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EVALUATION OF GENOTOXIC AND CYTOTOXIC EFFECTS OF ANORECTIC DRUGS IN LYMPHOCYTES OF OBESE PEOPLE

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ABSTRACT

Obesity is a major public health concern nowadays, as the risk of developing cancer and other chronic diseases has increased considerably in this population. Using in vitro tests (Comet Assay and Micronucleus Test), we evaluated whether lymphocytes from obese individuals (BMI: 40-62 kg / m2) are sensitive to genotoxic and cytotoxic damage when exposed to anorexigenics sibutramine, femproporex and phytotherapics Cordia ecalyculata and Echinodorus grandiflorus. We found that both possessed weak genotoxic activity and did not detect cytotoxic activity or interference in the cell division mechanisms of lymphocytes among obese individuals.

Key words: Genotoxic. Cytotoxic. Anorectic. Obese. Lympocytes.

RESUMO

Avaliação dos efeitos genotoxicos e citotóxicos de medicamentos anoréticos em linfócitos de pessoas obesas

A obesidade é uma das principais preocupações de saúde pública atualmente, pois o risco de desenvolver câncer e outras doenças crônicas aumentou consideravelmente nessa população. Utilizando testes in vitro (Ensaio Cometa e Teste de Micronúcleo), avaliamos se linfócitos de indivíduos obesos (IMC: 40-62 kg / m^2) são sensíveis a danos genotóxicos e citotóxicos quando expostos aos anorexígenos sibutramina, femproporex e fitoterápicos Cordia ecalyculata e Echinodorus grandiflorus. Descobrimos que ambos possuem fraca atividade genotóxica e não detectamos atividade citotóxica ou interferência nos mecanismos de divisão celular de linfócitos de indivíduos obesos.

Palavras-chave: Genotóxica. Citotóxico. Anoréxico. Obeso. Linfócitos.

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INTRODUCTION

Being overweight and obese worries health authorities on all continents. It is estimated that 30%-40% of the world's population is afflicted with this condition. In addition to this, in recent years, there has been a considerable increase in children and young people as part of this statistic.

Between 1980 and 2013, the prevalence of overweight or obese children increased by 47.1%, while that of adults increased by 27.5%. The number of individuals in this classification increased from 857 million to 2.1 billion people in that period (Ng and collaborators, 2013).

Obesity cannot be attributed to a single cause, as its development is associated with external factors such as eating habits, exercise practice, and internal factors such as genetic and hormonal composition. From a biomedical point of view, obesity can be classified as a multifactorial pathophysiology (Gustafson and Smith, 2015).

In addition to the discomfort and discrimination suffered by obesity, this condition is associated with the increased risk of occurrences of several pathologies. Chief among them are those related to the cardiovascular system, such as heart attack and stroke, and chronic diseases such as diabetes mellitus type II and cancer (Campbell, 2014).

Several scientists have carried out research on the relationship between increased body mass and increased incidence of certain tumours in the population. There are studies stating that with each increase of 5 kg / m2 body, there is a linear increase in the risk of developing certain cancers; in some cases, the risk increases by up to 63%. Observing these studies, it is possible to deduce that overweight or obese people body mass index > 24.9 kg / m2) are at higher risk of developing cancer than those with normal body mass (Bhaskaran and collaborators, 2014; Campbell, 2014).

The treatment of obesity involves a multidisciplinary effort. Natural methodologies, such as increased physical activity and changes in eating habits, should be prioritised. When the natural treatment does not produce effect, the anorectic drugs prescribed by health professionals are used responsibly. If all the attempt fails, more invasive interventions like bariatric surgery are conducted (Ravussin and Bouchard, 2000).

Some anorectic drugs released for marketing do not have long-term use studies, especially in relation to their genotoxic effects. Femproporex and Sibutramine are anorectics that are banned in some countries but are extensively used in others, especially developing countries. The use of these drugs generates much controversy and requires more comprehensive studies (da Silva and collaborators, 2010a, 2015).

Medicinal plants are also widely used to treat obesity. In some cases, these products are sold as natural medicines, but many of them are associated with allopathic anorectics. The Porangaba (Cordia ecalyculata) and the Chapéu-de-Couro (Echinodorus grandiflorus) are two native plants whose raw extract are widely used for treatment of obesity, mainly in Brazil (da Silva and collaborators, 2010b).

