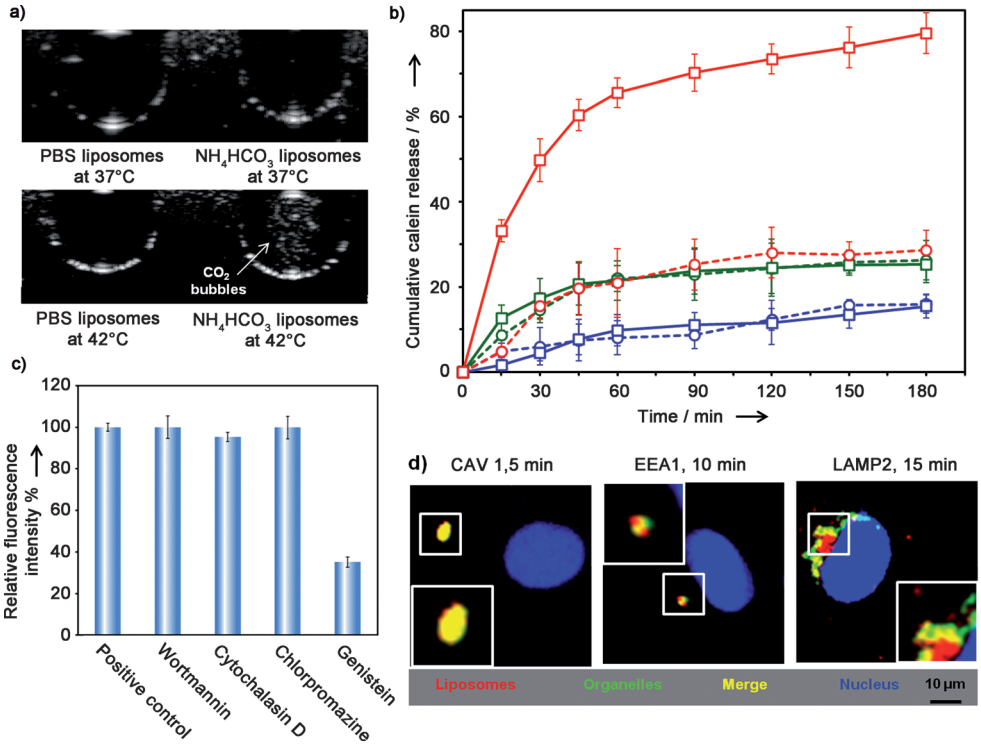

Figure 2. a) Ultrasound images of the PBS and NH_4HCO_3 liposomes suspended in aqueous media and heated to 37 and 42 °C. b) Release profiles of calcein from NH_4HCO_3 liposomes (——□——) and PBS liposomes (– – –○– – –) incubated at 25 (blue), 37 (green), and 42 °C (red). c) Effects of inhibitors on internalization of the NH_4HCO_3 liposomes ($n = 6$). d) Confocal images showing the intracellular trafficking of NH_4HCO_3 liposomes (red) taken at the indicated time points using the immunohistochemical stains to identify the caveosomes (CAV1; green), early endosomes (EEA1; green), and lysosomes (LAMP2; green).

We then investigated the ability of cavitation to destabilize the lipid-bilayer membranes of NH_4HCO_3 liposomes by incorporating calcein into the aqueous core. The cumulative calcein-release profiles of the liposomes at 25, 37, and 42 °C were then compared. The PBS liposomes containing calcein were used as a control. As shown in Figure 2b, the amount of calcein released from the PBS and NH_4HCO_3 liposomes was minimal at 25 °C. Notably, elevating the temperature to 37 °C induced a low level of calcein leakage from both types of liposomes; this leakage is probably due to thermal fluctuation of the membranes. When heated to 42 °C, the calcein-release profile of the PBS liposomes resembled that obtained at 37 °C. For the NH_4HCO_3 liposomes, however, the decomposition of NH_4HCO_3 at 42 °C generated a large number of CO_2 bubbles, which grew rapidly and then collapsed violently, thus producing a cavitation force that could perforate the lipid-bilayer membranes and trigger a significant release of the encapsulated calcein.

