Measuring oxidative DNA damage and DNA repair using the Yeast Comet Assay

Flávio Azevedo, Filipe Marques, Hanna Fokt, Rui Oliveira and Björn Johansson[1]

CBMA (Centre of Molecular and Environmental Biology), Department of Biology, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal, Phone: +351 253604310, Fax: +351 253678980, email: bjorn_johansson@bio.uminho.pt

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[1] Corresponding author

Abstract

Chromosomal DNA damage can be a result of several processes and agents of endogenous or exogenous origin. These cause strand breaks or oxidized bases that lead to strand breaks, which relax the normally supercoiled genomic DNA and increase its electrophoretic mobility. The extent of DNA damage can be assessed by single cell gel electrophoresis, where the chromosomal DNA migration distance correlates with the extent of DNA damage. This technique has been used for a variety of applications with several organisms, but only a few studies have been reported for *Saccharomyces cerevisiae*. A possible reason for this absence is that low cellular DNA content could hamper visualization. Here we report an optimization of the comet assay protocol for yeast cells that is robust and sensitive enough to reproducibly detect background DNA damage and oxidative damage caused by hydrogen peroxide. DNA repair was observed and quantified as diminishing comet tail length with time after oxidative stress removal in a process well described by first order kinetics with a tail length half life of 11 minutes at 37ºC. This is to our knowledge the first quantitative measurement of DNA repair kinetics in *S. cerevisiae* by this method. We also show that diet antioxidants protect from DNA damage as shown by a threefold decrease in comet tail length. The possibility of assessment of DNA damage and repair in individual cells applied to the model organism *S. cerevisiae* creates new perspectives for studying genotoxicity and DNA repair.

Keywords: Comet assay, Yeast, *Saccharomyces cerevisiae*, Oxidative stress, DNA repair, Hydrogen peroxide, Single Cell Gel Electrophoresis

Introduction

The single cell gel electrophoresis, or comet assay, is a technique to measure DNA damage in individual cells first described in 1984 by Östling and Johanson (1984). Cells are embedded in low melting agarose on a microscope slide, lysed and an electric field is applied, leading to electrophoresis of genomic DNA, which is then visualized by staining with a fluorescent dye and observed by microscopy. DNA will migrate towards the anode producing a shape resembling a comet with a tail. DNA strand breaks introduce a relaxation in the normally supercoiled chromosomal DNA, resulting in DNA that is more mobile [Östling and Johanson, 1984]. This is observable as pronounced migration of DNA towards the anode after electrophoresis (or longer comet tails). Determination of tail length, or the percentage of DNA in the tail, is a simple way of quantifying the degree of DNA damage in an individual cell. The comet assay is in principle simple and inexpensive to perform but is also sensitive and has been used in a number of different applications, such as testing for genotoxicity, ecological monitoring, human biomonitoring [Collins, 2004]. The substitution of in-vivo assays on vertebrates for in-vitro assays, such as the comet assay, whenever possible is stipulated by the European Union chemicals policy (Registration, Evaluation and Authorisation of Chemicals - REACH) [European Parliament, 2006].

The range of applications for this method was extended to include yeast [Miloshev *et al.,* 2002] by applying it to *Saccharomyces cerevisiae* cells. Despite the great potential of this model organism, this method has only been reported a few times [Miloshev *et al.,* 2002; Lah *et al.,* 2004; Nemavarkar *et al.,* 2004; Raspor *et al.,* 2005; Rank *et al.,* 2009]. In yeasts, the amount of DNA per cell is considerably lower than for higher eukaryotes, which has been suggested to pose a problem for the application of the comet assay [Rank

et al., 2009]. On the contrary, we managed to optimize the protocol to reproducibly measure oxidative DNA damage and to quantify DNA repair. We found that yeast cells display increased comet tail lengths as an actively growing culture proceed towards stationary phase. We also quantified the protective capacity of quercetin (a main diet flavonoid), ursolic acid and aqueous extracts of *Salvia fruticosa* and *Salvia officinalis.* Quercetin [Belinha *et al.,* 2007], ursolic acid and aqueous extracts of *Salvia fruticosa* and *Salvia officinalis* [Lima *et al.,* 2005] have been reported to increase resistance to oxidative stress. Our optimized yeast comet assay proved to be a very useful instrument for studying oxidative DNA damage and repair as well as the protective effect of various natural compounds.