Given that the obese population is at a greater risk of developing cancer, many substances that are used to treat obesity require refined studies on the genotoxic and cytotoxic potential, and cancer originates from damages in the genetic material, we conducted this work with the specific objective of verifying if the anorexigenic drugs Sibutramine and Femproporex and the crude extracts of Porangaba and Chapéu-de-Couro interfere in the genetic material and viability of lymphocytes of obese individuals.

MATERIALS AND METHODS

Plant material and chemicals

Fenproporex: (+)-3-(a-methylphenethylamino) propionitrile hydrochloride, CAS Registry: 15686-61-0 - Lot núm.: 7CG5010, Genix Indústria Farmacêutica Ltda., Anápolis-GO, Brazil.

Sibutramine:(+)-1-(p-chlorophenyl)-a-isobutyl-N, N-dimethylcyclobutaneme-thylamine hydrochloride monohydrate, CAS Registry: 106650-56-0 - Lot núm.: IF076506*2, Deg Importadora de Produtos Químicos Ltda. São Paulo-SP, Brazil.

Doxolem®: Chlorohydrate of doxorubicin, CAS Registry 23214-92-8; Zodiac Produtos Farmacêuticos S/A, Pindamonhangaba-SP, Brazil, was used as positive control.

The botanical medicines were purchased from specialised commercial establishments that are officially authorised for the commercialisation of these products: Echinodorus grandiflorus (Cham. & Schltdl.)

Revista Brasileira de Obesidade, Nutrição e Emagrecimento
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Periódico do Instituto Brasileiro de Pesquisa e Ensino em Fisiologia do Exercício

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Micheli Used part: Leaves Country of origin: Brazil Dried in: Oven Family: Alismataceae Lot nº: CHPCP01/0107 Phytochemical analysis: Positive test for flavonoids, steroids, saponins, and polyphenols (Duarte and collaborators, 2002; Manns and Hartmann, 1993) The plant (dried powdered leaves) was purchased in Santos Flora Comércio de Ervas Ltda. São Paulo-SP, Brazil. Cordia ecalyculata Vell. (sin. Cordia Salicifolia Cham.) Used part: Leaves Country of origin: Brazil Dried in: Oven Family: Boraginaceae Lot núm.: 164-F/05 Phytochemical analysis: positive test for saponins, anthocyanins, gums and mucilage, mineral salts, flavonoids, amide, tannins, amine groups, fixed acids, and steroids. The plant (dried powdered leaves) was purchased in, Hubert Comércio de Produtos Alimentícios Ltda. São José dos Pinhais-PR, Brazil.

Extract preparation

One kilogram of dried and powdered leaves from each plant (E. grandiflorus (Cham. & Schltdl.) Micheli. and C. ecalyculata Vell.) was submitted to exhaustive extraction by maceration at room temperature in a steel percolator using ethanol-water (7:3) solution. The obtained extracts were filtered and concentrated under reduced pressure in a rotary evaporator at 35-40 ºC, followed by lyophilisation to furnish the crude hydroacoholic extracts (15% and 17% (w/w) for E. grandiflorus and C. ecalyculata respectively). The extracts were stored in amber flasks protected against the light (da Silva and collaborators, 2010b).

Lymphocytes of obese subjects

The research material were obtained from eight obese subjets, 3 men and 5 women (BMI: 40-62 kg / m2) aged between 30-45 years, who did not present diagnosed genetic problems. This study was approved by the Research Ethics Committee of the Clinical Hospital and Medical School of Ribeirão Preto, University of São Paulo (HCRP No. 4684/2006).

Twenty cubic milliliters (20 mL) of venous blood was collected from each volunteer after the consent form was presented. The culture of human lymphocytes followed the methodology of Moorhead and collaborators (1960) with some modifications as described below: After venepuncture, the blood was transferred to tubes containing heparin and allowed to stand at room temperature for decantation of the cells and separation of the plasma. It was collected with the help of the Pasteur pipette, “leukocyte ring,” together with a small amount of plasma; after homogenisation of the material, approximately 12-15 drops are transferred to the culture flask containing 5 mL of complete culture medium, composed of 78% RPMI 1640 culture medium (Sigma), and supplemented with streptomycin (0.01mg / mL). These culture flasks are kept in an oven at 37 ° C until the culture time is over.

