

Original

Validation of an *in vitro* nutrition model using an enteral formula in aged neutrophils

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Abstract

The aim of the study is to validate a cell culture model appropriate for assessing the effects of standard nutritional formulas on neutrophil functionality *in vitro*. The model consists of aged cells exposed to a commercial nutritional formula containing solely LCT as lipid component. Preliminary experiments determined dosage of formula and culture interval. Neutrophils were isolated from a pool of whole blood in healthy volunteers (18-55 years old) and cultured with and without addition of a commercial enteral diet with 3.5% lipids (equivalent to 0.04, 0.08, 0.2 and 0.4 mM of intraassay LCT) for 18, 42 or 76 hours. Based on cell viability results, doses of 0.2 and 0.4 mM LCT and culture time of 18 hours were established for subsequent experiments. Neutrophil functionality was evaluated by phagocytosis (NBT test), MDA production (lipoperoxidation index) and DNA fragmentation. Optic microscopy showed higher percentages of pre-apoptotic cells and a significant increase in DNA fragmentation as compared to controls only with an LCT concentration of 0.4 mM ($p < 0.05$). Interestingly, cell cultures with both 0.2 and 0.4 mM of added LCT showed significant decreases in malonyldialdehyde (MDA) release as a lipoperoxidation marker. This nutrition model of neutrophils and *in vitro* complete enteral commercial diet is relatively simply to execute and can be applied to different pathological conditions in which the aim is to study changes in neutrophil functionality.

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VALIDACIÓN DEL MODELO *IN VITRO* DE LA NUTRICIÓN USANDO UNA FÓRMULA DE ENTERAL EN NEUTRÓFILOS ENVEJECIDOS

Resumen

El objetivo del estudio ha sido validar un modelo en cultivo celular, apropiado para valorar los efectos *in vitro* de las fórmulas estándar y completas de nutrición enteral en la funcionalidad de los neutrófilos. El modelo consiste en células envejecidas a las que se añade una fórmula comercial de nutrición enteral que contiene LCT como fuente de lípidos. En experimentos preliminares se determinó la dosis de la fórmula y el intervalo de tiempo del cultivo. Los neutrófilos se aislaron a partir de un pool de sangre procedente de sujetos voluntarios sanos (18 a 55 años) y fueron cultivados sin y con la adición de la fórmula comercial enteral que contiene un 3,5% de lípidos con una concentración intraensayo equivalente a 0,04, 0,08, 0,2 y 0,4 mM de LCT durante 18, 42 o 76 horas. En base a los resultados de viabilidad celular obtenidos se establecieron como adecuados unas concentraciones de 0,2, 0,4 mM y un tiempo de cultivo de 18 horas en posteriores experimentos. La funcionalidad de los neutrófilos se evaluó mediante la fagocitosis (test del NBT), producción de malonildialdehído (MDA) como índice de lipoperoxidación y la fragmentación del ADN. Por microscopía óptica se observó que la adición de una dosis 0,4 mM de LCT ($p < 0,05$) producía porcentajes elevados de células preapoptóticas y un incremento significativo en la fragmentación del ADN al comparar con los controles sin adición. En cambio, cultivos con adición de 0,2 y 0,4 mM de LCT mostraron un descenso significativo en la producción de malonildialdehído. Este modelo de nutrición *in vitro* en neutrófilos y con una dieta comercial enteral completa es de relativamente fácil ejecución y puede aplicarse a diferentes condiciones patológicas en las que se estudien los cambios en la funcionalidad de los neutrófilos.

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Palabras clave: Enteral. Envejecimiento. Modelo. Neutrófilos. Nutrición.

Introduction

Neutrophils die quickly *in vitro* and experience the morphological changes typical of cells undergoing programmed cell death¹. Culture of neutrophils implies a decrease in their viability within hours, making them excellent “test” particles for studies on cell aging. To determine the effects of nutrition on the changes brought about by cell aging, various nutritive elements, such as amino acids, vitamins, growth factors, lipids etc. are added to standard culture media, usually as single supplements, and their biochemical modulation of metabolic pathways is determined².

The non-specific response of the neutrophils to infection is complex. Among the many factors implicated it is known that the components of the cell environment play a large part. The lipid composition of the medium has a recognized role in the inflammatory response, although there is no consensus regarding the degree of involvement, or the significance of fatty acid chain length or saturation in this context³. In the studies on this subject, lipids are added as isolated supplements of MCT, LCT or mixtures of these fatty acids^{4,5}. However, to date there is no information on the metabolic changes occurring in neutrophil cell cultures after addition of commercial enteral diets with lipids and other nutrients.

