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ALKALINE WATER MODULES THE EXPRESSION OF INFLAMMATION GENES: *IN VITRO* MODEL FOR CANCER PREVENTION

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Abstract – *Objective:* *Many researches focus their attention on the behavior of cells to specific substances with alkalizing power. Each substance that we introduce with food, as well as water, contributes to conditioning the electrophilic balance (acid-base) of the extracellular matrix (MEC) of the whole organism. This systemic regulation of the MEC participates in modulating the expression of genes (transcriptome) and consequently participates in modulating the inflammatory response through the regulation of cellular signal molecules such as ROS (Reactive Oxygen Species) and RNS (Reactive Nitrogen Species). In this study, we investigated the influence of alkaline solutions (Alkawater and Alkacoffee) on one aspect of inflammation relating to the expression of immune modulation by tumor necrosis factor (TNF-α), interleukin 1β (IL-1β), interleukin 6 (IL-6), cyclooxygenase-2 (COX-2) on Caco-2 cell cultures.*

Materials and Methods: *In this paper, the transcriptome of human enterocyte-like Caco-2 cells, in a pH controlled environment with a mixture of alkalizing salts, AlkaWater® and AlkaCoffee®, has been studied. The cellular models used were Caco2 and LS174T cell lines. Isolation and purification of RNA from both Caco2 and LS174T cell lines were performed by TRIzol method. Transcriptome analysis was performed by RNA expression analysis of transcripts of the samples. The analysis was completed with the aid of information technology.*

Results: *When administered individually, Solution 1 (AlkaWater, alkaline water) and Solution 2 (AlkaCoffee, acidity corrector) decrease the expression levels of the four immune modulatory markers (TNF-α, IL-1β, IL-6, COX-2) in CaCo-2 cells (Figure 1), and even more pronounced effects were observed when alkaline solutions and coffee extract were administered in combination. About the influence of alkaline solutions on miR-146a expression, Solution 2 increases the expression of miR-146a gene, while the other solution combinations decrease its level.*

Conclusions: *Cells prevent pathogens through inflammatory responses. Alkaline solutions have been thought as adjuvants of a balanced diet rich of alkaline foods.*

KEYWORDS: *Dietary alkalinity, Acid-base balance, Inflammation.*

INTRODUCTION

Alkaline water is slightly basic containing minerals such as calcium, magnesium or bicarbonate that, binding to hydrogen ions in solution, make the water more basic. Natural alkaline water sources are usually springs, or a reservoir of natural water under the earth's surface. The rock structures holding

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the water may have basic minerals, such as calcium or limestone, that leak into the water, increasing the pH[1]. Water can also be ionized using concentrated alkaline liquid supplement rich of minerals and elements like boron, molybdenum, selenium and magnesium and calcium, both important for maintaining healthy bones. Alkaline waters have been reported as types of functional waters that may ameliorate various disease conditions; the water molecules in alkaline water are smaller and more readily absorbed by cells, which help body re-hydrate quickly and boosting immunity through neutralization of body's acidity caused by wrong diet, stress and environmental toxins. Alkaline water is known also to exert several anti-cancer effects, as well as to scavenge ROS and reduce blood-glucose levels[2].

AlkaCoffee is an acidity corrector, an alkaline product useful for balancing drinks. It is composed of distilled water, ascorbic acid, citric acid, sodium chloride, potassium hydroxide. It is an antioxidant product, designed to correct diets often composed of foods (in this case drinks) with a high percentage of acids like tea, infusions, animal milk, vegetable milk, coffee. The enrichment of negative ions (electrons) and hydrogen produces a strong antioxidant effect.

In this study we evaluated in Caco-2 cell line model several aspects in the field of cytokine property:

a) production (IL-1, IL-6, TNF-α);
b) evidences of pro-inflammatory cytokines are responsible of increased level of Cox-2 enzyme;
c) MiR-146a negatively regulates signal transduction pathways leading to NF-κB activation[3].