Materials and Methods

Yeast strain, culture and sample preparation

The yeast *Saccharomyces cerevisiae* strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) [Brachmann *et al.,* 1998] was used throughout this work. This organism was maintained on standard solid yeast extract (1% w/v), peptone (2% w/v), dextrose (2% w/v) and agar (2% w/v) medium (YPD). For experiments, the yeast cells were grown in liquid YPD medium at 30ºC using 500-mL or 50-mL Erlenmeyer flasks, with air-liquid ratio of 10:1 and agitation by a mechanical shaker at 200 revolutions per minute (rpm). Careful preparation of cells for the comet protocol was necessary for obtaining reproducible results. A liquid pre-culture of 5-10 mL was inoculated with a small amount of yeast cells and incubated overnight. Cells were then suspended in fresh medium to a density of $1.2*10^7$ cells per milliliter. The cells were harvested after two generations by centrifugation (2 min at 4500 g, 4ºC), washed twice with the same volume of ice-cold deionized water and diluted back to the same concentration in ice-cold S-buffer (1 M sorbitol, 25 mM KH_2PO_4, pH 6.5).

Viability assay

Yeast cells were serially diluted to 10^{-4} in deionized sterilized water and 100 µL were spread on solid YPD medium. Hydrogen peroxide was immediately added to the undiluted suspension (5 mM or 10 mM final concentration) and incubated at 30ºC, 200 rpm. The same procedure was followed at different time points and all plates were incubated at 30ºC for 48 h. Colonies were counted and viability was calculated as percentage of colony-forming units in relation to the untreated sample.

The yeast comet assay

Aliquots of this suspension with approximately 10^6 cells were harvested by centrifugation (2 min at 18000 g, 4ºC) and mixed with 1.5% (w/v) low melting agarose (in S buffer) containing approximately 2 mg/mL of zymolyase (20T; 20000U/g). Eighty microliters of this mixture were spread over an agarose-coated slide (slide coated with a water solution of 0.5% (w/v) normal melting agarose), covered with a cover slip and incubated for 20 min at 30ºC for cell wall enzymatic degradation, after which the cover slips were removed. All further procedures were performed in a cold room at 4ºC. Slides were incubated in lysis solution (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. The slides were rinsed three times for 20 min each in electrophoresis buffer (30mM NaOH, 10 mM EDTA, 10 mM Tris-HCl, pH 10) to remove lysis solution. 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 incubation in 76 and 96% ethanol. The slides were then air-dried and were visualized immediately or stored at 4ºC for later observation. For visualization in a fluorescence microscope the slides were stained with ethidium bromide (10 µg/mL) and 20 representative images of each slide were acquired at magnification of 400× using a Leica Microsystems DM fluorescence microscope. The images were analyzed with the help of the free edition of CometScore™ Software and the analytic parameter Tail Length (in µm) was chosen as the unit of DNA damage. In each slide, at least 20 comets were analyzed and error bars represent variability between the mean of at least three different slides obtained from biologically independent experiments.

Cell treatments for the comet assay

If the experiment involved pre-treatment with quercetin, ursolic acid or plant extracts prior to addition of the genotoxic agent on the slide, 50 µL of the natural compound or extract was placed on top of the gel-embedded spheroplasts, covered with cover slip and incubated at 30ºC for 20 min. The cover slip was removed and the slides were washed in S-buffer for 5 min.

If the experimental setup required treatment with hydrogen peroxide, about 80 uL of a hydrogen peroxide solution were placed on top of the gel-embedded spheroplasts after incubation for cell wall degradation. The solution was covered by a cover slip and incubated for 20 min at 4ºC. After this incubation the slides were washed once with S-buffer for 5 min before spheroplast lysis. For the study of DNA repair temperature dependence, the procedure was performed with 5 mM H_2O_2 and cells were incubated at 0ºC, 16ºC, 30ºC or 37ºC during 0-60 min after the washing step to allow DNA repair.

Alternatively, incubations were done with cells directly harvested from the culture (5 mL aliquots of the cell suspension in 50 mL Erlenmeyer flasks) for 20 min at 30ºC with different concentrations of the natural compounds or plant extracts, washed and subsequently incubated with a hydrogen peroxide solution for 20 min. At the end of this incubation, cells were washed, embedded in low melting agarose containing zymolyase, spread on glass slides, covered with cover slips and then incubated for 20 min to allow digestion of the cell wall by zymolyase. For background DNA damage recovery, the comet assay was applied with extended incubation of cells embedded in low melting agarose containing zymolyase to 90 min, instead with the usual 20 min.