The aim of this study is to validate a cell culture model appropriate for assessing the effects of standard nutritional formulas on neutrophil function *in vitro*. Specifically, we developed a model of aged cells in which a complete commercial formula containing solely LCT as the lipid component and often used in geriatric patients is evaluated.

Material and methods

In the preliminary step, we determined the optimum length of culture and dose of diet emulsion for the study purposes by examining cell viability and morphological changes. Once these parameters were established, we examined neutrophil functionality by measuring phagocytosis, DNA fragmentation and lipoperoxidation. All experiments were performed at least three times.

Culture of neutrophils

A pool of whole blood was collected after an overnight fast from healthy volunteers 18 to 55 years old and neutrophils were separated from whole blood with Polymorphrep™ (a sodium metrizoate/dextran solution, $d = 1.113$) at 1/2 (v/v) by centrifugation at 1750 rpm during 30-35 minutes at room temperature. The sample was washed with an equal volume of C1Na 0.45%, and twice more with equal volumes of saline 0.9%. Red blood cells were lysed by mixing the pellet in 10 mL of cold lysis buffer (155 mM de NH_4Cl ; 10 mM de KHCO_3 ; 0.1 mM de EDTA; pH 7.4) for 10 mi-

utes. Neutrophils were centrifuged, washed twice and seeded in RPMI-1640 culture medium with L-glutamine and FCS 10% without antibiotics. Cells with or without added diet emulsion were incubated for 18, 42 or 76 hours at 37 °C in a 5% CO_2 atmosphere.

Diet

We used a complete standard enteral diet (Cubitan; Nutricia Spain) containing 3.5 g of lipids per 100 mL, 14.2 g of carbohydrates and 10 g of proteins. The lipid composition was as follows: the 97.1% vegetable oil (canola and sunflower) and 2.9% milk fat resulting in 98.7% long chain triglycerides (LCT) and 1.3% medium chain triglycerides (MCT). Molarity was calculated assuming that the molecular weight of LCT was 865 daltons, as described⁴. The lipid concentrations used were adjusted according to the protocol and were equivalent to 0.04, 0.08, 0.2 and 0.4 mM of LCT. In some experiments only the higher doses of 0.2 and 0.4 mM LCT were added.

Cell viability and morphology

Cells were stained with Trypan Blue at 0.2% in saline solution and counted by light microscopy. Additionally, cells were stained with May-Grünwald/Giemsa and morphology was studied ($\times 100$ light microscopy). To determine cytosolic damage, the enzymatic activity of lactodehydrogenase (LDH) was measured in the supernatant by an automated spectrometry technique using pyruvate as substrate and adapted to the Hitachi 747 instrument.

Phagocytosis

For this and all the following determinations, cells were first centrifuged to obtain a pellet. The quantitative NBT test was carried out on aliquots of 250 μL (2.5×10^5 cells) mixed with 20 μL phosphate buffer (PBS; pH 7.4) for the non-stimulated samples or with 20 μL of latex bead suspension (particle diameter 1.094 μm , Sigma: LB-11) for the stimulated samples. A 250 μL volume of 0.1% nitroblue of tetrazolium (Sigma: N-6876) diluted in phosphate buffer (pH = 7.4) was added to all samples. After 30 min of incubation at 37 °C the reaction was stopped, tubes were centrifuged at 3000 $\times g$ during 30 min, the supernatants were discarded, and the reduced NBT was extracted with dioxan (Sigma). Optical density (OD) was measured in the supernatants at 525 nm using dioxan as a blank control, as described⁶.

Biochemical markers

LDH activity was determined in the supernatant (within-run CV: 3.8%) and ion concentrations of Na^+ , K^+ and Cl^- were analyzed by ion selective electrode automated methods (Hitachi 787).

Malonyldialdehyde production

For MDA release as a lipoperoxidation marker we used aliquots of 2×10^6 cells and the amount of protein in the sample was determined. Cells were lysed by freeze-thawing 3 times in liquid nitrogen and water bath at 37 °C. Thiobarbituric acid was added at 0.375% in 15% of TCA and 0.25N HCl and the sample was boiled for 15 min. It was then cooled on ice and MDA was extracted with butanol. The sample was read at 532 nm and calculations were performed considering a molar extinction coefficient of $\epsilon = 1.56 \times 10^5$, as described^{7,8}. Results are expressed per milligram of protein (Pierce).

