ORIGINAL ARTICLE

Diabetic Hyperglycaemia Protects Smooth Muscle Arterial Cells but Not Macrophages and Endothelial Cells from the Cytotoxic Effects of 7-Ketocholesterol: An *In Vitro* Study

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ABSTRACT

Plasmatic hyperglycaemia is responsible for positive and negative stimuli. Among responsive cells, smooth muscle cells respond actively, increasing their growth. Diabetes hyperglycaemia and lipidic disorders are frequently associated, resulting in the highest probability of cardiovascular disease, especially atherosclerosis. Endothelial dysfunction and initiation of inflammatory process mediated by macrophages cause the formation of atherosclerotic plaques, rich in oxidized cholesterol, mostly 7-ketocholesterol. In this study, we evaluated the effect of 7-ketocholesterol in rabbit aorta endothelial cells, murine macrophages (J774) and smooth muscle cells (A7R5) maintained in normoglycemic and hyperglycaemic cultures. The 7-ketocholesterol-induced-cell death was observed in the normoglycemic medium three times more than the hyperglycaemic medium in all cells. High glucose medium had a protective effect on arterial smooth muscle cell death.

Keywords: Atherosclerosis, hyperglycaemia, 7-ketocholesterol

INTRODUCTION

Coronary heart disease and diabetes are increasingly present in the World society. The association of these diseases is often observed by the medical community. Diabetes consisting of a metabolic disorder characterized by hyperglycaemia, which, besides other effects, alters the composition of the lipoprotein particles, making them more atherogenic and accelerating the formation of thrombi. The junction of these clinical conditions may induce several modifications in the immune system, inflammation and lipid metabolism (1).

The vascular endothelium is a relatively simple structure, composed of a thin layer of endothelial cells that cover the entire length of the circulatory system. It is semipermeable and responsible for controlling the flow of nutrients and other active molecules to the underlying cells, and have membrane surface receptors for hormones, proteins and lipids (2).

Arterial cardiovascular disease starts with endothelial dysfunction. Hypercholesterolemia induces thickness of intimal and medial layers of the artery; there is increased platelet activation with the release of prothrombotic substances (3). Diabetics have a lower release of vascular nitric oxide and high levels of dimethylarginine (nitric oxide inhibitor), contributing to endothelial dysfunction (1).

The subendothelial retention and apolipoproteins modification cause an inflammatory reaction mediated by macrophages. These cells become activated and begin to secrete proinflammatory cytokines and other substances which contribute to the formation of atherosclerotic lesions (4, 5).

The non-esterified cholesterol is an inducer of apoptosis in macrophages, which accumulate large amounts of lipids in its interior core, signalling to apoptosis. This event may promote destabilization of atherosclerotic plaque and vascular occlusion (6, 7).

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Oxygenated cholesterol degradation occurs naturally producing oxysterols, which play an important role in lipid homeostasis. Among the oxysterols stands the 7-ketocholesterol, abundantly found in foam cells and atherosclerotic plaques and high cytotoxicity (8, 9). The aim of this study was to evaluate the response of three cell types abundantly present in the atherosclerotic plaque, exposed to a high glucose medium and different concentrations of 7-ketocholesterol.

MATERIALS AND METHODS

The cells J774 line (murine macrophage), smooth muscle aorta cells (A7R5) and rabbit aorta endothelial cells (RAEC) were grown in sterile plastic bottles containing DMEM culture medium (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml amikacin. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 (10). The cells were purchased from American Type Culture Collection (ATCC).

Proliferation and cell viability by MTT method

The proliferation and cell viability of the strains studied were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The methodology consists in adding 0.5 mg/ml of MTT, a yellow tetrazolium salt that after incubation for 4 hours at 37°C is metabolized forming formazan crystals with dark blue colour. This reaction occurs only in metabolically active cells *via* mitochondrial succinate dehydrogenase enzyme (11, 12).

For tests with the J774 cell line were plated 4×10^3 cells per well, whereas for strain endothelial cells (RAEC) or A7R5, were plated 4.0×10^3 cells per well, in a 96 well plate in triplicate with different concentrations of 7-ketocholesterol (Sigma) and under different experimental conditions: normoglycemic culture medium (control group); and culture medium supplemented with glucose, prepared from normal medium with added 19.5 mM dextrose and 19.5 mM of mannitol, resulting in a concentration of 45.5 mM dextrose (glucose) (13).

Concentrations of 7-ketocholesterol used were 0.1875, 0.25, 0.5, 1, 2 and 4 µM (14). After the addition of oxidized cholesterol, plates were kept under culture conditions.