MicroRNAs (miRNAs) are a growing family of small noncoding RNAs (19–25 nucleotides) that regulate gene expression through binding to the 30 untranslated regions (30 UTR) of targeted messenger RNAs (mRNAs) to inhibit protein translation or degradation of mRNAs. Dysregulation of miRNAs and their pathogenic roles in various diseases have been demonstrated by abundant studies. By altering gene expression, miRNAs regulate cellular activities such as proliferation, apoptosis, differentiation and migration in multiple types of diseases[4]. MiR-146a is encoded on human chromosome 5q33.315 and is known to play a critical role in immune responses. The knowledge of MiR-146a function in the human context is limited, but some studies confirm its regulatory role in the inflammatory response. In Innate Immune Responses upon activation of a cell surface receptor such as TLR4, a molecular cascade including TRAF6 and IRAK1 leads to IκBα phosphorylation and degradation and to NF-κB activation and nuclear translocation[5]. Several pro-inflammatory cytokines and chemokines, such as TNF-α, IL-1, IL-6 and IL-8, produced upon NF-κB activation, are associated with tumor development and progression[6]. NF-κB activation induces transcription of many genes, including pri-miR146a. Once translocated to the cytoplasm and loaded onto the RISC complex (RNA-induced silencing complex), mature miR-146a contributes to attenuate receptor signaling through the down modulation of IRAK1 and TRAF6[3].

The aim of this study is to determine the expression of some immune molecules in a specific cell type, Caco2, human enterocyte-like, following treatment with alkalizing solutions. The distribution, physiology and polarization of these cells are similar to the cells of the mucosa of the small intestine and it is believed that the administration of solutions on the surface is comparable to the introduction of substances into the intestinal lumen. The yields obtained treated with both solutions (AlkaCoffee and AlkaWater), according to the concentrations indicated by the products, show a behavior similar to food intake in an organism. The next step was the evaluation of the expression of TNF-α, IL-1, IL-6 and COX-2, regulatory molecules of the inflammatory process, as well as that of miRNA-146a as a possible TNF-α regulator.

MATERIALS AND METHODS

Alkaline water supplementation (AWS[1]) was obtained adding 5 drops of AlkaWater® 279 in 250 ml of tap water, until pH 9.0. AlkaWater® 281 consists of boric acid, distilled water, potassium chloride, sodium chloride, 282 potassium hydroxide, sodium molybdate dihydrate, sodium selenite. AlkaCoffee® consists of distilled water, ascorbic acid, citric acid, sodium chloride and potassium hydroxide.

CELL CULTURES

LS174T and Caco-2 cells were purchased from the ATCC (American Type Culture Collection, Manassas, VA, USA). LS174T were cultured in Advanced Modified Eagle Medium (A-MEM, GIBCO, Carlsbad, CA, USA) containing 4.5 g/L glucose, supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 g/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (D-MEM, Sigma-Aldrich, St. Louis, MO, USA), containing 4.5 g/L glucose, supplemented with 10% FBS, 1% non-essential amino acids, 2 mM L-glutamine, 100 U/mL penicillin, and 100 g/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The cells were grown in a humidified atmosphere containing 5% CO_2, at 37°C.

TABLE 1. Experimental conditions.

Ctrl	Caco-2: control plate
EC	Caco-2 pH 7.4-8.4 + AlkaCoffee
Sol1	Caco-2 pH 7.4-8.4 +AlkaWater® (10µl in 1ml H_2O milli-Q®)
Sol2	Caco-2 +AlkaCoffee® (100µl in 1ml H_2O milli-Q®)
EC+Sol1	Caco-2 +AlkaWater® (10µl in 1ml H_2O milli-Q®)
EC+Sol2	Caco-2 +AlkaCoffee® (100µl in 1ml H_2O milli-Q®)

EXPERIMENTAL STUDY DESIGN

The transcriptome of human enterocyte-like Caco-2 cells, in a pH-controlled environment with a mixture of alkalizing salts, AlkaWater® and AlkaCoffee® , has been studied; the cells were treated using the follow procedure (Table 1).

