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Evaluation of Apoptosis Induced by Celecoxib Loaded Liposomes in Isolated Rat Hepatocytes

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Background: Apoptosis is an essential process for elimination of damaged and cancerous cells and is also a desired effect for the anti-cancer activity of drugs. Cyclooxygenase-2 (COX-2) inhibitors including celecoxib are a class of drugs with chemopreventive and anti-proliferative properties regardless of their routine anti-inflammatory effects.
Objectives: The objective of this study was to evaluate the effect of celecoxib loaded nano-liposomes on the isolated rat hepatocytes and comparing the results with what were obtained from usual form of this chemopreventive agent.
Materials and Methods: Freshly isolated rat hepatocytes were prepared by two step collagenase perfusion method and, following stabilization in rotary, were exposed to 0, 20, 40 and 100 μM of celecoxib in nano and usual form. Viability was obtained and apoptosis was determined by modified comet technique.
Results: At high concentrations (40 and 100 μM), apoptosis was demonstrated and it was clearly more prominent in hepatocytes exposed to celecoxib loaded liposomes.
Conclusions: Our results showed that celecoxib loaded liposomes robustly induced apoptosis, an action that can potentially make it more relevant than the usual form for chemopreventive strategies and also may raise concerns about its toxicity.

Keywords: Apoptosis; Celecoxib; Liposomes; Hepatocyte; Comet Assay; Cyclooxygenase-2

1. Background

Nanotechnology involves imaging, measuring, modeling and manipulating material at nanoscale (1). Nanoscale materials have unique physicochemical properties, such as high reactivity, ultra-small size, and larger ratio of surface area to mass, which are different from bulk materials (in microscale) of the same composition. These properties can be useful to decrease several of the limitations found in nanomedicine (2, 3). Nanomedicine has been defined as the application of nanotechnology to medicine. The difference between nano-drugs and conventional drugs is that nano-drugs are entirely based on small molecule chemistry. By manufacturing drugs in nanoscale, is possible to modify their pharmacokinetics and to improve the efficacy and safety of the therapy (4). Nanosized drugs, because of the large surface area to mass ratio, increased the effectiveness of drugs. The nanoparticles are good carriers for poorly soluble compounds (5). Current researches show that the bulk materials are more reactive and have more toxic effects than smaller particles (6, 7). Nanoparticles are not competently scavenged by macrophages, and therefore their blood circulation time is increased. Prolonged bioavailability extends the duration of controlled system drug delivery or improves the characteristics for nanoparticles to reach target sites, by extravasation (8). Nanotoxicology is rising as an important sub-discipline of nanotechnology. Nanotoxicology deals with the interaction of nanostructures with biological systems and shows the relationship between the physical and chemical properties of nanostructures and their toxic consequences (3, 4). Because of the importance of nanotechnology and its wide use, in the pharmaceutical industry, and the damage caused by the toxicity of nano-drugs, they are receiving increased attention. The coxibs are selective inhibitors of cyclooxygenase-2 (COX-2) (9). The COX_2 inhibitors are used as a treatment for inflammatory diseases, menstrual pain, headache, and patent ductus arteriosus, in premature infants.

Traditional non-steroidal anti-inflammatory drugs (NSAIDs) are prescribed for the treatment of mild to moderate pain, especially pain caused by inflammation. In diseases, such as rheumatoid arthritis and gout, non-selective NSAIDs and COX-2 inhibitors reduced the formation of polyps in susceptible patients. Also, they reduce the for-

mation, growth and metastasis, in vitro, and also reduced the number of intestinal tumors, in patients with adenomatous polyps (10-12). Apparently, by inhibiting the prostaglandin production and apoptosis induction, NSAIDs exert their anti-tumor effects (13). The NSAIDs reduce the incidence of carcinogen-induced colon tumors (14). Celecoxib is a sulfonamide NSAID and selective COX-2 inhibitor (1). Celecoxib enhances the induction of apoptosis in human pancreatic cancer cells, through a mechanism depending on the prostaglandin E 2 (PGE2) and COX-2 pathways (15), with improvement of colorectal polyps in familial adenomatous polyposis (13). As a chemoprevention of colorectal cancer, it induces apoptosis via calcium ion homeostasis (16). Celecoxib induces apoptosis in cervical tumor cells and colorectal cancer, by blocking the activation of Akt in human prostate cancer cells (17, 18). Target molecules for NSAIDs that participate at signaling pathways, include: 15-lipoxygenase-1, peroxisome proliferator-activated receptors (PPARs), P_{21} ras signaling and AKT/PKB kinase (19-21). Cerium oxide nanoparticles induced apoptosis and autophagy in human peripheral blood monocytes (22). Doxorubicin nanoparticles have the potential to serve as a beneficial healing approach to overcome the chemoresistance of adriamycin-resistant breast cancer (23).

