

Cholesterol induces fetal rat enterocyte death in culture

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Abstract

The effect of cholesterol on fetal rat enterocytes and IEC-6 cells (line originated from normal rat small intestine) was examined. Both cells were cultured in the presence of 20 to 80 μM cholesterol for up to 72 h. Apoptosis was determined by flow cytometric analysis and fluorescence microscopy. The expression of HMG-CoA reductase and peroxisome proliferator-activated receptor gamma (PPAR γ) was measured by RT-PCR. The addition of 20 μM cholesterol reduced enterocyte proliferation by 28 and 41% was observed after 24 h of culture in the presence and absence of 10% fetal calf serum, respectively, with the effect lasting up to 72 h. Treatment of IEC-6 cells with cholesterol for 24 h raised the proportion of cells with fragmented DNA by 9.7% at 40 μM and by 20.8% at 80 μM . When the culture period was extended to 48 h, the effect of cholesterol was still more pronounced, with the percent of cells with fragmented DNA reaching 53.5% for 40 μM and 84.3% for 80 μM . Chromatin condensation of IEC-6 cells was observed after treatment with cholesterol even at 20 μM . Cholesterol did not affect HMG-CoA reductase expression. A dose-dependent increase in PPAR γ expression in fetal rat enterocytes was observed. The expression of PPAR- γ was raised by 7- and 40-fold, in the presence and absence of fetal calf serum, respectively, with cholesterol at 80 mM. The apoptotic effect of cholesterol on enterocytes was possibly due to an increase in PPAR γ expression.

Key words

- Fetal enterocytes
- Cholesterol
- Intestine
- IEC-6 cells
- HMG-CoA
- PPAR γ

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Introduction

High cholesterol levels are a risk factor for arteriosclerosis and hypertension (1,2). In addition to diet, there are two endogenous sources of cholesterol: biliary cholesterol, which is always unesterified, and intestinal epithelial

sloughing. Cholesterol absorption requires emulsification, hydrolysis of the ester bond (when esterified) by a specific pancreatic esterase, micellar solubilization, absorption in the proximal jejunum, re-esterification within

the intestinal cells, and transport to the lymph in the chylomicrons (3). The intestine, however, has a limited capacity to absorb cholesterol. The absorption of dietary cholesterol ranges from 40 to 60% regardless of the amount ingested up to 600 mg per day (4). The absolute amount of cholesterol absorbed increases in parallel to intake within the normal range (5). Therefore, a significant proportion of dietary cholesterol remains in the intestinal lumen to be excreted (6).

Evidence has been reported that cholesterol can regulate cell function but can also cause cell death (7-11). Cholesterol is an important constituent of the membranes and plays a key role in the proliferation of rapidly dividing cells such as enterocytes (12). Also, cholesterol regulates cell differentiation (13), glutamine metabolism (14) and fatty acid composition (15). However, cholesterol can be toxic, being able to induce cell death (16). There is evidence that free cholesterol causes apoptosis of macrophages (7), which is recognized as a prominent feature of atherosclerotic lesions (8). Hypercholesterolemia leads to vascular smooth muscle cell death (9) and this process has been associated with plaque rupture in early and advanced atherosclerotic lesions (10).

On the basis of these considerations, we investigated the effect of cholesterol on enterocyte proliferation and death. The following parameters were examined in fetal enterocytes and IEC-6 cells cultured in the presence of cholesterol: cell growth, DNA fragmentation and chromatin condensation. The reason to work with IEC-6 cells is that measurements using flow cytometry analysis could not be performed with fetal enterocyte preparations. In an attempt to examine the possible mechanism involved, the expression of HMG-CoA reductase and peroxisome proliferator-activated receptor gamma (PPAR γ) was also determined under similar conditions. Inhibition of cholesterol synthesis (11) and activation of PPAR γ have been shown to induce apoptosis (17).

Material and Methods

Reagents

HF12 medium, DMEM, streptomycin, and penicillin were obtained from Invitrogen (New York, NY, USA). DMSO, collagenase type IA, hyaluronidase, and cholesterol were purchased from Sigma (St. Louis, MO, USA). Fetal calf serum (FCS) was obtained from Cultilab (Campinas, SP, Brazil) and trypsin was purchased from Adolfo Lutz Institute (São Paulo, SP, Brazil).