To elucidate their endocytosis pathways, we incubated the NH_4HCO_3 liposomes labeled with DOPE-rhodamine with HT1080 cells (human fibrosarcoma) that had been pretreated with chemical inhibitors for macropinocytosis (wortmannin and cytochalasin D), clathrin-mediated endocytosis (chlorpromazine), and caveolae-mediated endocytosis (genistein), and analyzed them by using flow cytometry.[9] Our experimental data showed that only genistein could effectively inhibit the cellular uptake of NH_4HCO_3 liposomes (Figure 2c; $P < 0.05$), thus indicating the involvement of caveolae-mediated endocytosis. Additionally, intracellular trafficking of the internalized NH_4HCO_3 liposomes was examined by colocalization of intracellular organelles and liposomes labeled with DOPE-rhodamine. Our results suggest that the NH_4HCO_3 liposomes entered the cells through caveolae and caveosomes (CAV1) at 5 min post culture and were transported to early endosomes (EEA1) at 10 min and, finally, to lysosomes (LAMP2) at 15 min (Figure 2d).

We also evaluated how the NH_4HCO_3 liposomes affected cell viability as a function of incubation time prior to heating at 42 °C. As shown in Figure 3a, the cell viability reached a minimum value when the culture time approached 15 min (that is, when the NH_4HCO_3 liposomes entered into lysosomes; see Figure 2d). Based on these results, we increased the temperature to 42 °C at 15 min post incubation in subsequent studies. Figure 3b summarizes the results of cell viability after the cells had been incubated with the PBS and NH_4HCO_3 liposomes at 25, 37, and 42 °C, with cells that were not treated with liposomes as the control groups. Similar to the controls, no apparent cytotoxicity was observed after the cells had been incubated with the PBS liposomes at 37 and 42 °C ($P > 0.05$), thus indicating that simple heating in the presence of plain liposomes would not cause cell death. For the group treated with NH_4HCO_3 liposomes at 37 °C, most cells were viable. However, raising the temperature to 42 °C significantly reduced the cell viability to $48.1 \pm 3.9\%$ ($P < 0.05$, $n = 6$). In this case, the cell death was believed to arise

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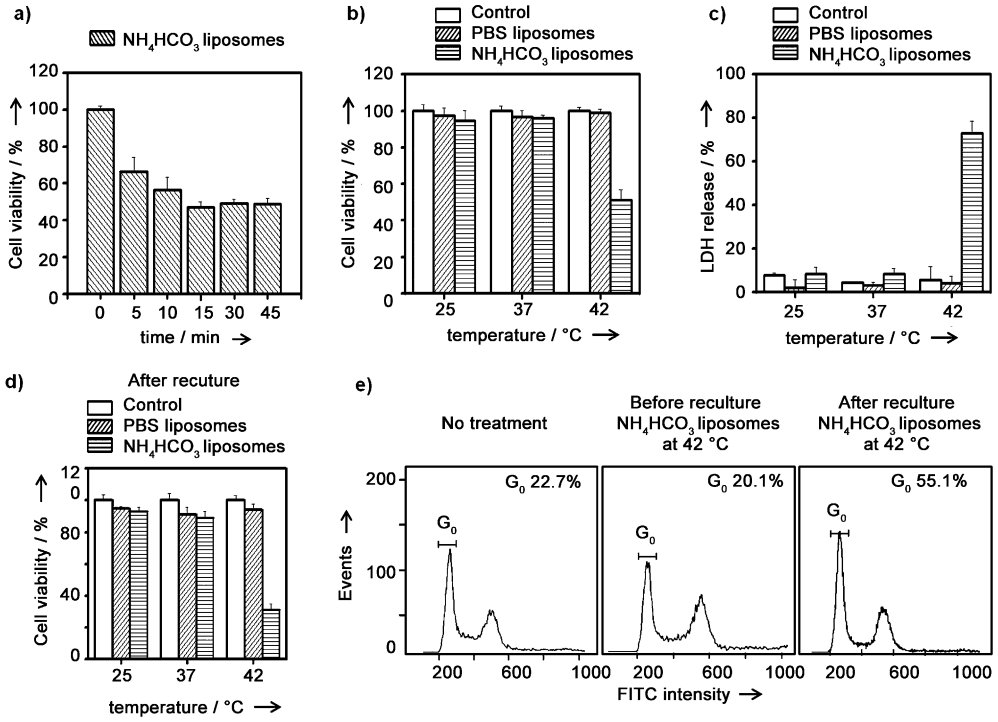