Chemicals

All reagents were of analytical grade. Quercetin and ursolic acid were obtained from Sigma and were dissolved in DMSO 1% (v/v) at the specified concentrations. Sage water

152 extracts (*Salvia officinalis* and *Salvia fruticosa*) were a kind gift from Cristina Pereira-
153 Wilson [Lima *et al.,* 2005]
154
155

Results

To set up the experimental procedure of oxidative DNA damage induction with hydrogen peroxide, we tested viability of yeast cells under several concentrations of the toxicant. Concentrations of 5 mM and 10 mM provoked aproximately 60% survival after 20 min incubation (Fig. 1A), which were chosen to use in the comet assay. We initially applied the yeast comet assay protocol as described by Miloshev and coworkers (2002). Comet-like images were obtainable by this method but, no differences were found between the incubation with various concentrations of hydrogen peroxide and control (data not shown). We optimized the protocol mainly by increasing the low melting agarose concentration, decreasing the detergent concentration and decreasing the pH of the cell lysis and electrophoresis buffers. The optimized protocol (see material and methods) produced visually different images of comets from cells treated with several concentrations of hydrogen peroxide (Fig. 1B and C). Comets are also visible in control, presumably a consequence of the comet preparation procedure and the presence of replication forks in S phase of the cell cycle. Replication forks are equivalent to single-strand breaks in electrophoretic mobility under alkaline conditions [Olive *et al.,* 1990].

We measured DNA damage along the growth of a batch culture to see if the efficiency of the procedure varies with culture density or phase. A yeast culture was followed from OD_{600} 0.2 to approximately 4. There is a two times increase of apparent comet tail length as the culture proceeds towards stationary phase (Fig. 2). However, a portion of cells from the culture at 10^8 cells per ml (OD_{600} = 3.3) did not show the comet features, possibly due to resistance to zymolyase. This situation was not reversed even with 10 times more zymolyase than the normal protocol (data not shown). Increased resistance to cell wall-degrading enzymes has been reported for cells in stationary phase [de Nobel *et al.,* 1990],

and it is commonly found that yeast cells do not spheroplast well at higher OD. The efficiency of the procedure decreased along with culture growth and at $1.5*10^8$ cells per ml (OD_{600} = 5), only 10-20% of cells formed comets. This means that the data cannot directly be interpreted as a general increase in tail length as the culture progresses, since only comets from a subpopulation of cells are visible at any one time.

Control cells prepared for comet assay display comet-like features even without treatment with hydrogen peroxide (Fig. 2A and 2B), possibly due to initial DNA damage present, replication forks or damage induced by the handling of cells during preparation of comets. We incubated cells embedded in agarose, prior to the cell wall digestion step, at 37ºC for one hour to allow DNA repair. Comet tails obtained from these cells were almost absent (not shown) as compared with the usual tail length of 18.2±1.6 μm, suggesting that this incubation allowed the activity of the DNA repair mechanisms in the cells while embedded in the agarose. In addition, cells were also able to decrease tails from comets provoked by 5 mM hydrogen peroxide (Fig. 3) in a temperature-dependent fashion. The rate of DNA repair gradually diminished from 37ºC and is not detected at 4ºC. We observed a first order decay of comet tail length with half-lifes of 11, 18 and 54 minutes at 37ºC, 30ºC and 15 ºC, respectively (Fig. 3). For a chosen temperature, the rate of decrease in comet tail length was independent of the initial tail length (results not shown). This rate of DNA repair is in agreement with the 3-30 min half life reported previously [Olive and Banáth, 2006].

We applied the yeast comet assay to the study of the protective capacity against oxidative stress of several natural compounds and plant extracts. Cells were pre-treated with different concentrations of quercetin, ursolic acid and aqueous extracts of *Salvia fruticosa* and *Salvia officinalis* and subsequently incubated with 5 mM H_2O_2. None of the natural