FLUORIMETRIC DETERMINATION OF REACTIVE OXYGEN SPECIES (ROS)

Fluorimetric determination of ROS levels was determined by the membrane-permeant ROS sensitive fluorogenic probe 2',7'-dichlorofluorescein diacetate, DCHF-DA (Molecular Probes, Leiden, The Netherlands). LS174T and Caco-2 cells were grown to semi-confluence in 24 multiwell plates and then incubated for 18 h before the experiments in medium complete in presence and in absence of 100 µM of α-glucans (AHCC), β-glucans and 25 µM vitamin C, alone or in combination (mix). The cells were washed twice with PBS and incubated with 10 µM DCHF-DA in the culture medium without serum for 10 min at 37°C. The cells were washed three times with PBS (Phosphate Buffer Saline). The fluorescence intensity was measured at different time intervals using the plate reader Fluoroskan Ascent FL (Waltham, MA, USA) fluorometer (485 nm excitation and 538 nm emission wavelengths) and data were analyzed by Ascent software (Luqa, Malta).

RNA EXTRACTION FROM LS174T AND CACO-2 CELL LINE

TRIzol (Invitrogen, no. 15596-026; Carlsbad, CA, USA) method has been used for isolation and purification of RNA[7]; 10 micrograms of total RNA from LS174T and Caco-2 cells were obtained. RNA was isolated including a DNase digestion step. These standardized RNA isolation procedures guarantee high-quality RNA. Using the Agilent 2100 Bioanalyzer platform (Agilent Technologies, Santa Clara, CA, USA) RNA samples were quality-checked documenting the identification of 18-S and 28-S ribosomal RNA (rRNA) peaks. The yields were 9–15 µg, and the RNA Integrity Number (RIN) was between 8.2 and 10.

QUANTITATIVE REVERSE TRANSCRIPTION-PCR (REAL-TIME RT-PCR)

To confirm the expression patterns of TNF-α, IL-1β and COX-2 genes, a quantitative RT-PCR using the comparative CT method was performed. Transcript levels of the target genes were normalized to G6PD (the internal control) after correcting for differences in amplification efficiencies. qRT-PCR reactions ($n = 3$) were performed for each gene of interest using a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). All genes investigated have previously been identified and sequences were available in GenBank. Primers for qRT-PCR analysis were designed using the Primer3 program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The final PCR reactions contained: 0.4 µM of each primer; 0.25 µl of SYBR Green (Invitrogen, Carlsbad, CA, USA); 4 mM $MgCl_2$ and as template 5 µl of cDNA reverse transcribed from a standardized amount of total RNA (0.3 µg). qRT-PCR was performed using Hotstart Taq polymerase (Qiagen, Hilden, Germany) in a final volume of 20 µl. All quantitative reactions were subjected to: 95°C for 15 min followed by 45 cycles at 94°C for 15 s, 59°C for 15 s and 72°C for 15 s. Melting curve analysis was applied to all reactions to ensure homogeneity of the reaction product. Potential contamination was assessed by including non-reverse transcribed total RNA (genomic DNA contamination) and controls without template, observing no products in these reactions. To detect the expression patterns of miR-146a, a quantitative RT-PCR using the cDNA obtained either with oligo-dT primers and stem-loop reverse transcriptase (RT) primers was performed, respectively. RNU6B was used as control for miRNAs. Real-Time qPCR was performed under the following conditions: 94°C for 4 min followed by 40 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 1 min. Relative expression levels of hsa-mir-5588-5p, hsa-mir-3658, hsa-mir-567 and hsa-mir-3908 were calculated using the $2^{-\Delta\Delta Ct}$ method.

RESULTS

We evaluated the effects of Solution 1(AlkaWater, alkaline water) and Solution 2 (AlkaCoffee, acidity corrector) both alone and in association with coffee extract on mRNA levels of COX-2, TNF-α, IL-1β and IL-6 measured by qRT-PCR. When administered alone, all the substances decrease the expression levels of the four immune modulatory markers in CaCo-2 cells (Figure 1), and even more pronounced effects were observed when alkaline solutions and coffee extract were administered in combination.