2. Objectives

The objective of this study was to evaluate the effect of celecoxib loaded nano-liposomes on the isolated rat hepatocytes and comparing the results with what were obtained from usual form of this chemopreventive agent.

3. Materials and Methods

3.1. Chemicals

Triton X-100, dimethyl sulfoxide (DMSO), ethidium bromide, NaCl, KCl, $MgSO_4$, KH_2PO4, Na_2HPO_4, $NaHCO_3$, HEPES buffer, BSA, collagenase, $Na_2Cr_2O_7$, NaOH and disodium ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), KH_2PO_4, tris base, $CaCl_2$, sodium lauryl sarcosinate ($C_{15}H_{28}NO_3Na$) and, Na_2SO_4, low melting point agar, normal melting point agar were obtained from Sigma (Darmstadt, Germany), celecoxib loaded liposomes were prepared in the School of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, IR Iran. Liposomes were prepared by thin film method. Briefly, soya lecithin and cholesterol were dissolved in chloform-methanol (1:1) and 5 mg celecoxib was added in the solution, then the mixture was evaporated in a rotary evaporator (24). Hepatocytes extraction solution: Hanks × 10 buffers: composed of KH_2PO_4 (0.3 g), NaCl (40 g), Na_2 HPO_4 (0.3 g), KCl (2 g), $MgSO_4$, $7H_2O$ (1 g) and dH_2O q.s. to 500 mL. Hanks I buffer: composed of dH_2O q.s. to 500 mL, HEPES (1.5 g), $NaHCO_3$ (1.05 g), and Hanks × 10 (50 mL). Krebs solution: composed of $MgSO_4$ (0.295 g), NaCl (6.95 g), KCl (0.355 g), (0.287 g), KH_2 PO_4 (0.16 g), $CaCl_2$ $NaHCO_3$ (2.1 g), and dH_2O q.s. to 1000 mL. Perfusion buffer: composed of BSA (1.3 g), EGTA (0.039 g), and Hanks buffer q.s. to 200 mL. Collagenase solution: composed of $CaCl_2$ (0.03 g), collagenase (0.065 g), and Hanks I buffer q.s. to 100 mL. Washing solution: composed of Krebs buffer q.s. to 200 mL, BSA (2 g), and HEPES (0.06 g). Incubation solution: composed of HEPES (0.75 g) and Krebs buffer q.s. to 250 mL. Neutral comet assay solution include: 1) phosphate solution: composed of NaCl (0.8 mg), KCl (0.02 g), $Na_2HPO_4.2H_2O$ (0.216 g), KH_2PO_4(0.02 g), and dH_2O q.s. to 100 mL; 2) lysis buffer: composed of NaCl (14.61 g), Na_2EDTA (3.72 g), Tris base (0.121 g), Sodium lauryl sarcosinate 1% (1 g), NaOH (0.9 g), DMSO 10% (10 mL), Triton X-100 1% (1 mL), and dH_2O q.s. to 100 mL. Electrophoresis solution: composed of tris/borate/EDTA (TBE) solution and dH_2O, at a ratio 1:5 (V/V).

3.2. Animals

Male Wistar rats, weighing 230 - 270 g were obtained from Jundishapur University of Medical Sciences, Ahvaz, Iran, and housed in ventilated plastic cages and at temperature of 20 - 24˚C, 12 hour night - 12 hour day light cycle, with controlled humidity and were fed ad libitum.

3.3. Isolated Rat Hepatocytes

Animals were anesthetized by intraperitoneal injection of ketamine 90 mg/kg and xylazine 10 mg/kg. After cannulation of portal vein, liver was perfused, with perfusion solution for 10 minutes and then, with collagenase solution, for 12 minute. Cellular suspensions were prepared and filtered and finally were centrifuged at 1500 rpm, for 3 minutes. Hepatocyte viability was assessed microscopically, by the trypan blue test. The hepatocytes used were at least 85–95% viable. Isolated hepatocytes (10 mL, 10^6 cells/mL) were placed in a bioreactor bath, under an atmosphere of 95% O_2, 10% N_2, and 5% CO_2, in a 37˚C water bath, for 30 minutes.