Animals

Male and female Wistar rats (3-4 months of age) obtained from the Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, were maintained (3 females and 1 male) under a 12-h light/dark cycle for 7 days. Pregnant female rats were housed in separate cages up to the 20th day of pregnancy. Female rats were then killed by cervical dislocation and fetuses were collected after abdominal incision. The experimental procedure used in this study was approved by the Ethics Committee of the Institute of Biomedical Sciences, University of São Paulo.

Isolation and culture of enterocytes from rat fetuses

This protocol is a modification of the method described by Fukamachi (18) and Evans et al. (19). Fetuses were washed with 70% alcohol and the small intestine was then dissected and washed in Hank's balanced salt solution (HBSS) at 37°C. Mesenteric tissue was removed and the intestine was opened laterally under a light microscope. The intestinal mucosa was treated with collagenase type IA (458 U/ml) and hyaluronidase (320 U/ml) in HBSS, at 37°C, for 60 min, in a shaker bath and then centrifuged at 1200 g for 5 min at 4°C. The pellet was

resuspended in sterile HF12 medium by repeated pipetting with a Pasteur pipette. The cells were decanted by gravity in 50-ml polycarbonate tubes and centrifuged for periods of up to 10 min. Cells were then cultured in HF12 medium at 37°C, in an atmosphere of 5% CO₂ and 95% air.

IEC-6 cell culture

The IEC-6 cell line (ATCC No. CRL 1592) was obtained from the American Type Culture Collection (Rockville, MD, USA). This line originated from the normal small intestine of *Rattus norvegicus* (20). The cells were stored in DMEM with 44 mM sodium bicarbonate, 4 mM L-glutamine, 25 mM glucose, 1 mM sodium pyruvate, 0.1 U/ml insulin, 10% (v/v) FCS, 10 U/ml penicillin, and 10 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ and 95% air, at 37°C. For the experiments, the cells were taken up using a 0.2 trypsin solution plus 0.02% versene, diluted in DMEM containing 10% FCS and cultured at 37°C in an atmosphere of 5% CO₂ and 95% air.

Cell growth conditions

The growth curves for enterocytes and IEC-6 cells were constructed by counting the cells at different times, i.e., 3, 6, 12, 24, 48, and 72 h. Cells were diluted in medium only or in medium containing 10% FCS to achieve a final concentration of 1.0 x 10⁵ cells/ml. Cells were plated onto 24-well dishes at the times listed above and maintained at 37°C in an atmosphere at 5% CO₂ and 95% air. Cells were removed from the plates by gentle scraping and cell viability was determined in a Neubauer chamber using 0.1% Trypan blue dye in saline.

Cholesterol treatment

The sterol was solubilized in ethanol. For the control, the same volume of ethanol was

added and the final concentration was always less than 0.5%. Preliminary experiments using fluorescent labeled cholesterol showed that in this condition the sterol is rapidly incorporated by enterocytes and IEC-6 cells. The effect of cholesterol on cell growth was tested at 20 µM. The concentrations of 20, 40, and 80 µM cholesterol were used for the analysis of DNA fragmentation, chromatin condensation and expression of HMG-CoA reductase and PPAR γ . The concentrations of cholesterol used were determined in preliminary experiments.

Internucleosomal DNA fragmentation - flow cytometric analysis

Cells (1 x 10⁶) were incubated for 24 h in the dark in a solution containing 0.1% citrate, 0.1% Triton X-100 and 50 µg/ml propidium iodide (21). Cell propidium iodide fluorescence was then evaluated by flow cytometry with a Becton Dickinson (San Jose, CA, USA) cytometer using the CellQuest software. Ten thousand events were evaluated per experiment. Propidium iodide fluorescence is reported in histograms.

Chromatin condensation assay

After treatment with 20, 40 or 80 µM cholesterol for 48 h, cells were treated by the method of Pompeia et al. (22). Cells (1 x 10⁶) were suspended in 20 µl 0.9% NaCl containing 10 µg/ml Hoechst 33342 (Molecular Probe, Eugene, OR, USA). After 10 min, the cells were observed under a fluorescence microscopy (Axiovert 100 M microscope, Zeiss, Zepplinstrasse, Germany) under UV light (365/380 nm) and images were analyzed with the Axio Vision software (Zeiss). Fluorescence intensity was calculated using the KS300 3.0 Image System software (Zeiss).