206 compounds tested showed cytotoxic effects, as measured by counting colony-forming
207 units as compared to untreated cells, at any of the concentrations tested after 20 min of
208 exposition (data not shown). Since these natural compounds are only available in low
209 amounts, we added both hydrogen peroxide and protective compounds in small volumes
210 on top of the embedded cells on the microscope slide. This method has the added benefit
211 of removing initial DNA damage by incubation of the slides at 37ºC prior addition of the
212 genotoxic toxic agent. Pre-treatment with each one of these natural compounds and
213 extracts significantly reduced the genomic DNA damage in contrast to the control
214 experiments with H_2O and DMSO after the incubation with 5 mM of H_2O_2 (Fig. 4). This
215 protection against the oxidative stress is consistent with the published results for these
216 natural compounds and extracts in yeast [Belinha *et al.,* 2007] and in animal cells [Lima *et*
217 *al.,* 2005]. The mechanism of the protective action is not known.
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Discussion

In this work we present an improved protocol for the comet assay applied to yeast *Saccharomyces cerevisiae* cells, that proved to be reproducible, robust and with potential of application to a variety of research fields. This combination is interesting, since the combination of *S. cerevisiae* and the comet assay may be one of the most economically accessible techniques for genotoxicity testing in terms of consumables. The performance improvements were possibly due to the generally milder conditions of our comet protocol. In addition, increasing low melting agarose concentration could provide a more stable electrophoresis matrix for migration of damaged DNA from cells with low chromatin content.

A dose-response relationship was found between hydrogen peroxide concentration and tail length until 10mM (Fig. 1C). Comet tail length did not increase when cells were shocked with 50mM H_2O_2 (not shown), which might be a consequence of a limited capacity of the genomic DNA to unwind and migrate in an electric field. Another possible explanation is that tail formation is at least partly dependent on a catalysed process. Toxicity of hydrogen peroxide is mediated by the Fenton reaction, in which reactive oxygen species are generated by the hydrogen peroxide-mediated oxidation of Fe^{2+} [Henle and Linn, 1997; Jeon *et al*., 2002]. Recycling of Fe^{2+} from Fe^{3+} is mediated by a NADH-dependent enzymatic reaction [Henle and Linn, 1997], which could be limiting to hydrogen peroxide genotoxicity when in excess.

The modification of the comet assay method to allow cells to recover DNA damage embedded in agarose, after the toxic treatment, has been proved to be useful in the assessment of the DNA damage repair ability. Tail length varied inversely with incubation time and, at 4ºC, tail length was constant regardless of the time of incubation. The studies

of DNA repair with the comet assay have also been performed in animal cells, but a very long period of repair is needed in these experiments. In addition, complete repair is not attained because the exposition to atmospheric oxygen of animal cells is sufficient to induce oxidative stress [Collins, 1999]. To our knowledge, this is the first time the comet assay is applied to assess the DNA damage repair activity in yeast cells. Taking in consideration that yeast is a good model for such studies due to its tractability, we propose the use of the comet applied to yeast cells as a valuable approach for these and other studies involving DNA integrity monitoring.

The yeast comet assay developed in this work proved to be a useful tool in the study of genome integrity. This method is sensitive and versatile, since it can be easily adapted to assess toxicity to DNA by compounds, DNA damage, DNA repair and DNA protection by natural compounds.