Figure 3. a) Viability of HT1080 cells after they had been incubated with the NH_4HCO_3 liposomes for different periods of time prior to heating to 42 °C ($n=6$). b) Viability of the cells treated with PBS and NH_4HCO_3 liposomes, at 25, 37, and 42 °C, as evaluated by the MTT assay. c) Results of LDH assay for the same samples as (b). d) Viability results for the cells in (b) after they had been cultured again for 24 h, determined by the MTT assay. e) Cell-cycle progression in HT1080 cells after they had been treated with the NH_4HCO_3 liposomes at 42 °C before and after reculture, analyzed by flow cytometry.

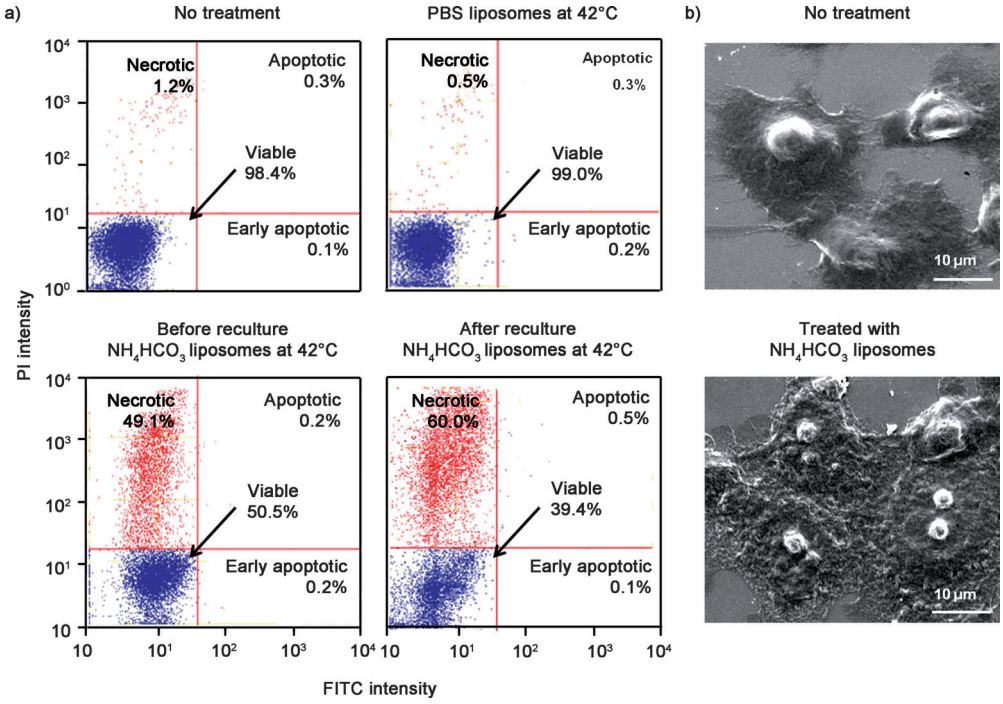

Figure 4. a) Flow cytometry analysis of necrosis in HT1080 cells after treatment with the PBS and NH_4HCO_3 liposomes at 42 °C. b) SEM micrographs of HT1080 cells before and after being treated with the NH_4HCO_3 liposomes at 42 °C.

from the formation, growth, and collapse of CO_2 bubbles during the heating of NH_4HCO_3 liposomes. Through a process known as transient cavitation,[10] the lysosomes were ruptured to release their contents into the cytosol, ultimately resulting in cell death. A prior study found that lysosomes, the organelles containing numerous hydrolases, played a profound role in cell necrosis.[11]