Extraction of total RNA

Total RNA was obtained from 0.5 to 1 x

10^7 cells by the guanidinium isothiocyanate extraction method. Briefly, the cells were lysed using 1 ml Trizol reagent (Life Technologies, Rockville, MD, USA). After 5 min incubation at room temperature, 200 μ l chloroform was added to the tubes and centrifuged at 12,000 *g*. The aqueous phase was transferred to another tube and the RNA was pelleted by centrifugation at 12,000 *g* with cold ethanol and dried in air. RNA pellets were redissolved in RNase-free water and treated with DNase I. RNAs were then stored at -70°C until the time of the experiment. The RNA was quantified by measuring absorbance at 260 nm. The purity of the RNAs was assessed by the 260/280 nm ratio and by electrophoresis on 1% agarose gel stained with ethidium bromide at 5 $\mu\text{g}/\text{ml}$ (23). These samples were used for RT-PCR analysis.

RT-PCR

Two micrograms total RNA was treated with 1 U DNase for 25 min at 25°C and then the enzyme was inactivated with 2.5 mM EDTA. Next, cDNA was synthesized using oligo (dT)₁₂₋₁₈ containing 1 mM of each dNTP, 10 mM DTT and 200 U SuperScriptTM II RNase H⁻ reverse transcriptase at 42°C for 50 min according to manufacturer instructions. The reaction was stopped by heating at 70°C for 15 min. RT-PCR was performed using parameters described by Innis and Gelfand (24). The reaction was carried out in a total volume of 25 μ l containing 2.5 μ l

buffer, DNA polymerase enzyme (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 9.0), 10 pmol primer, 200 μ M dNTP, 2.5 U Taq DNA polymerase, and 2 μ l cDNA. RT and PCR were carried out in a Techne Touchgene Gradient thermocycler (Princeton, NJ, USA). The sequences of the primers were designed using information from the GeneBank of the NCBI (Table 1). The number of cycles used was selected to obtain linear amplification of the cDNA under study. The amplification cycle, annealing temperature, and MgCl₂ concentration were determined for each gene (Table 1). For semi-quantitative PCR analysis, the housekeeping β -actin gene was used as reference. Published guidelines were followed to guard against bacterial and nucleic acid contamination (25).

Analysis of the PCR products

For the analysis of PCR amplification products, aliquots of the reactions containing sample buffer (0.25% bromophenol blue, 0.25% xylene cyanol-FF and 30% glycerol) were applied to 1.5% agarose gel containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide and electrophoresed for 1 h at 100 V. The fragment lengths of the PCR products were 350 pb for HMG-CoA reductase, 474 pb for PPAR γ and 549 pb for β -actin genes. The gels were photographed using a DC120 Zoom Digital Camera System from Kodak (Life Technologies). The images were processed and analyzed us-

Table 1. Conditions for RT-PCR analysis. The sequences of the primers, the PCR fragment lengths, the temperature, and the number of cycles are shown for each gene under study.

| Gene | Primer sense | Primer antisense | MgCl ₂ (mM) | Annealing temperature ($^\circ\text{C}$) | PCR cycles | PCR fragment lengths (bp) |
|-------------------|-------------------------------|------------------------------|------------------------|--|------------|---------------------------|
| HMG-CoA reductase | 5'TCGGGCAGAGCTACGTTTGTG3' | 3'CAGCAAGCTCCCATCACCAAGG5' | 1.5 | 60 | 45 | 350 |
| PPAR γ | 5'GACGAATGCCAAGATCTGAGAAAGC3' | 3'CGTCTCCTTTGTAGTGCTGTACGC5' | 1.5 | 55 | 50 | 474 |
| β -actin | 5'GTGGGGCGCCCCAGGCACCA3' | 3'CTCCTTAATGTACGCACGATTTC5' | 1.5 | 55 | 25 | 549 |

ing the Kodak Digital Science 1D Image Analysis software (Life Technologies), confirming the amplification fragments (Table 1).

