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Stimulation of DNA repair in *Saccharomyces cerevisiae* by *Ginkgo biloba* leaf extract

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ABSTRACT

Many extracts prepared from plants traditionally used for medicinal applications contain a variety of phytochemicals with antioxidant and antigenotoxic activity. In this work we measured the DNA protective effect of extracts of *Ginkgo biloba* leaves from oxidative stress using *Saccharomyces cerevisiae* as experimental model. The extract improved viability of yeast cells under oxidative stress imposed by hydrogen peroxide. In accordance with previous reports on antioxidant properties of *G. biloba* extracts, pre-incubation of yeast cells promoted a decrease in intracellular oxidation. We assessed DNA damage by our recently developed yeast comet assay protocol. Upon oxidative shock, DNA damage decreased in a dose-dependent manner in experiments of pre-incubation and simultaneous incubation with the extract, indicating a direct protective effect. In addition, the extract improved DNA repair rate following oxidative shock as measured by faster disappearance of comet tails. This suggests that the extract stimulates the DNA repair machinery in its DNA protective action in addition to directly protect DNA from oxidation. The observed DNA repair depends on the DNA repair machinery since no DNA repair was observed under restrictive conditions in a conditional mutant of the *CDC9* gene (Accession No. Z74212), encoding the DNA ligase involved in the final step of both nucleotide and base excision repair.

1. Introduction

Extracts prepared from plants traditionally used for medicinal applications have received considerable attention owing to their potential health benefits as therapeutic agents, especially for aging and age-related diseases (Zheng and Wang, 2001; Silva et al., 2005; Manach et al., 2009; Tosetti et al., 2009). Phytochemicals found in these extracts are often difficult to purify and synthesize and biological activity often depends on synergistic effects between compounds.

Ginkgo biloba leaf extracts, some of the best-selling antioxidant medicinal products worldwide, contain ginkgo flavone glycosides (quercetin, kaempferol, isorhamnetin) and ginkgo terpene lactones (ginkgolides, bilobalide), which are believed to be responsible for most of the biological properties (Wei et al., 2000; Tendi et al., 2002; Smith and Luo, 2004; van Beek, 2005; Altiok et al., 2006). Extracts from *G. biloba* leaves have been suggested by several studies to possess numerous beneficial properties, including antioxidant or free radical scavenging (Silva et al., 2005; Wei et al., 2000; Tendi et al., 2002; Altiok et al., 2006; Thiagarajan et al., 2002; Stromgaard and Nakanishi, 2004; Liu et al., 2006; Yeh et al., 2009), antiapoptotic (Wei et al., 2000; Altiok et al., 2006; Thiagarajan et al., 2002; Yeh et al., 2009; Schindowski et al., 2001; Wu et al., 2008), antiaging (Schindowski et al., 2001) and antigenotoxic (Vilar et al., 2009). They have also been described to regulate gene expression (Tendi et al., 2002; Stromgaard and Nakanishi, 2004; Gohil, 2002; Augustin et al., 2009; Bidon et al., 2009).

Reactive oxygen species (ROS) such as the superoxide radical ($O_2^{\cdot}$), hydroxyl radical ($^{\cdot}OH$) and H_2O_2, pose a significant threat to cellular integrity. In the presence of redox-active metal ions, such as Fe^{2+}, $O_2^{\cdot}$ and H_2O_2 can undergo Fenton chemistry, generating the extremely reactive $^{\cdot}OH$, which attacks almost all cell components, including DNA (Henle et al., 1996). High levels of ROS, formed through both endogenous and exogenous routes, and the DNA damage it produces contribute to genetic instability. The majority of endogenous ROS are produced through leakage of these species from the mitochondrial electron transport chain that diffuse out freely through membranes and attack other cellular components, while exogenous ROS occur through exposure to numerous exogenous agents including ionizing radiation, ultraviolet radiation, chemotherapeutic drugs, environmental toxins and hyperthermia (Salmon et al., 2004). Cells are exposed permanently to oxidative challenge imposed by the environment and by the oxidative metabolism in mitochondria. Incapacity of the cellular defences to cope with the oxidative challenge generates oxidative

Abbreviations: BER, base excision repair; GBE, *G. biloba* leaf extract; H_2DCF, dichlorofluorescein; H_2DCFDA, dichlorofluorescein diacetate; LMA, low-melting agarose; NER, nucleotide excision repair; OD_{600}, optical density at 600 nm; ROS, reactive oxygen species; rpm, revolutions per minute; SD, standard deviation.