We also confirmed the presence of cavitation-induced cell necrosis by studying the release of enzyme lactate dehydrogenase (LDH) into the culture medium. According to a previous study, the amount of LDH in the culture medium is proportional to the extent of lysosomal leakage.[12] Compared with the other cell groups, only the cells treated with the NH_4HCO_3 liposomes at 42 °C released a significant amount of LDH into the medium (Figure 3c), thus suggesting lysosomal destabilization. This process could significantly damage the cells, causing them to die or enter a stable quiescent state (the G_0 phase).[13] To test this hypothesis, the cells were cultured again without further stimulation. After 24 h, the viability of cells originally treated with NH_4HCO_3 liposomes at 42 °C was further reduced to $31.3 \pm 3.1\,\%$ ($P < 0.05$, Figure 3d). Our analysis based on flow cytometry revealed that the diminished cell growth was related to the inhibition of cell cycle progression at the G_0 phase. As shown in Figure 3e, there was a significant increase in cell population from $20.1 \pm$

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6.7% (before cell reculture) to 55.1 ± 5.1% (after cell reculture) in the G_0 phase.

Apoptosis and necrosis are two major modes of cell death. Flow cytometry can be used to enumerate apoptotic and necrotic cells. The percentage of cells undergoing necrosis was significantly increased from 1.2 ± 0.5% (control) to 49.1 ± 3.5% after treatment with NH_4HCO_3 liposomes at 42 °C (Figure 4a). Additionally, after 24 h of reculture, the percentage was further increased to 60.0 ± 2.5%. Our results based on flow cytometry analysis were also confirmed by carefully examining the cells under a scanning electron microscope. Compared to the untreated control cells, the cells treated with NH_4HCO_3 liposomes at 42 °C were characterized by discontinuities and crumpling in plasma membranes, as well as profound nuclear changes culminating in nuclear dissolution, an indication of cell necrosis.[14] It is well established that toxic chemicals generally induce cell apoptosis while lysosomal leakage may cause cell necrosis.[15] Apoptosis is a reversible process, but necrosis, being an irreversible process, can start only and exclusively when the cell dies.[16]

One of the major problems with the conventional chemotherapy is that the treatment may also damage adjacent normal tissues. The cavitation method demonstrated in this work may provide a potential solution to this problem. In a proof-of-concept experiment, cells were grown on a Transwell device consisting of two chambers, with the upper and lower chambers representing the cancer and neighboring healthy cells, respectively (Figure 5). The NH_4HCO_3 liposomes were only added to the upper chamber and incubated with the cells. The two chambers were separated by a 200 nm microporous membrane to prevent the liposomes (300 nm in diameter) from passing through. However, the products from decomposition during heating (e.g., lipids and CO_2) could freely diffuse into the lower chamber. The results of our live/dead staining and MTT assay (Figure 5) indicate that only the cells directly treated with the NH_4HCO_3 liposomes in the upper chamber underwent necrosis, while their neighboring cells in the lower chamber remained unharmed.

In summary, we have developed a thermal-responsive liposome system containing NH_4HCO_3 that is able to kill cancer cells through transient cavitation. Our results demonstrate that using NH_4HCO_3 liposomes can substantially amplify the induction of necrosis in cancer cells, resulting in a clinically important reduction in adverse outcomes on healthy tissues.