Then we evaluated the influence of alkaline solutions on miR-146a expression always measured by qRT-PCR. Mostly the Solution 2 increases the expression of miR-146a gene, while the other solution combinations decrease its level (Figure 2). The results obtained in these experiments show that the expression of pro-inflammatory molecules is reduced in the samples treated with alkalizing solutions. These results demonstrate a better control of cellular homeostasis and inflammation, after treatment with alkalizing solutions. By means of qRT-PCR analysis, a significant decrease in the expression levels of pro-inflammatory cytokines was found, demonstrating an anti-inflammatory effect of alkalizing solutions (AlkaWater and AlkaCoffee).

DISCUSSION

Enterocyte like-Caco 2 cells were cultured under different experimental conditions: with coffee extract, in presence of alkaline water, in presence of an acidity corrector and in the presence of an association of both alkaline solutions to evaluate how level of cytokines is modified. We used this kind of cell culture because it offered valuable information about the absorption of drugs and dietary components and because has the property of cytokines production[8]. Cytokines are produced in response to infection, inflammation, and trauma. Some cytokines act to make disease worse (pro-inflammatory), whereas others reduce inflammation (anti-inflammatory). IL-1 and TNF are pro-inflammatory cytokines that produce fever, inflammation, tissue destruction, and, in some cases, shock and death[9]. IL-6 acts as either a pro-inflammatory cytokine or an anti-inflammatory cytokine. IL-6 triggers its receptors CD130 and CD126 proteins to form a complex, thus initiating a signal transduction cascade through certain transcription factors like JAKs and STATs[7]. COX-2 is an inducible early response gene and is activated in response to various extracellular or intracellular physiological stimuli: lipopolysaccharide (LPS), IL-1, TNF and others[10].

Enterocyte like-Caco 2 cells produce a basal level of proinflammatory cytokines, as we can see in untreated control, because they are colorectal