3.4. Drug Treatment

Three doses of celecoxib loaded liposomes and celecoxib including, 20, 40, and 100 μM (test groups) were prepared and hepatocytes were exposed to different doses of celecoxib loaded liposomes and celecoxib for 60 minute. Incubation buffer was used, as negative, respectively. To determine whether the use of liposome nanoparticles would affect hepatocytes they were exposed to concentrations of 20, 40, and 100 μM of liposomes without the drug, and the results were compared with negative controls.

3.5. Neutral Comet Assay

A volume of 1 mL of cell suspension was mixed with 10 mL of low melting point agarose and 100 μL of this suspension were transferred to a microscope slide, covered with a layer of normal-melting-point agarose. After putting a coverslip on the slides, they were put in the freezer for 10 minute, to allow the agarose to solidify. Then, the coverslip

was carefully removed. Slides were immersed in cold lysing buffer for 1 hour, in the dark. Then, slides were placed side-by-side in a horizontal gel electrophoresis tank, which was filled with fresh electrophoresis buffer. Electrophoresis was conducted for 25 minutes at 25 V. After all these steps, the slides were neutralized in the neutralization buffer and then stained with 50 μL of ethidium bromide (20 μg/mL) for 5 minutes, before being analyzed with a fluorescence microscope (25). A total of 50 individual cells were analyzed by the comet assay. An intact cell resembles an undamaged nucleus, without a tail, whereas an injured cell has the appearance of a comet. The apoptotic cells were classified by scoring them as 2 or 3. Score of 0 and 1 reflected intact cells.

3.6. Statistical Analysis

All data were analyzed using the statistical with SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). The results were expressed as means ± standard deviation (SD) and analysis performed using the non-parametric X_2 test to compare groups of data.

4. Results

In this study, the neutral comet assay was used for the comparative assessment of the apoptotic induction effect of the usual form of celecoxib with celecoxib loaded liposomes in the rat hepatocytes. Totally, 85.98% of cells were apoptotic after treatment with 100 μg/mL celecoxib loaded liposomes (Table 1). From images of the fluorescence microscope, apoptotic cells showed comet like images, with long tail and small head, while intact cells were without any tail.

Celecoxib loaded liposomes induced an apoptotic response in hepatocytes and produced a comet pattern, similar to the usual form of celecoxib. The percentage of normal and apoptotic cells are shown in Table 1. In this study, hepatocytes were initially exposed to 20, 40, and 100 μM of celecoxib loaded liposomes. As shown in Table 1, and Figure 1 and 2, the highest percentage of apoptotic cells was achieved at 100 μM. Celecoxib loaded liposomes, in this concentration, caused an 85.98% apoptotic response and apoptotic cells showed no statistically significant differences, compared to usual form of celecoxib ($P < 0.0001$). Also, there were significant differences in apoptosis induction at 20 and 40 μM dose, between the usual form of celecoxib and celecoxib loaded liposomes ($P < 0.005$). Apoptotic effect of 20 μM of usual form was evident in 2% of cells. At the lowest concentration of celecoxib loaded liposomes, 20 μM, the apoptotic effect was evident in >10% of cells. For a more complete evaluation, hepatocytes were treated with 40 and 100 μM of celecoxib loaded liposomes. The maximum apoptosis was observed in the presence of 100 μM dose. This concentration caused a significant increase in apoptotic cells and the percentage of apoptotic cells increased to 85.98%, after treatment ($P < 0.005$).

Table 1. Percentage of Intact and Apoptotic Cells After Exposure to Usual Form of Celecoxib and Celecoxib Nanoparticles [a]

Groups	Intact Cell	Apoptotic Cell
Usual form of celecoxib 20 μm	97.98 + 4.14	2 ± 0
Usual form of celecoxib 40 μm	95.98 ± 3.18	3.98 + 1.1
Usual form of celecoxib 100 μm	19.98 ± 1.72	79.98 ± 2.67
Celecoxib loaded liposomes 20 μm	87.32 + 4.57	12.66 + 2.15
Celecoxib loaded liposomes 40 μm	85.98 + 4.6	13.98 + 2.3
Celecoxib loaded liposomes 100 μm	11.98 + 5.56	85.98 + 5.03

[a] The values are presented as mean ± SD.

