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Reliability of plant root comet assay in comparison with human leukocyte comet assay for assessment environmental genotoxic agents

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ABSTRACT

Comet assay is an efficient test to detect genotoxic compounds based on observation of DNA damage. The aim of this work was to compare the results obtained from the comet assay in two different type of cells extracted from the root tips from *Lactuca sativa* L. and human blood. For this, Spent Pot Liner (SPL), and its components (aluminum and fluoride) were applied as toxic agents. SPL is a solid waste generated in industry from the aluminum mining and processing with known toxicity. Three concentrations of all tested solutions were applied and the damages observed were compared to negative and positive controls. It was observed an increase in the frequency of DNA damage for human leukocytes and plant cells, in all treatments. On human leukocytes, SPL induced the highest percentage of damage, with an average of 87.68%. For root tips cells of L. *sativa* the highest percentage of damage was detected for aluminum (93.89%). Considering the arbitrary units (AU), the average of nuclei with high levels of DNA fragmentation was significant for both cells type evaluated. The tested cells demonstrated equal effectiveness for detection of the genotoxicity induced by the SPL and its chemical components, aluminum and fluoride. Further, using a unique method, the comet assay, we proved that cells from root tips of *Lactuca sativa* represent a reliable model to detect DNA damage induced by genotoxic pollutants is in agreement of those observed in human leukocytes as model. So far, plant cells may be suggested as important system to assess the toxicological risk of environmental agents.

1. Introduction

Environmental pollution is a question of major concern that affects all living organism, including human. Thus, it is of great importance studies that clarified the effects of environmental pollutants (industrial waste, heavy metals, and pesticides) in living beings and how they interact. The mechanism of toxicity of those compounds also have to taken into account (Bianchi et al., 2015). To achieve this goal, tests with different models are available, allowing the access of a range of endpoints of the toxic agents.

In this context, Environmental Protection Agency of United States (US EPA) point out the higher plants as efficient for the identification of toxicity of environmental pollutants (Grant, 1999). The plant bioassays usually focus on the evaluation of chromosome alterations during mitotic cell cycle (Andrade-Vieira, 2012). However, the reliability of the plant chromosome aberration bioassay is still questioned for some that wonder if it could be extrapolate for animal and even human ones (Palmieri et al., 2016). Apart from this, few studies compared plant cells with animal cells considering the same assay for both cell types. Thus, we aimed to evaluate whether *Lactuca sativa* L. root tips cells is effective to detect DNA damage induced by environmental genotoxic agents as far as human leukocytes trough the comet assay.

In this sense, the objective of the present research is in agreement with the Toxicology' guidelines for the twenty-first century, which claims for bioassays that could substitute the ones that use animals as models (Hartung, 2009). Moreover, this is the first work considering a comparative approach between human and plant cells, applying the treatments in entire cells and not in the isolated nucleus, in order to validate plants as an alternative model to animal cells.

Of the assays available for this end, the comet assay is considered an ideal tool in toxicological studies (Kuroda et al., 1992; Avishai et al., 2002; Collins, 2015), standing out for its rapidly, simplicity, sensitivity and relatively low cost for detecting and analyzing DNA breaks in individual cells. In addition, comet assay allows qualifying and quantifying the severity of the damage on the DNA by analyzing the drag of nuclei containing fragmented molecules (Brendler-Schwaad

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et al., 2005). With this in mind, in the present study the SPL a solid waste from industry of aluminum mining and processing, and its components, aluminum (Al) and fluoride (Fl), will have their genotoxic potential evaluated on model cells.

Our research group have studied the effect of SPL in plant models and we previously described the toxicity of this waste on *Allium cepa, Zea mays* and *Lactuca sativa* evaluated through seed germination, root growth and cell cycle alterations tests (Andrade et al., 2008; Andrade-Vieira et al., 2010, 2012; Palmieri et al., 2014). Point mutation in *Tradescantia* clone #4430 (Trad-SHM) was also an endpoint assessed (Andrade-Vieira et al., 2011). All the works above-mentioned show the toxicity of SPL and its components fluoride (Fl), cyanide (CN) and aluminum (Al) salts previously referred by the US EPA.

Recently Palmieri et al. (2016) checked the *Allium* chromosome aberration assay against the comet assay in human leukocytes. It was demonstrated a correlation between clastogenic alterations (fragments, bridges, and condensed nucleus) and comet arbitrary units value Thus, here we aimed to go far: the same test and nucleus' stage will be considered, demonstrating that the DNA damage is Universal independently of the physiology of the organism itself.

2. Material and methods

2.1. Preparation of experimental solution

The treatment solutions of SPL and its components (Al and Fl) used in the present work were the same prepared previously by Palmieri et al. (2014, 2016). Here, we used three concentrations for each treatment (SPL, Al and Fl) given in Table 1. The concentration of Al and Fl applied here were determined in SPL solutions by Palmieri et al. (2014). The negative control applied was a 0.01 M $CaCl_2$ solution, according Palmieri et al. (2014). A solution of 10^{-4} M of metil metanosulphonate (MMS), a genotoxic agent that induced breaks on DNA (Mauro et al., 2014) was used as positive control for *Lactuca sativa* root tips cells, while a doxorubicin (DXR) solution at 10 µg/mL, an antitumoral drug (Barcelos et al., 2009), was used as positive control for human leukocytes.