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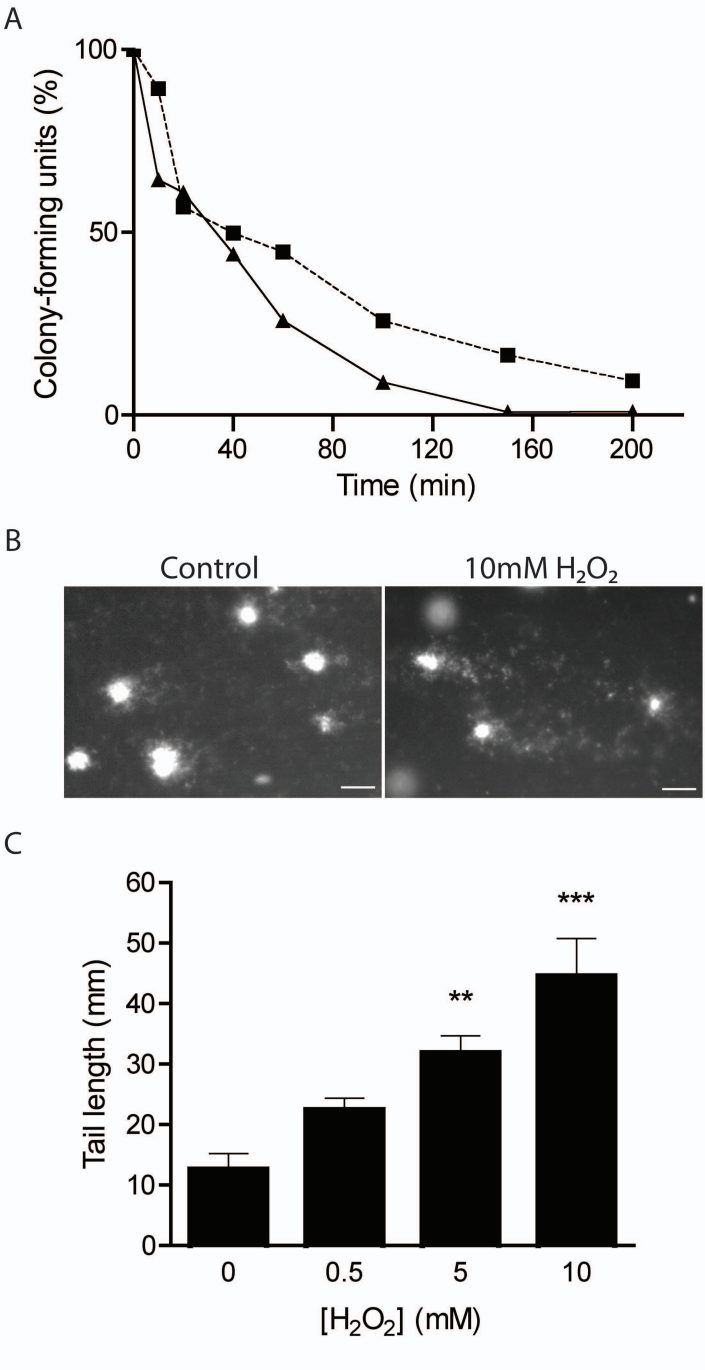
Figure 1 – Toxicity and DNA damage induced by the exposure of *S. cerevisiae* cells to H_2O_2. A: survival curves of yeast cells exposed to 5 mM (squares) and 10 mM (triangles) H_2O_2. At each time point, an aliquot of the suspension was harvested, serially diluted to 10^{-4} and 100 μL were spread on YPD agar plates. Percentage of colony-forming units was calculated taking as reference the cell suspension before addition of H_2O_2. One representative experiment is presented from at least three replicas. B: image samples obtained by the application of the yeast comet assay developed in this work in untreated cells (control) and treated with 10 mM H_2O_2. The images were acquired with fluorescence microscopy at 400x magnification. White bar corresponds to 10 μm. C: DNA damage is represented as mean (±SD) Tail Length of three independent experiments, with at least 50 comets scored per experiment for each concentration. Asterisks represent significance from control by One-way Anova test (** represents $p < 0.01$ and *** represents $p < 0.001$). The control experiment (0 mM H_2O_2) reflects the amount of DNA damage that cells have without exposure to H_2O_2 or incubation for DNA damage recovery.

Figure 2 – Cells were collected from a YPD culture at the specified culture densities. DNA damage was assessed by the yeast comet assay and tails were measured for at least 50 comets per sample (A) based on image samples acquired with a fluorescence microscope at 400x magnification (B). Arrows indicate cells, which did not form comets. Data shown corresponds to one representative experiment from three independent experiments.