Diphenylamine reaction

Oligonucleosomal DNA fragments produced in apoptosis can be separated from non-fragmented DNA by centrifugation. This is the basis of the technique modified by Wyllie⁹ which gives a quantitative indication of the percentage of fragmented DNA in a cell population. Apoptosis was assessed in aliquots of 12×10^6 cells. Briefly, the cell pellet was resuspended with lysis buffer (10 mM Tris, 20 mM EDTA, 0.5% Triton; pH 8.0) and after several steps including hydrolysis for 15 min at 90 °C, we added 240 mM of diphenylamine in glacial acetic acid and 0.01% (v/v) of paraldehyde were added. Samples were incubated at 30 °C overnight and measured absorbance (595 nm) was compared from a standard curve (calf thymus DNA sodium salt; Sigma D-1501).

Statistics

Analysis of variance and the Wilcoxon test were used to analyze the data (SPSS, 8.0). Significance was set at a p level of < 0.05.

Results

LDH enzymatic activity and cell viability were determined to establish optimum incubation time. Mean LDH activity in the culture supernatant was 90 ± 3 UI/L at 18 hours and 108 ± 4 at 42 hours, as compared to 88 ± 3 and 104 ± 2 respectively in the controls. The comparison of LDH results at 18 and

Table I
Lactodehydrogenase levels in culture supernatants

<i>LCT in diet (mM)</i>	<i>LDH (18 hours) (UI/L)</i>	<i>LDH (42 hours) (UI/L)</i>
No diet (control) . . .	88 ± 3	$104 \pm 2^*$
0.04	93 ± 2	$110 \pm 2^*$
0.08	90 ± 2	$108 \pm 1^*$
0.2	88 ± 3	$108 \pm 2^*$
0.4	88 ± 3	$109 \pm 7^*$

Data expressed as mean \pm S.D. (n = 4).
* Statistically significant differences vs. 18 hours (p < 0.05).

42 hours showed statistically significant increases (ANOVA; p < 0.05). The fact that there was no increase in enzymatic activity at 18 hours with the various initial diet concentrations confirms the excellent viability obtained. The LDH data from all groups are shown in table I.

Mean cell viability was $98.2 \pm 1.6\%$ at 18 hours, $78.3 \pm 6.6\%$ at 42 hours and $76.2 \pm 4.4\%$ at 76 hours. Since viability was highest at 18 hours, we established this time interval for later measurements.

Observation of neutrophil morphology by optic microscopy after 18 hours of culture showed different percentages of pre-apoptotic cells among the groups: 21-23% without diet addition, 28-30% with added 0.2 mM LCT, and 37-39% with 0.4 mM LCT.

There was a slight, non-statistical decrease in phagocytosis at both levels of added diet, $141.2 \pm 8.8\%$ (0.4 mM LCT) and $157.5 \pm 4.2\%$ (0.2 mM LCT) as compared to the control group ($174.3 \pm 9.8\%$).

The ion concentration results are shown in table II. There was a significant increase in extracellular potassium concentration after addition of the higher diet dose (0.4 mM), a sign indicating a decrease in the "cell vitality" index¹⁰.

The increase in DNA fragmentation in cultures with the higher concentration of LCT indicated an increase in cell apoptosis (table III).

Regarding MDA production as an index of lipoperoxidation, cell cultures with 0.2 and 0.4 mM of added LCT showed significant decreases that were greater at the higher dose (table III).

Table II
Ion levels in culture supernatants after 18 hours

<i>LCT in diet (mM)</i>	<i>Sodium (mEq/L)</i>	<i>Potassium (mEq/L)</i>	<i>Chlorine (mEq/L)</i>
No diet (control)	133.4 ± 6.3	6.07 ± 0.21	122.9 ± 4.6
0.2	129.7 ± 11.7	6.27 ± 0.50	119.7 ± 9.1
0.4	131.3 ± 5.4	$6.49 \pm 0.22^*$	121.1 ± 3.9

Mean \pm S.D (n = 8). * Statistically significant differences vs. control (p < 0.01).

Table III
DNA fragmentation and TBARS after 18 hours

LCT in diet (Mm)	DNA Total	DNA Supernatant	Fragmentation (%)	MDA/mg prot (nmol)
No diet (control)	55.0 ± 26.5	6.45 ± 3.9	10.8 ± 5.0	0.075 ± 0.04
0.2	58.8 ± 22.6	7.16 ± 2.5	11.4 ± 1.5	0.071 ± 1.60**
0.4	1.5 ± 6.2	12.7 ± 4.7*	22.2 ± 2.8**	0.007 ± 0.006**

Mean ± S.D (n = 6 or 8). Statistically significant differences vs. control. * p < 0.01 and ** p < 0.05. Units of DNA fragmentation: µg/12 × 10⁶ cells.