Lymphocyte treatments

From the preliminary data, the following test concentrations were established: negative control (culture medium), positive control (Doxorubicin 0.02 µg / mL culture medium), Femproporex (5, 10 and 20 µg / mL culture medium), Sibutramine (5, 10 and 20 µg / mL culture medium), C. ecalyculata (25, 50.80 µg / mL culture medium), and E. grandiflorus (25, 50, 80 µg / mL medium of culture). Each individual blood sample was fractionated in 14 flasks of culture medium and the treatments were distributed.

After 24 hours of culture, an aliquot was taken for the accomplishment of the Comet Assay (T0), and the treatments were added, returning the flasks to the culture chamber for another 24 hours. After this period, aliquots (500 µL) were withdraw to perform the Comet Assay and the remaining cell suspension was concentrated by centrifugation (1000 rpm / 5 minutes), the supernatant discarded, and 5 mL of complete culture medium added with Cytochalasin B in concentration of 5.0 µg / mL culture medium. After generous homogenisation, the culture flasks returned to the culture chamber for another 28 hours. After this period, the cells are collected for the Micronucleus Test.

Comet assay

The “Single-Cell Electrophoresis Assay” or Comet Assay was performed under alkaline conditions as described in classical works. The slides were evaluated in a “blind test” and 100 nucleoids were analysed by treatment (50 nucleoids per slide and 2 slides per treatment). The nucleoids are classified according to the size of the tail visualised into 5 classes, with the ones not being damaged categorised as class zero (0) and the totally

Revista Brasileira de Obesidade, Nutrição e Emagrecimento
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Periódico do Instituto Brasileiro de Pesquisa e Ensino em Fisiologia do Exercício

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damaged nucleoids categorised as class four (4). The value (damage index) is assigned to each nucleoid, which is analysed according to its class. The damage index varies from zero (0), when all nucleotides present no damage (100 nucleoids x 0), to 400, when all nucleoids have a maximum level of damage (100 nucleoids x 4). Nucleoids with atypical abnormalities were disregarded in the count, since they may originate from dead cells (Hartmann and Speit, 1997; Tice and collaborators, 2000).

Micronucleus test

Micronucleus quantification was determined in 1,000 binucleate lymphocytes (of each individual) with well-preserved cytoplasm. The criteria for preparation of slides and identification of the micronucleus are those recommended by Fenech (2000).

Nuclear Division Index (NDI)

The nuclear division index (NDI) was determined by the analysis of 1,000 cells per slide. Only cells with a well-preserved cytoplasm containing 1 to 4 nuclei were counted with the aid of a digital cell counter and light microscopy. The NDI was calculated according to Eastmond and Tucker (1989), using the following formula:

$$NDI = [M1 + 2(M2) + 3(M3) + 4(M4)]/ N$$

Where M1-M4 are cell numbers with 1, 2, 3 and 4 nuclei respectively; N is the total number of cells analysed (Eastmond and Tucker, 1989).

Tripan blue cell viability test

For each treatment, 10 µL of cell suspension is collected from the culture medium; mixed with 10 µL of Trypan blue solution (0.04 mg / mL); after 3 minutes, this material is deposited in a Neubauer chamber; 200 cells are counted in two observation fields; viable cells do not stain, inviable cells turn blue; the result is given as a percentage of live (viable) cells. Cultures with a cell viability above 70% are considered viable for the Comet test.

Statistical analysis

The data were analysed using The One-Way ANOVA Test and the Student's t-test (SigmaStat to Windows-1.0. Copyright© 1992-1994, Jandel Corporation.). All of the tests considered a 5% significance level. The results are expressed as means + SD of three independent experiments. A result was considered positive when there was a statistically significant increase ($p < .05$) in values for at least one dose that exceeded the negative control range.

RESULTS

Evaluation of the Drugs Sibutramine and Femproporex

Lymphocytes obtained from obese subjects exposed for 24 hours to Sibutramine or Femproporex treatments at different concentrations (5.0, 10 and 20 µg / mL of medium) did not significantly increase ($P> 0.05$) the number of binucleate cells with micronucleus, when compared with the negative control treatment, with the exception of treatment with Sibutramine at 20 µg / mL concentration of medium. In this dose, the number of binucleated cells with micronucleus was significantly higher ($P <0.05$) than that observed for the negative control (Fig. 1).