The quantification of viable cells was counted 24, 48 and 72 hours after the start of incubation. The resulting crystals formazan MTT mitochondrial metabolism were solubilized in 100 ml of isopropyl alcohol and reading of absorbances were done in a spectrophotometer at a wavelength of 565 nm with a reference filter at 655 nm.

Cell viability by Trypan-Blue method

The staining technique by Trypan Blue was performed to confirm cell viability by the MTT results. The colourant is able to distinguish viable cells (refractile) from non-viable cells (stained in blue).

Cells were plated in 24-well plates under the same conditions MTT assay. The J774 strain was plated in a proportion of 3×10^5 cells per well; RAEC and A7R5 were plated in a proportion of 2.5×10^5 cells per well.

Staining was performed after centrifugation at 2000 rpm for 4 minutes for sample concentration, then resuspended in 2 ml medium. Cells were counted in a Neubauer chamber.

RESULTS

The growth and cell viability were analysed by MTT assay, at different concentrations of 7-ketocholesterol and at different periods of incubation time, the hyperglycaemic and normoglycemic means. Hyperglycaemia did not result in increased cell viability, which was similar to normoglycaemic means. The addition of 7-ketocholesterol was shown to be harmful to cells, both in normal medium and in medium supplemented with glucose. The results for the J774 are shown in Fig. 1, and the results of RAEC are shown in Fig. 2.

Hyperglycaemic medium protects A7R5 cells from the deleterious effects of 7-ketocholesterol. These results are shown in Fig. 3.

The Trypan Blue exclusion method was also performed in order to confirm the results obtained by the MTT method. The growth and cell viability were evaluated under the same conditions of culture and incubation previously used. The results had the same arrangement (data not shown).

DISCUSSION

Chronic diseases are the leading cause of mortality worldwide. An increase in the incidence of diabetes associated with obesity and metabolic syndrome, factors that increase the risk of cardiovascular disease, especially atherosclerosis (15, 16).

The concentration of cytoplasmic and plasma antioxidants is often lower in diabetic patients (17). The association of this hypercholesterolemia framework results in increased oxysterols formation from non-esterified cholesterol, among them 7-ketocholesterol,