Discussion

Neutrophil culture allows the study of biochemical mechanisms and pharmacological and nutritional effects related to the immunological response. The present study validates an *in vitro* model designed to investigate the effects of a standard nutritional formula (containing LCT lipids), on neutrophil functionality. After testing several culture periods, maximum culture time was established at 18 hours, the time after which cell viability begins to decline¹¹. Significant increases in spontaneous apoptosis are seen after 20 hours of culture¹². In agreement with previous observations, the absence of cytosolic damage at 18 hours was confirmed by the fact that extracellular levels of LDH, a cytosolic marker used in numerous cell models, showed no alterations at this time¹³.

Analysis of ions revealed a significant increase in extracellular potassium without changes in sodium concentrations. It has been described that the intracellular potassium/sodium ratio is an index of cell vitality¹⁰. Potassium excretion to the medium with maintained sodium concentration would lower this ratio, which could be interpreted as an indication of decreased cell vitality, in keeping with the aged status of the cells.

In human *in vitro* cell studies testing the immunomodulatory properties of lipids, various lipid emulsions are added to cell cultures as supplements. The types used (LCT, MCT, LCT/MCT or structured) and the amounts vary considerably: 1.25 to 5.0 mmol/L¹⁴, 0.00188 to 0.03%⁵ (according to our calculations, the equivalent of 0.075 to 1.2 mM), 2.5 mM of triglycerides per liter¹⁵, etc. In the present study the lipids provided were the LCT present in the commercial enteral diet. The intraassay concentration of enteral diet was difficult to establish because the literature contains no information on the use of complete diets *in vitro*. We chose doses of 0.2 to 0.4 mM, equivalent to approximately 1/3 the concentration used by Kruimel¹⁴. Basing his estimation on data from patients treated with TPN¹⁶, Kruimel considered *in vitro* concentrations of 1.25 mmol/l to be physiological. In preliminary experiments (data not shown) we tested this concentration in culture and found that it was excessively high, precluding LDH determination. Thus, we decided on a lower concentration.

The phagocytosis capacity of cultured neutrophils showed a non-significant decrease with addition of the various amounts of LCT-containing diet. This finding is in keeping with published results in which addition of MCT or MCT/LCT mixtures produced notable inhibition of phagocytosis, but LCT alone resulted in a more moderate decrease¹⁵.

The diphenylamine method separating oligonucleosomal DNA fragments produced during apoptosis from non-fragmented DNA gives a measure of the percentage of fragmented DNA in the cell population. This technique does not determine the exact count of apoptotic cells, but, when combined with controls, it gives a reliable indication of the magnitude of cell death occurring in the cultures. The results of this assay showed that at 18 hours of incubation without the addition of diet, 10% cell fragmentation had occurred spontaneously. With the addition of 1% of Cubitan (v/v), equivalent to 0.4 mM LCT, the percentage of fragmentation (often considered the biochemical hallmark of apoptosis) doubled, but a highly significant decrease in lipoperoxidation was also registered; thus we encountered two apparently contradictory findings. Nevertheless, a recent study has suggested a dissociation of DNA fragmentation from other indicators of apoptosis in neutrophils, which could explain this apparent discrepancy. The authors contend that measurement of DNA fragmentation alone is not a good method for evaluating the changes prompted by inductors of apoptosis, such as nitric oxide donors¹⁷.

Paradoxically, there was a decrease in lipoperoxidation with addition of a higher concentration of diet. The explanation for these results is uncertain, but we believe it may be related to the presence of larger amounts of antioxidants in the enteral diet (vitamins, selenium, taurine, etc.), which would have a protective effect against oxidative stress produced during the 18 hours of neutrophil aging.

In conclusion, this is the first study in which a complete commercial enteral diet is directly added to cultures in order to investigate changes in cell function. The results for phagocytosis, DNA fragmentation and lipoperoxidation obtained with 0.4 mM of LCT provided as *in vitro* enteral diet indicate that this neutrophil model adequately reports the changes in neutrophil functional capacity taking place during cell aging as a result of nutritional modulation. The culture condi-

tions established are not overly demanding, making this system relatively simple to execute and potentially applicable to different age groups and pathological conditions.

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