2.2. Seed germination and root growth of Lactuca sativa

We chose L. *sativa* as plant model for this study. Although L. *sativa* is not generally used as plant model in cytogenotoxic researches, it has been proven to be an efficient system for ecotoxicological studies (Aguiar et al., 2016) with fast germination (upon to 20 h) (Andrade et al., 2010) and chromosomes with great length (2,8 to 5,5 µm according to Matoba et al., 2007).

Seeds from L. *sativa* var. Veronica were placed into Petri dishes over a filter paper moistened with 3 mL of distilled water. Seeds grown in BOD at 24 °C for 16 h. After germination (upon 16 h), thirty seeds with roots of 1 mm in length were transferred to new Petri dishes containing filter paper impregnated with 3 mL of each treatment solution (Table 1). The experiment was done in triplicate (3 repetitions – Petri dish - per treatment).

After 48 h of exposure, 15 roots per treatment solution, were collected and chopped in 300 µL of 1X phosphate buffered saline (PBS) at 4 °C with a razor blade, in order to isolate the nucleus. The obtained suspension were filtered on a CellTrics (Partec®) of 50 µm in diameter. Then, 30 µL of the filtered was mixed with 30 µL of 1% low melting point agarose (v/v) at 42 °C. A drop of this mixture (agarose + nuclei) was spread with a cover slip under a slide with a thin layer of 1% normal melting point agarose. After agarose solidification, on ice, nuclei were denatured on 30 mM sodium hydroxide (NaOH) with 0.5 mM ethylenediaminetetraacetic acid (EDTA) in pH 11.8 for 10 min at room temperature (20 ± 2 °C). The slides were washed on cold TBE 1X (90 mM Tris-borato, 2 mM EDTA, pH 8.4) for 5 min (adapted of Jovtchev et al., 2001).

Table 1
Concentrations of the solutions applied in comet assay with human leukocytes and root tip cells.

Solutions	½ IC_{50}	IC_{50}[a]	$^3/_2$ IC_{50}
SPL	13.25 g/L	26.50 g/L	39.75 g/L
Aluminum	1.67×10^{-6} g/L	4.59×10^{-6} g/L	1.20×10^{-6} g/L
Fluoride	0.06×10^{-3} g/L	0.39×10^{-3} g/L	0.43×10^{-3} g/L

[a] IC_{50}: concentration responsible for the inhibition of 50% of the root growth in developing seedlings according to Palmieri et al. (2014).

2.3. Preparation of human leukocytes samples

Blood (10 mL) of six healthy volunteers 20–40 years old, male or female) were collected after signature of the consent term approved by the Research Ethics Committee from the Federal University of Lavras (COEP, UFLA, number 212). The preparation of the samples was conducted according to Marcussi et al. (2011, 2013) as brief described below.

The assays were carried out using cultures of lymphocytes in total blood and then the leukocytes were selected during slide treatment with lisys solution. The blood used in this test was obtained from 6 healthy volunteers between 20 and 35 years of age (3 male and 3 female). The blood of each volunteer corresponded to an individual experiment. Three flasks of cell culture were used for each sample/concentration evaluated. The treatment was then carried out for 4 h (considering the fases of lymphocyte cycle; 7 h in G1; 24 h in S and 48 h in G2) at 37 °C. After incubation, the cells were then used to prepare the slides. A cellular suspension containing approximately 10^6 cells/mL was used to obtain 5–10 million cells per slide; three slides were prepared for each flask of each treatment per experiment, evaluating 100 nucleoids per flask, with a total of 300 nucleoids/treatment. Approximately 30 µL of each cell culture were mixed with 100 µL of LMP (low melting point) agarose. The mixture was homogenized and put on the slide with NMP (normal melting point) agarose and covered with the coverslips until the agarose solidification. Then the slides were immersed in lyses solution [solution: 0.25 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100% and 5% dimethyl sulfoxide (DMSO), pH 10] for 12 h, to obtaintion of the nucleoids. The procedures described above were carried out in the dark.

Before the eletrophoresis, the slides were kept for 20 min at 4 °C in electrophoresis solution (300 mM NaOH and 1 mM EDTA, pH 13) for exposure of alkali-labile sites.

2.4. Electrophoresis and nuclei evaluation

The DNA breaks [mainly single-strand breaks (SSB)] were detected by alkaline denaturation followed by neutral gel electrophoresis (A/N), one of the variants of the comet assay (Menke et al., 2001).

The slides obtained from samples of L. *sativa* root tips cells (2.2 section) and human leukocytes (2.3 section) were subjected to electrophoresis on a horizontal chamber filled with the run solution (300 mM NaOH, 1 mM of EDTA at pH 13). The electrophoresis was performed at 25 V and 300 mA for 15 min (root tipscells) or 30 min (leukocytes sample). The slides with root tips were immediately dehydrated on 96% ethanol solution, while slides with leukocytes samples remained in neutralizing solution (0.4 M Tris-HCl, pH 7.4) for 25 min and then in 96% ethanol solution for DNA precipitation.

The slides were stained with propidium iodide solution (2 µg/mL), covered with a glass coverslip and evaluated under epifluorescence microscope (Olympus BX 60) at wavelength of 530–550 nm and a magnification of 400x. For plant cells, 100 nucleoids per slide and three slides per treament (one of each replicate) were counted, totalizing 300 nuclei evaluated. For the human leukocytes it was evaluated 300 nuclei