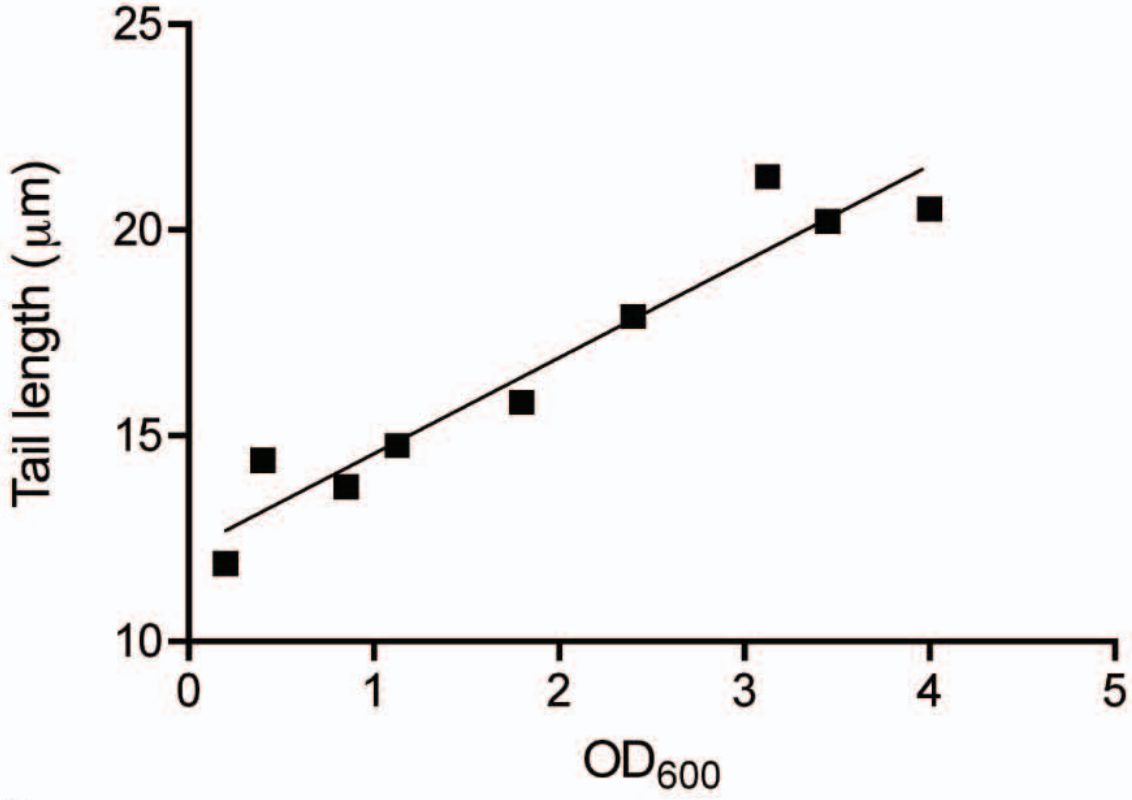
Figure 3 – Length of comet tails decrease by incubation of samples, after H_2O_2-induced DNA damage in a temperature-dependent fashion. After the incubation with 5 mM H_2O_2 for 20 min, cells were embedded in low melting agarose containing zymolyase, spread on

a glass slide, covered with a cover slip and incubated 20 min to allow digestion of the cell wall by zymolyase. After this incubation, slides were further incubated at 0ºC (circles), 16ºC (squares), 30ºC (triangles) or 37ºC (diamonds) for 0-60 min. Data represented are the average of at least three independent experiments.

Figure 4 – Cells from an exponentially growing culture were pre-treated 20 min at 30ºC with different concentrations of the natural compounds or plant extracts, washed and subsequently incubated with 5 mM H_2O_2 for 20 min. "C" represents the control with untreated cells allowed to recover background damage; "No pre-treatment" represents DNA damage due only to exposure to 5 mM H_2O_2 for 20 min; "H_2O" and "DMSO" (dimethyl sulfoxide) represent control experiments of pre-treatment with solvents of quercetin and ursolic acid (1% v/v DMSO) and *Salvia* extracts (H_2O) before exposure to 5 mM H_2O_2 for 20 min; Experiments were made with 20 min pre-treatment of quercetin (white bars), ursolic acid (grey bars) and *Salvia fruticosa* (SF) and *Salvia officinalis* (SO) extracts (dark bars) before exposure to 5 mM H_2O_2 for 20 min. Values are presented as mean (±SD) Tail Length of three independent experiments, with at least 20 comets scored per experiment for each concentration. Asterisks symbolize significance from control by One-way Anova test (*** $p < 0.001$).


A
Colony-forming units (%)
100
50
0
0
40
80
120
160
200
Time (min)
B
Control
10mM H_2O_2
C
Tail length (mm)
60
50
40
30
20
10
0
**
***
0
0.5
5
10
[H_2O_2] (mM)