The different doses of Sibutramine treatment resulted in no statistically significant differences ($P> 0.05$) in the number of binucleate cells with micronucleus. With regard to the treatment with Femproporex, a similar result was observed (Fig. 1).

There were no differences in the nuclear division indices in the lymphocytes of obese subjects exposed for 24 hours to treatments with the following: doxorubicin (0.02 µg / mL medium), Sibutramine (5.0, 10 and 20 µg / mL medium), Femproporex (5.0, 10 and 20 µg / ml medium), and negative control (Fig. 2).

When evaluating the possibility of genotoxic damage in lymphocytes obtained from the venous blood of obese subjects exposed in vitro to the drugs Sibutramine or Femproporex, the following results were obtained: after 24 hours of treatment with the drug Sibutramine at concentrations of 5, 10 and 20 µg / mL, the lymphocytes presented greater damage scores ($P <0.05$) than that of the negative control treatment. The different doses of Sibutramine treatment resulted in no

Revista Brasileira de Obesidade, Nutrição e Emagrecimento
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Periódico do Instituto Brasileiro de Pesquisa e Ensino em Fisiologia do Exercício

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considerable variation (P> 0.05) in the damage scores (Fig. 2).

The Femproporex drug, evaluated in the same treatment conditions, also presented a similar result to that presented by Sibutramine. That is, the lymphocyte damage scores was higher at all concentrations (P <0.05) than that observed in the negative control treatment. The different doses of Femproporex treatment also resulted in no significant variations (P> 0.05) in the damage scores (Fig. 2).

When comparing the results of damage scores of the lymphocytes evaluated in time (T0) with those from the negative control, no significant difference (P> 0.05) was detected. (Fig. 2).

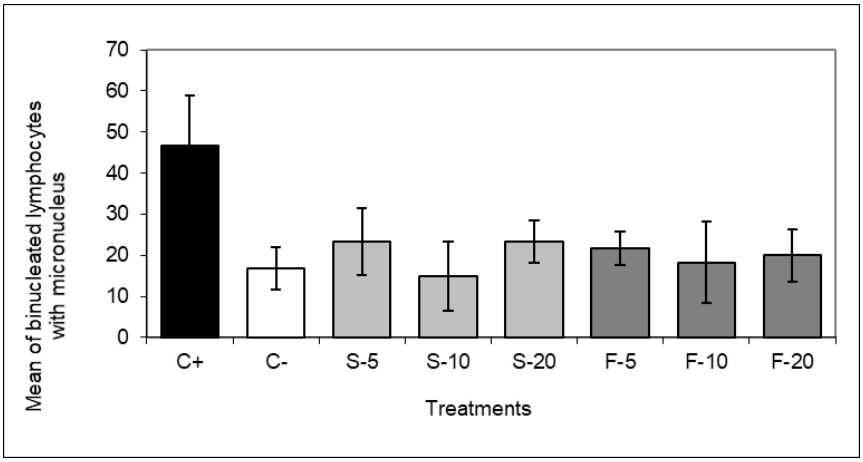

Figure 1 - Mean of binucleated micronucleus lymphocytes exposed for 24 hours to the following: C + doxorubicin (0.02 µg / mL culture medium); C- (culture medium); S-5 (Sibutramine 5.0 µg / mL culture medium); S-10 (Sibutramine 10 µg / mL culture medium); S-20 (Sibutramine 20 µg / mL culture medium); F-5 (Femproporex 5.0 µg / mL culture medium); F 10 (Femproporex 10 µg / mL culture medium); and F-20 (Femproporex 20 µg / mL culture medium). A thousand cells were analysed from each subject by treatment.