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stress, which can cause oxidative damage in macromolecules, including genomic DNA (Boiteux and Guillet, 2004). DNA lesions such as DNA base modifications, single- and double-strand breaks, and the formation of apurinic/apyrimidinic sites may be formed (Salmon et al., 2004). Once DNA damage is sensed, cell cycle is arrested so that repair mechanisms can operate, or induction of programmed cell death may take place if damage cannot be repaired. The major DNA repair pathways are base excision repair (BER) in the removal of damage of single bases caused by oxidation and nucleotide excision repair (NER), which is involved in repairing bulky DNA lesions caused by ultra-violet light (Friedberg, 2003). A DNA ligase encoded by the mammalian *LIG1* (Accession No. NG_007395.1), orthologous of the budding yeast *CDC9*, completes the repair process for both BER and NER (Wu et al., 1999).

The antigenotoxic activity of many phytochemicals and plant extracts can be attributed to their antioxidant properties, which allow protection of DNA from oxidative damage. Despite the fact that a large number of studies concern the antioxidant properties of *G. biloba* extracts, only few have associated these extracts with antigenotoxicity (Vilar et al., 2009). We investigated viability, intracellular oxidation as well as DNA damage and repair by our recently developed protocol of comet assay applied to yeast (Azevedo et al., 2011), in cells treated with *G. biloba* extract and exposed to oxidative stress imposed by H_2O_2. Our results show that *G. biloba* leaf extract protects genomic DNA against oxidative stress and suggest that the two modes of action are present, direct protection from oxidation and stimulation of DNA repair.

2. Materials and methods

2.1. Plant material and extract preparation

G. biloba leaves were collected in autumn (October) from a specimen located in an urban area of Braga, Portugal (University of Minho in Braga, Campus map coordinates 41.559223,-8.397503). Preparation of the water extract (GBE) was performed as described elsewhere (Ding et al., 2004). Leaves were washed with deionized H_2O, cut to exclude petioles and air-dried at room temperature in the dark for one week. Dried leaves were pulverized with a pestle into a fine powder and stored in nontransparent glass bottles until used for extraction. Five grams of powder were transferred into 200 mL polypropylene centrifuge tubes with 30 mL of boiling deionized H_2O, heated in a water bath at 100 °C for 5 min and centrifuged at 2000*g* for 15 min. The extraction process was repeated once with the pellet, the supernatants were pooled, cleaned by filtration with 0.5 μm filters, adjusted to pH 6.5 with NaOH and stored in aliquots at −20 °C. The standardized *G. biloba* extract EGb 761 (kindly provided by Schwabe Pharmaceuticals, Germany) was used as benchmark in the comet assay experiments.

2.2. Yeast strains, culture media, and growth conditions

The haploid *Saccharomyces cerevisiae* strains BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (Brachmann et al., 1998), *S. cerevisiae* NK427 (*MATa ura3-52 trp1-289 leu2-3,112 bar1::LEU2 cdc9-1*) carrying a temperature-sensitive mutation in the *CDC9* gene and its reference strain, NK1 (*MATa ura3-52 trp1-289 leu2-3,112 bar1::LEU2*) (Makovets et al., 2004) were used in this work. Stocks of these strains were maintained on standard solid YPD medium (1% w/v BD Bacto™ yeast extract, 2% w/v BD Bacto™ peptone, 2% w/v glucose) with 2% (w/v) agar at room temperature. Yeast cells were grown on 50 mL liquid YPD medium in an Erlenmeyer flask with air–liquid ratio of 5/1 at 30 °C (strain BY4741) or 23 °C (for strains NK1 and NK427) and 200 rpm. Growth was monitored by optical density at 600 nm (OD_{600}).

2.3. Viability measurement

A liquid pre-culture of 5–10 mL was prepared with a single yeast colony and grown overnight. The culture was diluted with fresh medium to a density of 1.2×10^7 cells/mL and harvested by centrifugation (2 min at 5000 rpm, 4 °C) after two generations. Cells were subsequently washed twice, each time with one culture volume of ice-cold deionized H_2O, and diluted back to 1.2×10^7 cells/mL in ice-cold S buffer (1 M sorbitol, 25 mM KH_2PO_4, pH 6.5). Pre-treatment with GBE was made by addition of one fifth of a volume GBE in S buffer to the cells suspended in S buffer. Cells and GBE were incubated at 30 °C, 200 rpm for 20 min, washed with one volume of deionized H_2O at 4 °C and resuspended in an equal volume of S buffer. One hundred microlitres of this suspension was harvested, serially diluted to 10^{-4} in deionized sterilized H_2O and 100 μL were spread on solid YPD medium. Hydrogen peroxide (Merck, Germany) was immediately added to the undiluted suspension (5 mM final concentration) and incubated at 30 °C, 200 rpm. The same procedure for harvesting and plating cells was followed at different time points, all plates were incubated at 30 °C for 48 h and the colonies counted. Survival rates were calculated as percentage of colony forming units at each time point in relation to the beginning of the experiment (0 min).