A


25
20
15
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Tail length (μm)
0
1
2
3
4
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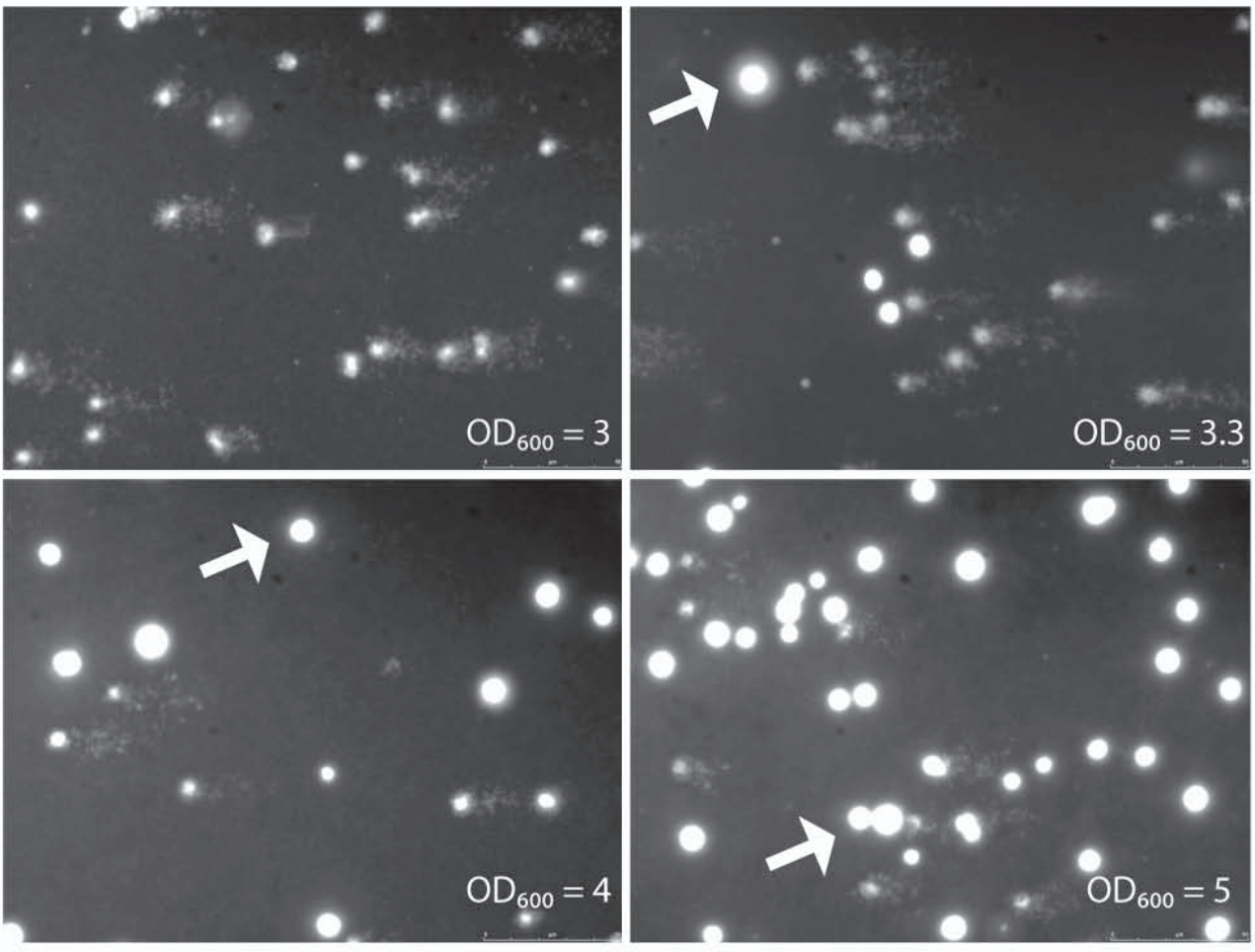
OD600