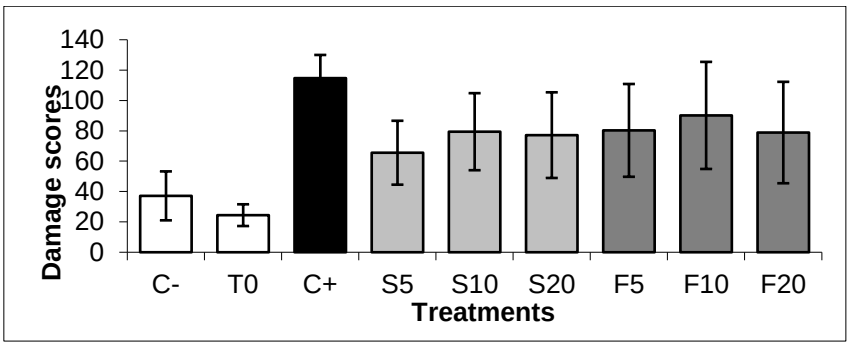

Figure 2 - Damage score verified in venous blood lymphocytes from obese individuals after 24 hours treatment: C- (culture medium), T0 (Comet Assay done immediately after blood collection), C + (Doxorubicin 0.02 µg / mL culture medium), S5 (Sibutramine 5.0 µg / mL culture medium), S10 (Sibutramine 10 µg / mL culture medium), S20 (Sibutramine 20 µg / mL culture medium), F5 (Femproporex 5.0 µg / mL culture medium), F10 (Femproporex 10 µg / mL culture medium), and F20 (Femproporex 20 µg / mL culture medium). Hundred nucleoids of each individual were analysed by treatment.

Evaluation of extracts of C. ecalyuculata and E. grandiflorus

In the lymphocytes obtained from obese subjects, after exposure for 24 hours to the hydroalcoholic extracts of C. ecalyculata or E. grandiflorus at concentrations of 25, 50 and 80 µg / mL of culture medium, there were no differences in the number of binucleate cells with micronucleus between treatments with different doses of phytoterapic and the negative control (Fig. 3).

There were also no variations between the different doses within the same treatment for both C.ecalyculata and E.grandiflorus(Fig. 3).

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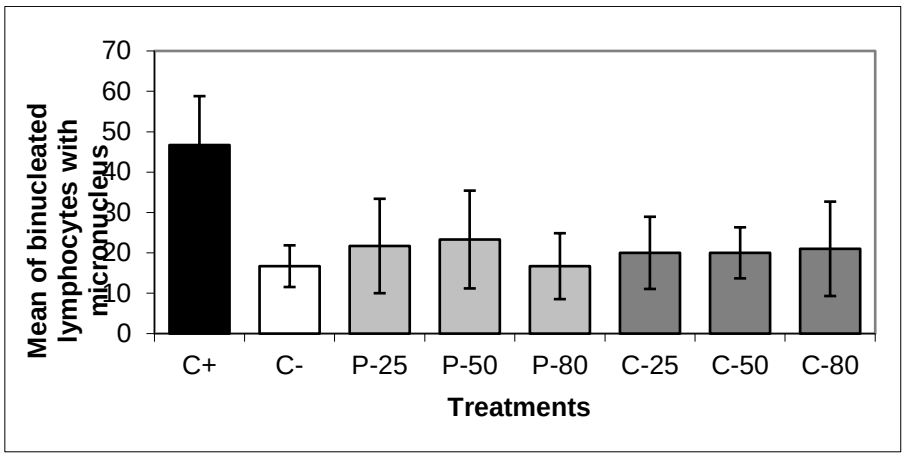

Figure 3 - Mean of binucleated micronucleus lymphocytes exposed for 24 hours to the following: C + Doxorubicin (0.02 µg / mL culture medium); C- (culture medium); P-25 (C. ecalyculata 25 µg / mL culture medium); P-50 (C. ecalyculata 50 µg / mL culture medium); P-80 (C. ecalyculata 80 µg / mL culture medium); C-25 (E.grandiflorus 25 µg / mL culture medium); C-50 (E.grandiflorus 50 µg / mL culture medium), and C-80 (E.grandiflorus 80 µg / mL culture medium). Thousand cells were analysed from each subject by treatment.