The results of gene expression are presented as the ratio of the relative absorbance values (relative expression) of the gene of interest and of β -actin (gene of β -actin; Table 1).

Statistical analysis

Data are reported as means \pm SEM. The effect of cholesterol was analyzed statistically by ANOVA plus the Tukey-Kramer test for comparison with control conditions (ethanol addition).

Results

Fetal enterocytes presented a higher number of cells when cultivated in the absence of serum (Figure 1A,B). After 12 h in culture, the total number of cells was about two times higher in the absence than in the presence of bovine serum. This observation may be related to the fact that these cells are on the luminal side of the intestine. The addition of 20 μ M cholesterol to the culture medium caused a significant reduction in the total number of fetal enterocytes (Figure 1). This effect was clearly more pronounced in the absence of serum (Figure 1A). Cholesterol addition led to a reduction in the total number of cells as early as after 3 h in culture but the effect became more evident after 12 h.

Addition of cholesterol to the culture medium also led to fragmentation of enterocyte DNA (Figure 2). Twenty-four hours after cholesterol addition (Figure 2A), a significant proportion of cells with fragmented DNA was observed for 40 μ M (9.7%) and 80 μ M (20.8%) concentrations. When the culture period was extended to 48 h, the effect of cholesterol was still more pronounced, with the percentage of cells with fragmented DNA reaching 53.5% for 40 μ M and 84.3% for 80 μ M. It is interesting to note that cho-

lesterol at 20 μ M concentration had no marked effect on DNA fragmentation.

The addition of cholesterol also led to an increase in chromatin condensation as observed by Hoechst staining (Figure 3). The intensity gradually increased with the amount of cholesterol added to the medium. The effect of cholesterol on chromatin condensation was already visible at 20 μ M concentration. These findings suggest that, under these

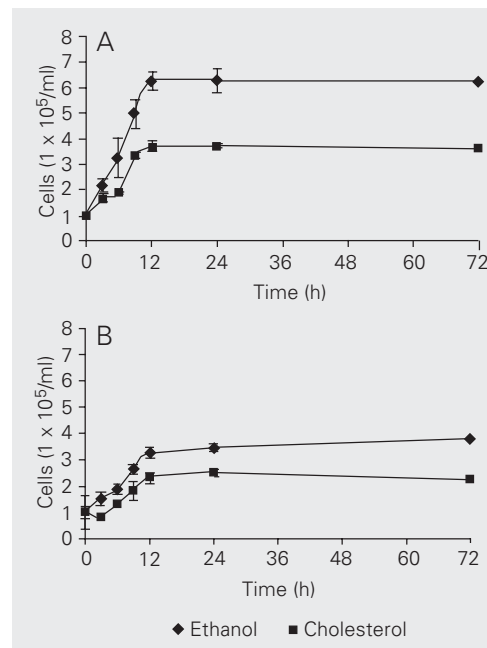


Figure 1. Growth curve of fetal enterocytes cultured in the presence of 20 μ M cholesterol or 0.5% ethanol (control) in medium without (A) or with (B) 10% fetal calf serum. Data are reported as means \pm SEM for nine determinations for three different cell preparations. The cholesterol was dissolved in 0.5% (v/v) ethanol.

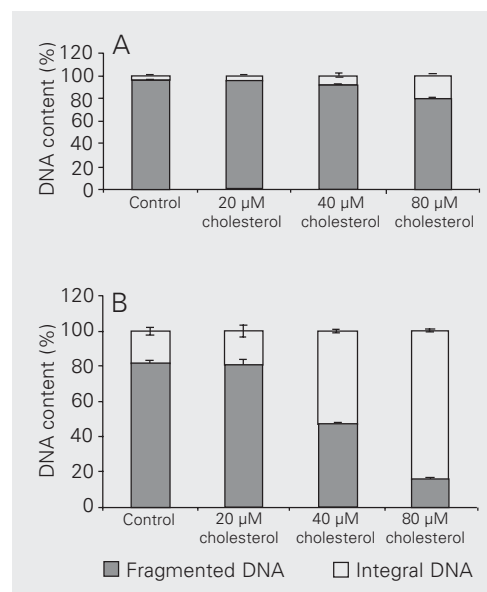


Figure 2. A, DNA fragmentation analysis in IEC-6 cells after 24 (A) or 48 h (B) in culture in the presence of cholesterol concentrations of 20, 40 and 80 μ M or solvent (control). The percentage of cells with DNA fragmentation, determined by flow cytometry, is indicated. Data are reported as means \pm SEM of 5 experiments carried out in duplicate.