B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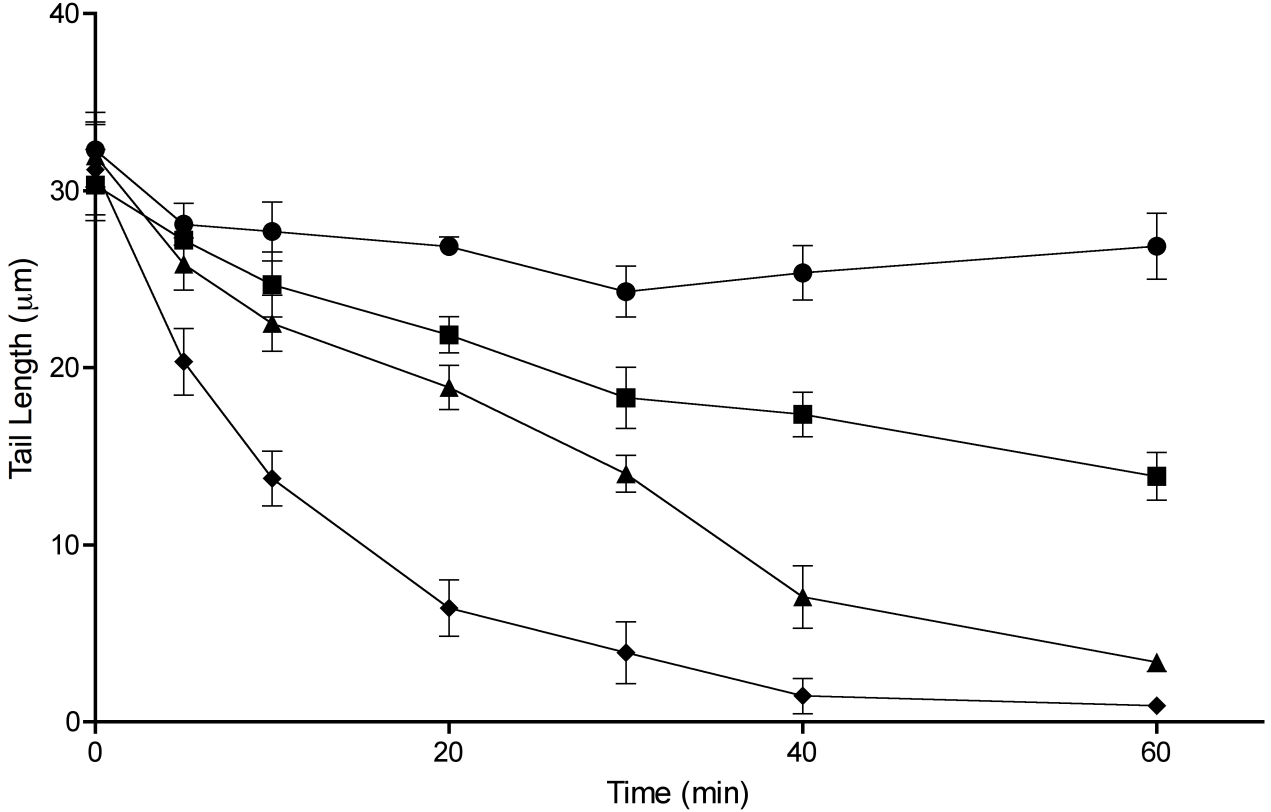
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30
20
10
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Tail Length (μm)
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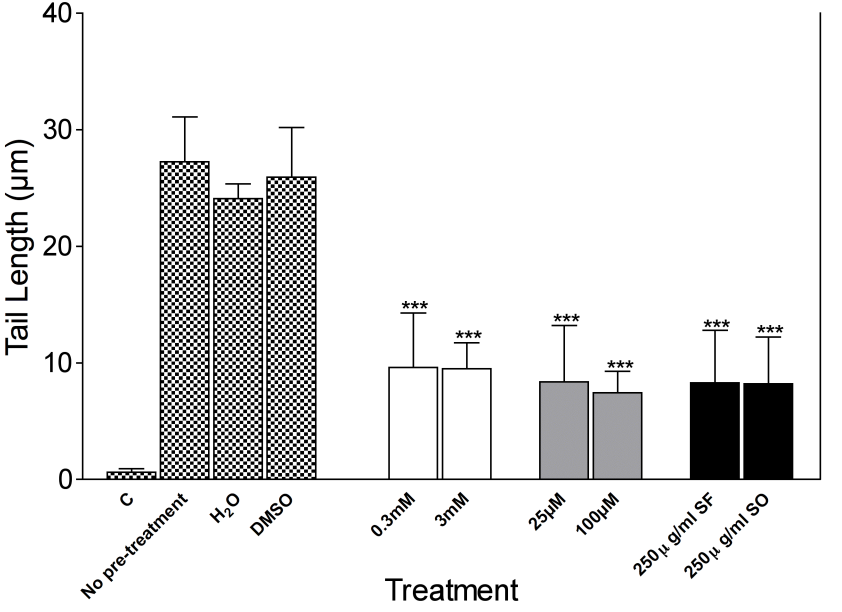
60
Time (min)


40
30
20
10
0
Tail Length (µm)
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***
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***
***
C
No pre-treatment
H2O
DMSO
0.3mM
3mM
25µM
100µM
250µg/ml SF
250µg/ml SO
Treatment