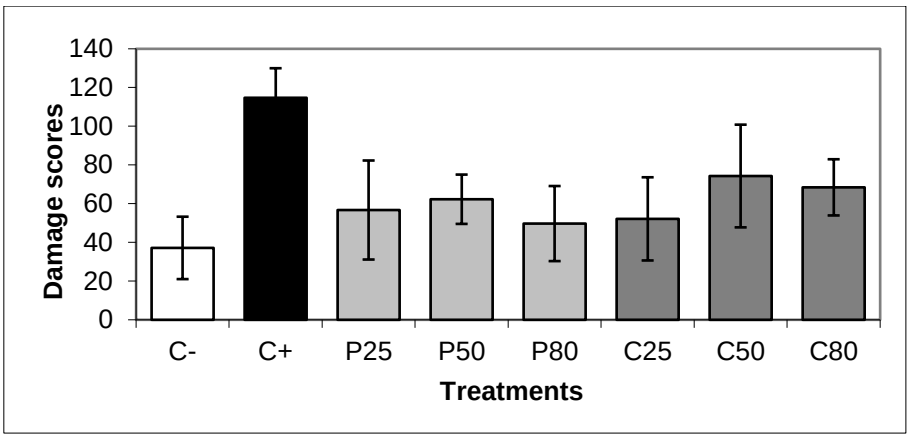

Figure 4 - Damage scores verified in venous blood lymphocytes of obese individuals after 24 hours of treatment with the following: C- (culture medium), C + (Doxorubicin 0.02 µg / mL culture medium), P25 (C. ecalyculata 25 µg / mL culture medium), P50 (C. ecalyculata 50 µg / mL culture medium), P80 (C. ecalyculata 80 µg / mL culture medium), C25 (E. grandiflorus 25 µg / mL culture medium), C50 (E. grandiflorus 50 µg / mL culture medium), and C80 (E. grandiflorus 80 µg / mL culture medium). Hundred nucleoids of each individual were analysed by treatment.

With respect to the possible interferences of the extracts of C.ecalyculata and E. grandiflorus in the nuclear division indices of lymphocytes, which were obtained from the venous blood of obese individuals, it was verified that there were no statistically significant differences (P> 0.05) across all evaluated treatments, including the positive and negative controls.

When evaluating the venous blood lymphocytes of obese individuals, after 24 hours of treatment with C. ecalyculata extract at concentrations of 25, 50, and 80 µg / mL of culture medium, it was verified by the Comet Assay that the damage scores in treatments with concentrations of 25 and 80 µg / mL are not, statistically, different (P> 0.05) from negative control. Treatment with C. ecalyculata at a concentration of 50 µg / mL showed a higher lymphocyte damage score than the negative control treatment (P <0.05). There were no differences (P> 0.05) detected between lymphocyte damage scores within the same treatment of C. ecalyculata but with different doses (Fig. 4).

In the treatment of lymphocytes with the extract of E. grandiflorus at concentrations of 50 and 80 µg / mL, the damage scores were higher (P <0.05) than the negative control. At the concentration of 25 µg / mL, there was no

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Periódico do Instituto Brasileiro de Pesquisa e Ensino em Fisiologia do Exercício

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difference (P> 0.05) noticed. Within the treatment, there were no differences (P> 0.05) detected in the damage scores between the three evaluated doses (Fig. 4).

DISCUSSION

Peripheral blood lymphocytes are important indicators present in the internal compartment of the human body. Although they are produced in the hematopoietic system, they are later released and circulate through all organs of the body. This particularity makes them important tools for the evaluation of genotoxic risks caused by substances administered to humans, or even those that are assimilated due to environmental exposure.

Based on the premise that the internal environment is rich in factors with potential to provoke cellular instability in obese individuals, this study sought to certify if the lymphocytes obtained from these individuals, when exposed to the anorexigenic drugs Femproporex or Sibutramine and phytotherapics, present genotoxic damages.

It was verified by means of the Comet Assay that both the drug Sibutramine and the drug Femproporex at the evaluated concentrations of 5, 10 and 20 µg / mL caused an increase in the lymphocyte damage scores of obese individuals, who were exposed to different concentrations of these drugs for a period of 24 hours under culture conditions. This increase was recorded by comparing the mean values of the DNA damage scores of the lymphocytes treated with the drugs with the mean score of the negative control treatment.

It was also found that there is a no dose-effect response between the different concentrations of Sibutramine; the same occurs in the concentrations of Femproporex. To confirm whether the result of an in vitro genotoxic test is positive or negative, some considerations become necessary. An essential consideration is that test substances present a damage score higher than that found in the negative control; this prerogative our study meets. Also recommend the verification of the dose-response relationship, a fact not evidenced in our evaluation. In addition, the physicochemical properties of test substances should be taken into account as well as the relevance of the results (OECD Guideline 476, 1997).

In observance of our data, we can affirm that Sibutramine and Femproporex cause damage to the lymphocyte DNA of obese individuals. This in vitro result is in agreement with the study that we did in vivo, where we also verified positive results for both drugs (da Silva and collaborators, 2010a). As in our in vitro study, we did not use metabolic activation system, and we can affirm that the damages detected are due to the direct action of the drugs. To certify this fact, we recommend the evaluation of the metabolites originating from these drugs. We also found it important to verify the behaviour of these drugs exposed to treatment for 2 and 6 hours, since our treatment was for 24 consecutive hours, and this long period of exposure may perhaps influence the results.