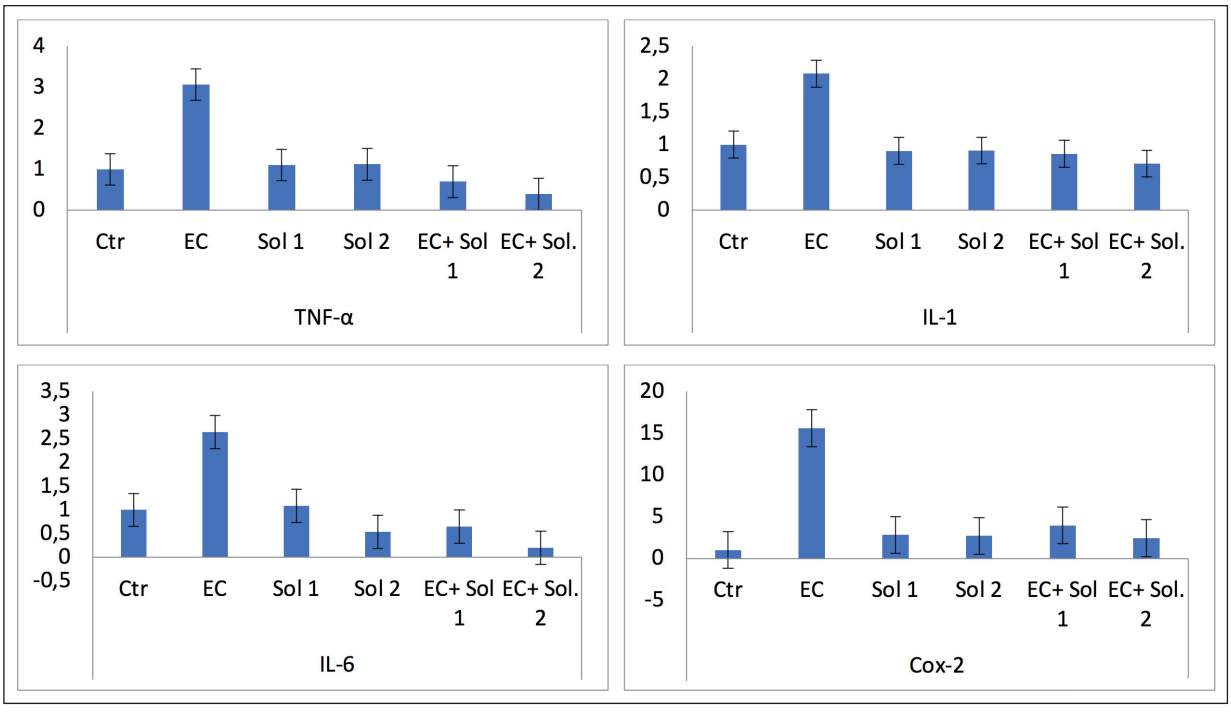

Fig. 1. Levels of TNF-α, IL-1, IL-6 and COX-2 in Caco-2 cells after treatment with AlkaWater and AlkaCoffee.

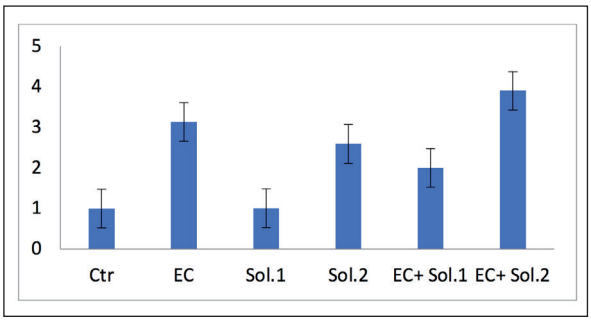

Fig. 2. Levels of miR-146a in Caco-2 cells after treatment with AlkaWater and AlkaCoffee.

adenocarcinoma cells and the same cytokines are responsible of Cox-2 rising. The presence of coffee extract exacerbates the inflammatory state of cells and induces a higher expression of coding gene for the inducible isoform of Cyclooxygenase2. The most important aspect of work was to demonstrate that alkaline solutions improve the inflammatory state with an overall lowering of cytokines and proinflammatory enzyme. Cytokines block causes an inhibition of several pathways that normally trigger an increase in expression of proinflammatory genes. Another important aspect of work is the role of miR-146a. Cytokines activate nuclear factor kappaB (NF-kB), which is frequently detected in tumors, through TLR4, a protein receptor. This receptor detects cytokines and continues through a single transduction pathway that uses IRAK1 and TRAF6, other protein receptors. After going through the transduction pathway, kB is ultimately activated. When kB is activated, NF-kB detects genes that have a complementary binding site. One of the genes that has a binding site for NF-kb is microRNA-146a. MicroRNA l46a then inhibits the translation of IRAK1 and TRAF6 as demonstrated in experiment of Tagonov et al[5] that demonstrated a targeted deletion of the miR-146a gene in mice. They discovered that the deletion of the miR-146a gene caused a decrease in concentration of miR-146a in the spleen, bone marrow and thymus. This caused the increase of inflammatory cytokines (IL-6). Tagonov et al[5] concluded that miR-146a deletion could cause an over-response to inflammatory stimuli or an extended response to inflammatory stimulus. A decrease in the presence of miR-146a in the cell, could lead to chronic inflammatory states and tumor growth. Our results are in agreement with Tagonov et al[5], because a lowering inflammatory state due to the presence of alkaline solutions reduces the activation of the NF-kb pathway by pro-inflammatory cytokines and, therefore, there will be a lower expression of gene that codes for miR-146a.

CONCLUSIONS

Cells prevent pathogens through inflammatory responses; however, uncontrolled inflammatory response disrupts the natural function of cells and their homeostasis. In conclusion, the alkaline solutions have been thought as adjuvants of a balanced diet rich of alkaline foods.

Conflict of interest:
The Authors declare that they have no conflict of interests.

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