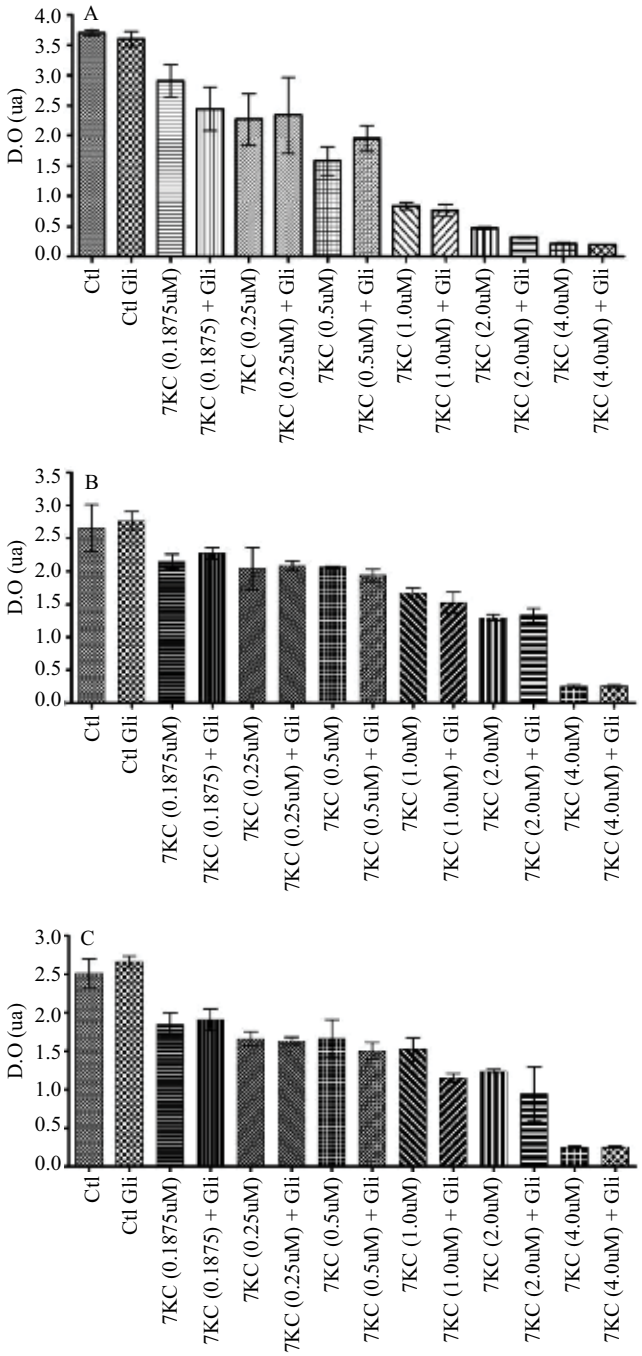

Fig. 1: Dose–response evaluation of 7-ketocholesterol in culture normal medium and medium supplemented with glucose. J774 macrophage cells. (A) 24 hours of treatment; (B) 48 hours; (C) 72 hours. Data are expressed as mean and standard error of the mean. Statistical analysis was performed using ANOVA followed by Bonferroni´s Multiple Comparison Test.

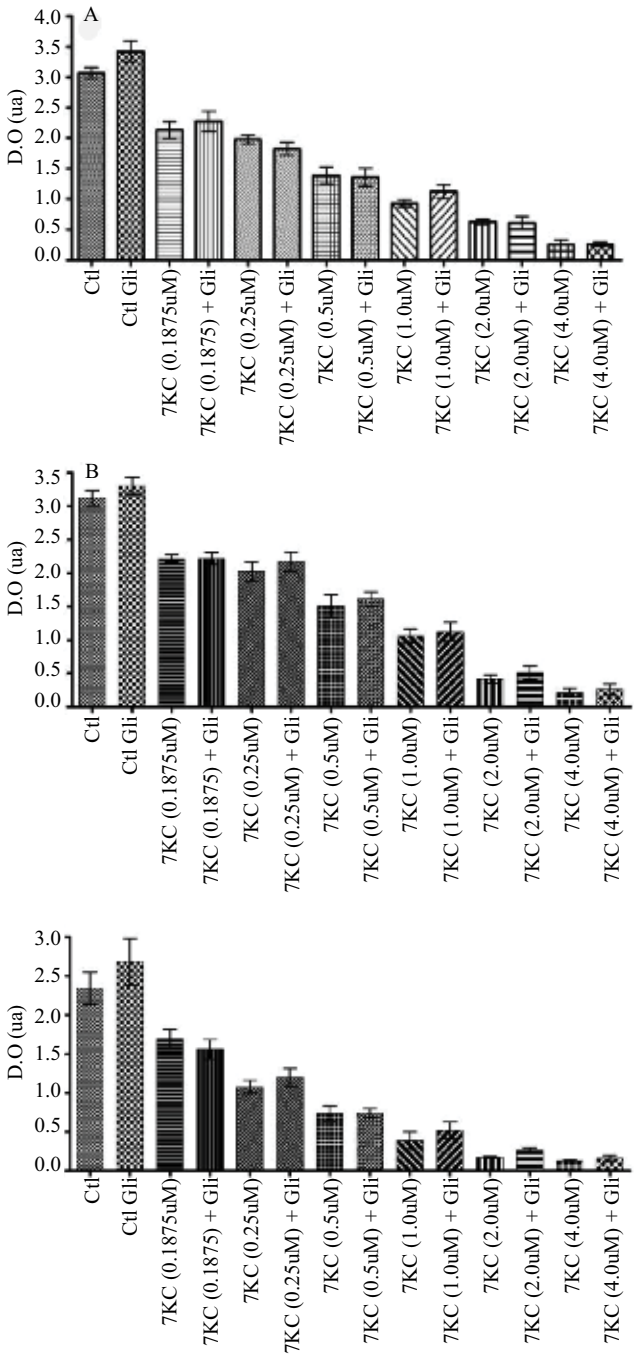

Fig. 2: Dose–response evaluation of 7-ketocholesterol in culture normal medium and medium supplemented with glucose. RAEC endothelial cells. (A) 24 hours of treatment; (B) 48 hours; (C) 72 hours. Data are expressed as mean and standard error of the mean. Statistical analysis was performed using ANOVA followed by Bonferroni´s Multiple Comparison Test.

the most oxidized cholesterol was found in atherosclerotic plaques (18).

The cytotoxic effects of 7-ketocholesterol were shown in this study. The analysed cell lines did not resist the deleterious action of oxysterol, not even in the shortest period of time (24 hours).

There were no significant differences regarding the composition. The use of the hyperglycaemic medium was not able to provide protection for macrophages or endothelial cells, *ie*, cell death was seen in both normal media added and the means of glucose when compared to control groups.