The Micronucleus Test detects unrepaired damage that interferes with the dynamics of cell division or genetic material. In our study, Femproporex at the doses and conditions evaluated did not increase the frequency of micronucleated lymphocytes compared to the negative control. Sibutramine at the dose of 20 µg / mL caused a slight increase in the frequency of micronucleated lymphocytes when compared to the negative control. Since both drugs cause increased lymphocyte DNA damage scores, and only Sibutramine at this concentration has led to micronucleus formation, we may suggest that for Femproporex and the two other doses of Sibutramine, repair mechanisms and cellular homeostasis may reverse, while at the highest dose of Sibutramine (20 µg / mL), there is saturation of these mechanisms, which allows the fixation of the damage in the form of micronucleus.

NDI is an important tool to evaluate possible interferences of test substances in the cytokinesis and karyokinesis mechanisms (Fenech, 2000). In our study, we verified that both the drug Sibutramine and the drug Femproporex do not present differences in the NDIs of lymphocytes when compared to the negative control. This shows that the drugs neither block cell division nor stimulate it. An intriguing fact is that our positive control also had NDI similar to the negative control. A similar phenomenon was also observed in the study by Dhawan and collaborators (2003). We can attribute this behaviour to the low dose used (0.02 µg / mL) or overtime of exhibition

Plant extracts are composed of a mixture of various chemicals present in a greater or lesser number and amount, depending on the plant species or even the conditions of the environment of origin. Both

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Periódico do Instituto Brasileiro de Pesquisa e Ensino em Fisiologia do Exercício

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the extracts of C. ecalyculata and E. grandiflorus were already identified substances with potential protection of the cellular genetic material, such as flavonoids.

In our work, we verified that the extract of C. ecalyculata at the concentration of 50 µg / mL and the extract of E. grandiflorus at concentrations of 50 and 80 µg / mL increased the DNA damage scores of lymphocytes when compared to the negative control. The possible explanation for the action of the extract of C. ecalyculata only at the concentration of 50 µg / mL and in the composition of the extract is that there may be some genotoxic substance that, isolated or in association, finds in this concentration favourable conditions to cause damage in the DNA. At lower or higher doses, this balance is broken and the substance loses or decreases the genotoxic action.

In the case of E. grandifloras, we verified that the damage score is higher than the negative control in the two highest concentrations. The explanation for this may be the same as previously provided for C. ecalyculata, with the difference that for E. grandifloras, the range of genotoxic action of the substance is broader. Possibly, the dose increase will interfere with the effect, because at the concentration of 80 µg / mL, a slight reduction in the damage score is observed compared to the concentration of 50 µg / mL.

In relation to possible clastogenic / aneugenic effects, the phytotherapeutic extracts showed no activity and also did not interfere in human lymphocyte cytokinesis.

We interpret the genotoxic action of the extracts of C. ecalyculata and E. grandiflorus as weak positive, since we only detected effects with the help of Comet Assay, and this method is not only very sensitive, but also sometimes detects repairable damages. Associating this information with the study that we did in vivo (to quote my works), we can deduce that the crude extracts evaluated should present little genotoxic risk for humans. Despite this, we recommend the study of the constituent fractions of both extracts to better elucidate their actions.

Regarding the lymphocyte study of obese individuals, we suggest enlarging the sample and conducting studies that explore molecular aspects related to mitochondria.

CONCLUSION

With respect to the conditions and methodology of this study, both Sibutramine and Femproporex increase the frequency of genotoxic damage in lymphocytes of obese individuals;

Sibutramine at the dose of 20 µg / mL caused a slight increase in the frequency of micronucleated lymphocytes when compared to the negative control;

In our study, we verified that both the drugs Sibutramine and Femproporex do not present differences in the NDIs of lymphocytes when compared to the negative control;

We can conclude from our study that the drugs Femproporex and Sibutramine present genotoxic activities in vitro, although it is discrete in terms of magnitude;

Under the conditions of our study, the extracts of C. ecalyculata and E. grandiflorus induce weak genotoxic activity in lymphocytes of obese individuals;

The phytotherapics studied in accordance with the methodology of this work did not present interference in the mechanisms of cell division or cytotoxic activity.

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