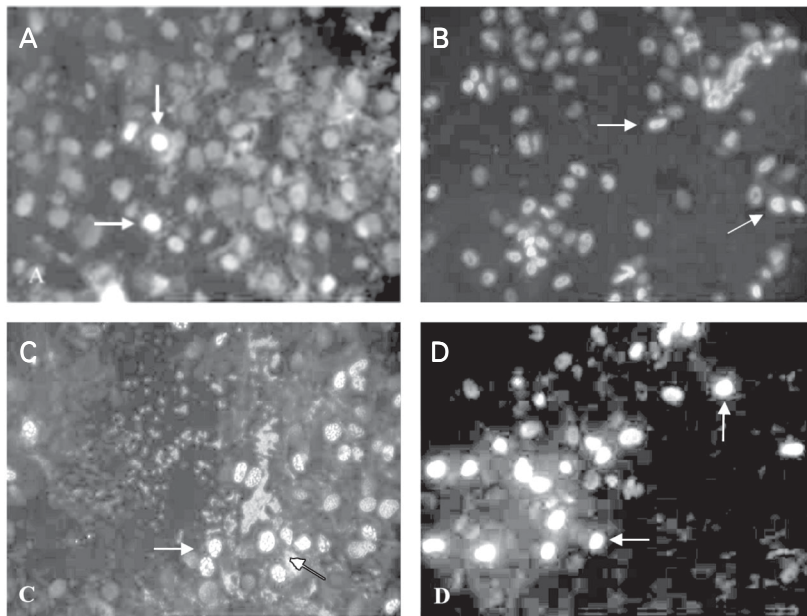
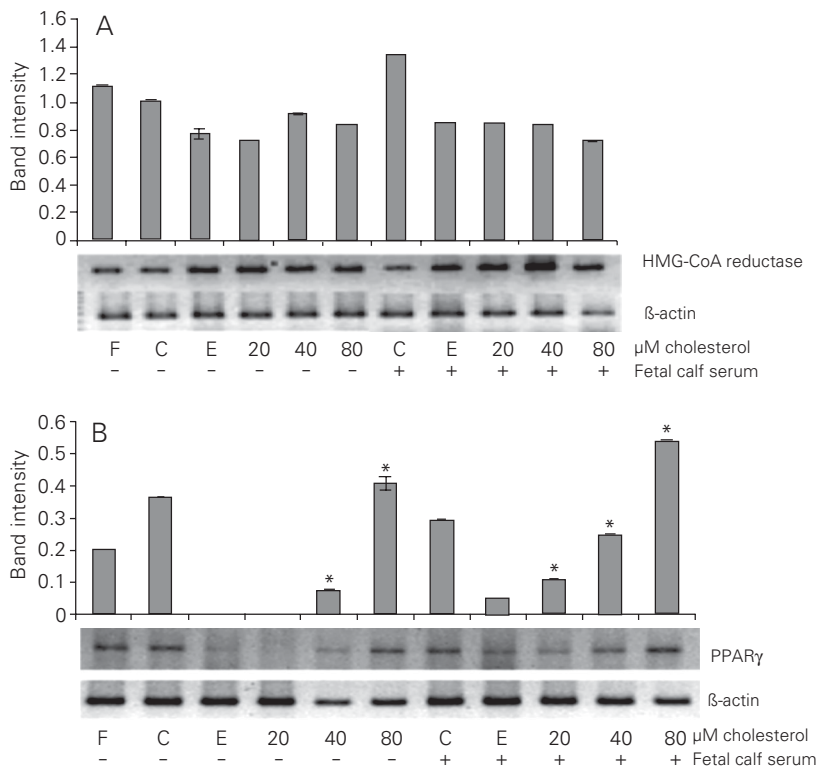


Figure 3. Analysis of chromatin condensation in IEC-6 cells by Hoechst stain in the presence of different concentrations of cholesterol or 0.5% ethanol. Fluorescence intensity under UV light of 365/380 nm was calculated using the KS300 3.0 Image System software (Zeiss): A) control - 66.29 ± 1.83 ; B) 20 μM - 116.04 ± 2.67 ; C) 40 μM - 172.75 ± 0.97 ; D) 80 μM - 220.75 ± 3.56 . Arrows indicate cells presenting DNA fragmentation.



conditions, chromatin condensation may occur earlier than DNA fragmentation.