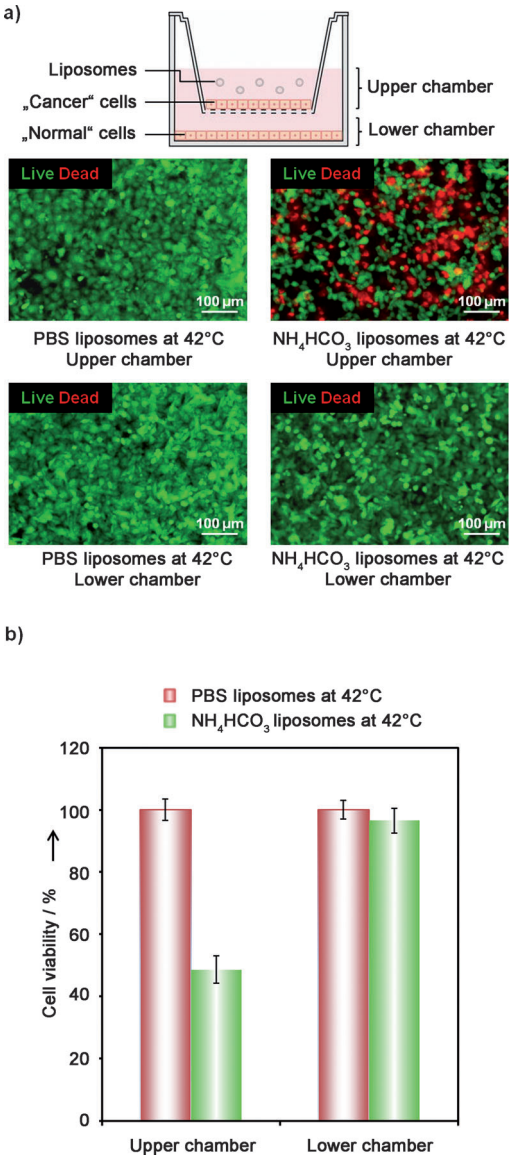

Figure 5. a) Fluorescence images showing the viability of cells following treatment with the PBS or NH_4HCO_3 liposomes (1 mg mL^{-1}) at 42 °C in a Transwell device. The live cells were stained green while the dead cells were stained red. The distance between the two layers of cells was 1 mm. b) The quantitative results as evaluated by the MTT assay ($n = 6$).

Experimental Section

Materials: HSPC, DOTMA, cholesterol, and DOPE-rhodamine were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Anti-CAV1, anti-EEA1, and anti-LAMP2 antibodies were purchased from Abcam (Cambridge, MA, USA).

Liposome preparation: Liposome colloidal suspensions were prepared by dissolving the lipid mixture (40 mg) in chloroform. The organic solvent was removed with a rotavapor to generate a thin lipid film on the glass vial. The lipid film was then hydrated with an aqueous NH_4HCO_3 solution (0.2 g mL^{-1}) by sonication at room temperature. The excess NH_4HCO_3 was removed by dialyzing against PBS.

Calcein release from liposomes: An aliquot of the calcein-loaded liposomes was added to a quartz cuvette at various temperatures. Fluorescence intensity of the solution was monitored over time by using a fluorescence spectrometer.

Intracellular trafficking: After incubation with the liposomes, the cells were washed twice with PBS before they were fixed in 4% paraformaldehyde. The fixed cells were examined using immunohistochemical stains to identify caveosomes, endosomes, and lysosomes, followed by examination with a confocal microscope (TCS SL, Leica, Germany).

Cell viability assay: HT1080 cells were co-cultured with the liposomes for 15 min at distinct temperatures; the culture medium was heated up to the predetermined temperature within 5 min. After treatment, samples were aspirated and cells were incubated in a medium containing 1 mg mL^{-1} MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent for 4 h, and 1 mL of

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DMSO was then added. Optical density readings were obtained using a multi-well scanning spectrophotometer.

Cell cycle analysis: After treatment, cells were gathered and washed with PBS, fixed in precooled (-20°C) 70% ethanol, and stored at −20°C. After fixation, the cells were washed with PBS and stained with propidium iodide (PI) and anti-cyclin A2-FITC. The cells were analyzed using a flow cytometer (Beckman Coulter, CA, USA). Necrosis was observed by using the Roche Annexin-V-FLUOS Staining Kit (Roche, Indianapolis, IN, USA).

Statistical analysis: Two groups were compared by the one-tailed Student's *t*-test using statistical software (SPSS, Chicago, IL, USA). Data are presented as mean ± standard deviation. A difference of $P < 0.05$ was considered statistically significant.

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