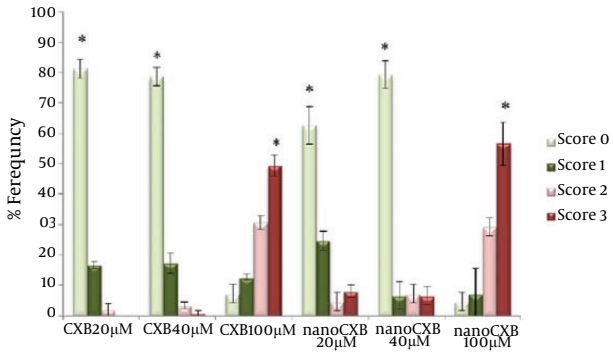

Figure 1. Frequency of Comet Patterns of Hepatocytes After Exposure to Usual Form of Celecoxib and Celecoxib Loaded Liposomes.

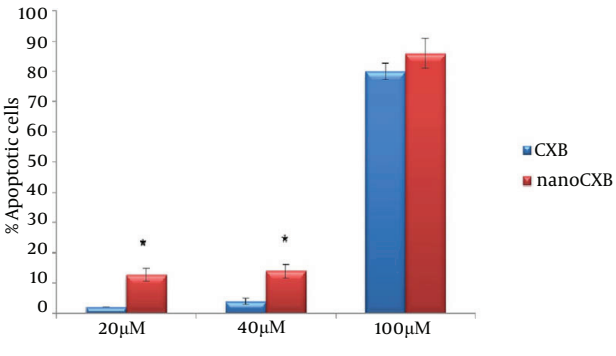

Figure 2. Percentage of Apoptotic Cells After Exposure to Usual Form of Celecoxib and Celecoxib Loaded Liposomes.

5. Discussion

Many chronic diseases were linked to the defects in the apoptosis process (28). Coxibs specifically inhibit COX-2 (29). The results of Steinbach et al. showed celecoxib can be employed to improve colorectal polyps in familial adenomatous polyposis (13). The results of Vaish showed that celecoxib and sulindac, as the chemoprevention, caused induction of apoptosis in colorectal carcinomas, via the calcium ion homeostasis (16). Kim et al. (30) showed that caspase-8 and -9 executed the apoptotic effect of celecoxib, in cervical cell line. This necessitates the fas-associated death domain-dependent pathway, in a cell type-specific manner. Also, nuclear factor kB (NFkB) may play a key role in celecoxib-induced apoptosis. Piplani et al. (31) studies have shown that, dolastatin 15, a mollusk linear peptide, and celecoxib induced apoptosis via inducible nitric oxide synthase, therefore, revealing the potential of this inflammatory protein, as an antineoplastic target in colon cancer. Hsu et al. (18) data demonstrate that inhibition of Akt activation, in human prostate cancer cells, may play a crucial role in the induction of apoptosis by celecoxib. Investigations showed that several other molecular targets of the NSAIDs, except for COX-2, may exist, such as the signaling pathway that contains, 15-lipoxygenase-1, P21 Ras, PPAR delta, and AKT/PKB Kinase (19, 20, 32). The results of this study are comparable with other researches on cell death effects of celecoxib. The results of this study indicated a dose-dependent increase in apoptosis in rat hepatocytes. Our results showed that the usual form of celecoxib (100 μM) treatment caused 79.98% cell death in rat hepatocytes. By high concentrations (40 and 100 μM), apoptosis was demonstrated and it was clearly more prominent in hepatocytes exposed to celecoxib loaded liposomes. Zhou et al. (34) showed that high doses of celecoxib have dose dependently induced apoptosis in the human glioma cell line U251. Li et al. (35) Our results showed that celecoxib loaded liposomes robustly induced apoptosis, which can potentially make it more relevant than the usual form for chemopreventive strategies.

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Authors' Contributions

Study concept and design: Mohsen Rezaei, Haibatolla Kalantari. Analysis and interpretation of data: Mojtaba Kalantar, Golnaz Varnaseri. Drafting of the manuscript: Mojtaba Kalanatar, Arash Frouzan. Liposome preparation and Characterization: Eskandar Moghimipour, Neda Bavarsad. Critical revision of the manuscript for important intellectual content: Mohsen Rezaei .

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