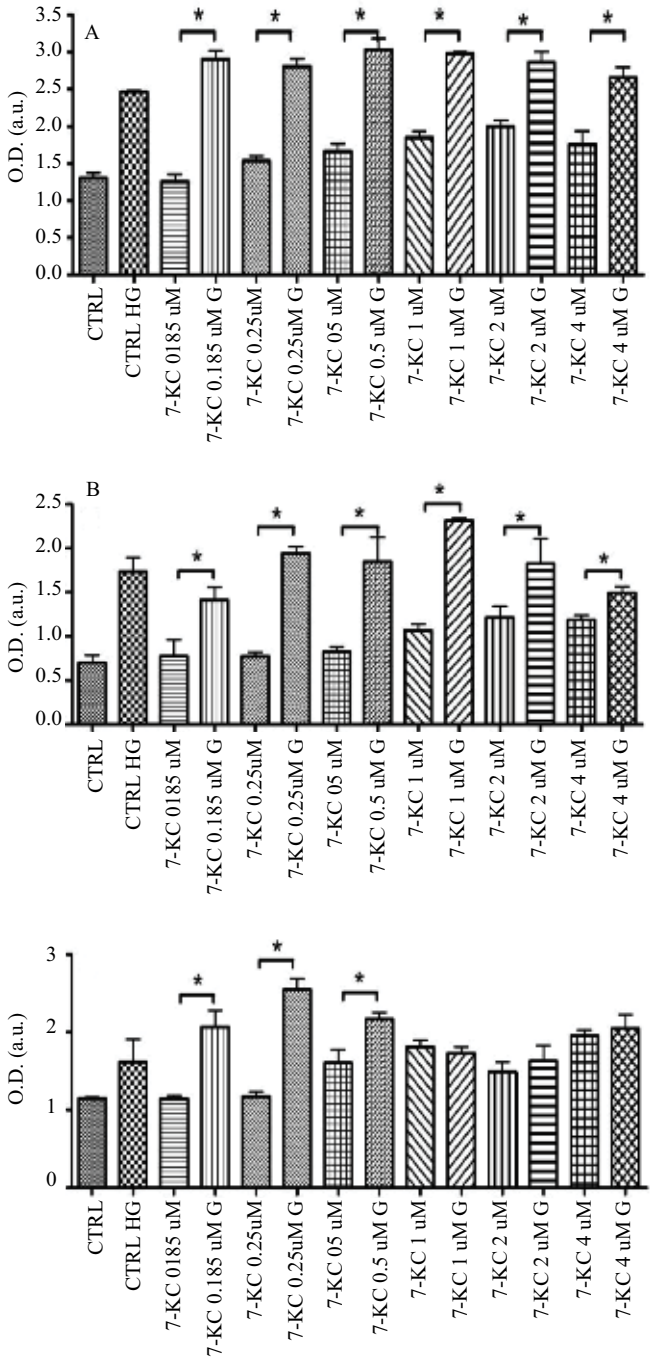

Fig. 3: Dose–response evaluation of 7-ketocholesterol in culture normal medium and medium supplemented with glucose. A7R5 smooth muscle cells. (A) 24 hours of treatment; (B) 48 hours; (C) 72 hours. Data are expressed as mean and standard error of the mean. Statistical analysis was performed using ANOVA followed by Bonferroni´s Multiple Comparison Test. * $p < 0.05$ compared with the control group.

In atherosclerotic plaque the deleterious effects of oxysterols are higher on the foamy macrophages, leading to its death and increasing the inflammatory process, so presenting a more intense tissue disorder. The smooth muscle plays an important role in the process of obstructive disease, and one responsible for the formation of a stable and potentially more occlusive plate. What we see here, at this moment, is that the hyperglycaemic means protects the arterial smooth muscle cells from cell death exposed to 7-ketocholesterol, this phenomenon should lead to a more stable and potentially fatal card. Further studies should strengthen our work.

CONCLUSION

7-Ketocholesterol is the principal product of a reaction between cholesterol and oxygen and the most abundant oxysterol in the atherosclerotic plaque. In this study, we observed their cytotoxic action on macrophages, aorta endothelial cells and aorta smooth muscle cells in high glycaemic medium. Furthermore, it is concluded that hyperglycaemia media was not able to protect macrophages and endothelial cells but can protect smooth muscle cells from harmful effects of oxysterol. The fact that glucose has a protective effect on arterial smooth muscle cell death may cause a more stable and occlusive disease, potentially fatal.

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