The addition of cholesterol (at 20, 40 and 80 μM concentrations) did not affect the expression of HMG-CoA reductase in fetal enterocytes cultivated in the absence or in the presence of serum (Figure 4A). In contrast, the expression of PPAR γ was gradually increased by cholesterol addition under similar conditions (Figure 4B). This effect was clearly more pronounced in the absence of serum.

Discussion

It has been recognized that cholesterol plays an important role in modulating fluidity and phase transitions in the plasma membranes (26,27). In addition, cholesterol, together with sphingomyelin, forms plasma membrane microdomains, rafts and caveolae that are sites where signaling molecules are concentrated (28). Therefore, it is not surprising to find out that this sterol can affect cell growth. In the present study, we obtained evidence that cholesterol reduces enterocyte proliferation. This effect was clearly more pronounced in the absence of serum, which mimics the luminal side of the intestinal epithelium. Therefore, free cholesterol in the intestinal lumen may act to provide a constant control of the cellularity of the epithelium. In fact, intestinal epithelium function is the result of a balance be-

Figure 4. Effect of cholesterol on the expression of HMG-CoA reductase (A) and PPAR γ (B) in fetal enterocytes cultured for 4 h. The cells were cultured in the absence (-) or in the presence (+) of serum. Cholesterol was added to the medium at concentrations of 20, 40 or 80 μM . The intensities of the bands were compared to those of β -actin and presented as bars. F = enterocytes before treatment, immediately after isolation; C = enterocytes cultivated in medium only; E = enterocytes treated with the solvent (ethanol 0.5%) as control. Data are reported as the mean \pm SEM of 4 experiments. *P < 0.05 compared with the control group (ANOVA and Tukey-Kramer test).

tween apoptosis and proliferation (29).

As previously shown for macrophages (12) and vascular smooth muscle cells (14), we show here that cholesterol is toxic for enterocytes. The sterol also caused a marked increase in DNA fragmentation of IEC-6 cells as early as after 24 h in culture. Extension of the period of exposure induced a more pronounced effect, possibly explaining the reduction in the total number of cells. The high content of cholesterol in the intestinal lumen is present for a few hours before being metabolized by intestinal bacteria and excreted (4).

Our demonstration of DNA fragmentation and chromatin condensation support the proposition that the toxic effect of cholesterol on enterocytes is likely to occur by apoptosis. Apoptosis is especially relevant in the gastrointestinal tract because the intestinal mucosa undergoes continuous epithelial regeneration (30). Cell detachment induces apoptosis in normal human intestinal epithelial cells through a sequential cleavage of focal adhesion kinase by caspases (31). Evidence is presented here that free cholesterol in the intestinal lumen plays a role by maintaining intestinal epithelial function.

Previous studies have shown that oxidized products of cholesterol cause apoptosis of several cell types such as corneal endothelial and lens epithelial cells (32),

microglial cells (33), human U937 cells (34), human neuroblastoma cells (35), and aortic smooth muscle cells (36,37). It remains to be determined if the results presented here were due to oxidized products of cholesterol.

The pathway of *de novo* cholesterol synthesis generates active intermediates that control cell division (38). The toxic effect of cholesterol on enterocytes is unlikely to be mediated by inhibition of this pathway since no marked change in HMG-CoA reductase expression was observed.

Previous studies have also shown that PPAR γ expression is regulated by lipids (39). The PPAR γ expression in enterocytes induced by cholesterol was dose dependent. PPAR γ has been implicated in the death of several cell types (11,17,40). This may be an important mechanism for the toxic effect of cholesterol reported here. Fatty acids that are PPAR γ ligands (such as linoleic acid) may increase the effect of the sterol when present at high proportions in the diet.

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