2.4. Analysis of antioxidant activity

Cells were prepared in the same way as for viability measurement, except that they were diluted to a density of 1.0×10^6 cells/mL and suspended in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4), instead of S buffer. Five hundred microlitres of untreated cells were removed for autofluorescence measurement. Dichlorofluorescein diacetate (H_2DCFDA; Sigma–Aldrich) (50 μM final concentration) was added to the reminder of the cells and cell suspension was further incubated at 30 °C, 200 rpm for 1 h in the dark, washed twice with the same volume of PBS and distributed in aliquots for the different assay conditions. Cells were subsequently sediment by centrifugation at 5000 rpm, 4 °C for 2 min and suspended in GBE diluted in PBS. Samples were incubated at 30 °C, 200 rpm for 20 min, washed twice with the same volume of PBS and treated with 10 mM H_2O_2 and incubated at 30 °C, 200 rpm for 20 min. Twenty thousand cells of each sample were analyzed by flow cytometry in an Epics® XLTM cytometer (Beckman Coulter) equipped with a 15 mW argon-ion laser emitting at 488 nm. Green fluorescence was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 225 nm band-pass filter. Data were analyzed and histograms were made with the WinMDI 2.8 software.

2.5. Analysis of DNA damage

Analysis of DNA damage was performed with the comet assay as described before (Azevedo et al., 2011). Briefly, cell walls were digested with 2 mg/mL zymolyase (20,000 U/g; ImmunO™ – 20T), spheroplasts were suspended in GBE diluted in S buffer, so that osmotic protection by 1 M sorbitol is maintained, incubated at 30 °C for 20 min and collected by centrifugation at 15,300 rpm, 4 °C for 2 min. Treated spheroplasts were then washed, embedded in 1.5% (w/v) low melting agarose (LMA) at 35 °C and distributed by glass slides. Spheroplasts were then exposed to the oxidant solution (10 mM H_2O_2) for 20 min at 4 °C, washed with S buffer for 5 min and submerged in the lysing buffer (30 mM NaOH, 1 M NaCl, 0.05% w/v laurylsarcosine, 50 mM EDTA, 10 mM Tris–HCl, pH 10) for 20 min in order to lyse spheroplasts. Samples were washed with electrophoresis buffer (30 mM NaOH, 10 mM EDTA, 10 mM Tris–HCl, pH 10) for 20 min and samples were then submitted to electrophoresis in the same buffer for 10 min at 0.7 V/cm. After electrophoresis, the slides were incubated in neutralization buffer (10 mM Tris–HCl, pH 7.4) for 10 min, followed by consecutive 10 min incubations in 76% and 96% (v/v) ethanol. Then, the slides were dried at room temperature and were visualized immediately or stored at 4 °C until visualization. For visualization in a fluorescence microscope (Leica Microsystems DM fluorescence) slides were stained with GelRed (10 μg/mL; Biotium) and representative images were acquired at magnification of 400× in order to obtain at least 20 random comets per sample that were analyzed with the CometScore software for the tail length. Error bars represent variability between the mean of at least three different slides obtained from biologically independent experiments.

2.6. Analysis of DNA repair

Analysis of DNA repair was performed with the comet assay as described before (Azevedo et al., 2011). Here, treatments were all performed with spheroplasts in suspension before embedding in LMA. After an incubation of 20 min at 4 °C with 10 mM H_2O_2 (in S buffer), spheroplasts of each sample were collected by centrifugation at 15,300 rpm, 4 °C for 2 min, washed with 80 μL of S buffer and then resuspended in 80 μL of GBE diluted in S buffer (or only S buffer for the control). Samples were incubated at 37 °C for different periods of time until 20 min to allow DNA repair, collected by centrifugation at 15,300 rpm, 4 °C for 2 min and incorporated in LMA. The described procedure for the comet assay (Azevedo et al., 2011) was followed afterwards. When using the *cdc9* conditional mutant, incubation at the restrictive temperature of 37 °C (Makovets et al., 2004) for 1 h was performed before treatment with H_2O_2 to allow complete inactivation of Cdc9p.

2.7. Statistical analyses

The experiments were done at least in triplicate and results are presented as mean value ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for comparison of more than two means and Tukey's test to multiple comparisons. All asterisks indicate differences considered statistically significant: * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$, when compared to the respective control.