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The *Ginkgo biloba* extract EGb761 reduces stress sensitivity, ROS accumulation and expression of catalase and glutathione *S*-transferase 4 in *Caenorhabditis elegans*

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Abstract

The standardised extract EGb761 from the leaves of *Ginkgo biloba* is a popular herbal dietary supplement and it is used as a phytopharmacon for the therapy of diverse cerebral insufficiencies. The beneficial impact of EGb761 is believed to be conferred by diverse biological actions under physiological conditions as well as in response to stress. In this study we examined effects of EGb761 in the model organism *Caenorhabditis elegans*. EGb761 reduced the body size but did not affect the reproduction of *C. elegans*. In fluorescence-based assays performed in microtiter plates we demonstrated the protective action of EGb761 by the increase of resistance to thermal stress and the attenuation of ROS accumulation under conditions of thermal stress in single living worms. Under normal conditions the lifespan of the worms was extended by the EGb761 supporting the beneficial effects found under stress conditions. In a reporter gene approach using individual living worms the expression of the stress-inducible glutathione *S*-transferase 4 was shown to be reduced by EGb761 under physiological conditions as well as under oxidative stress. EGb761 also led to a decrease in transcription of the stress-inducible catalase genes. These results suggest that the beneficial impact of EGb791 on resistance to thermal stress and lifespan in *C. elegans* is at least partially due to its ability to relieve oxidative stress.
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Keywords: Catalase; *Caenorhabditis elegans*; EGb761; Glutathione *S*-transferase 4; Stress

1. Introduction

Reactive oxygen species (ROS) are produced by the incomplete reduction of oxygen at the respiratory chain in the mitochondria and as side-products of diverse normal metabolic reactions in the cytoplasm, in the endoplasmic reticulum, in the plasma membrane and in peroxisomes [1,2]. Additionally ROS are also generated by a variety of environmental threats like drugs, metal ions, radiation, UV and heat [3–6]. Since ROS can react with and damage physiologically important cellular macromolecules all aerobic organisms have evolved antioxidative enzymatic and non-enzymatic defence mechanisms to decompose ROS. Oxidative stress occurs when the balance between ROS production and antioxidant defences is shifted to the prooxidative side and in consequence of this cellular proteins, lipids and DNA are modified [7]. Oxidative stress is regarded as an important aspect in the pathophysiology of various diseases and in ageing [8]. In diseases like atherosclerosis and rheumatoid arthritis the excess of ROS causes or boosts the involved inflammation. The development of mutations and chromosomal aberrations by oxidative damage of genomic DNA is considered as a substantial factor of tumour initiation. Furthermore, oxidative stress is assumed to play an important role in neurodegenerative diseases like Alzheimer's and Parkinson's disease by the generation of pathological protein aggregates [9–16]. Ageing is also at least partially caused by oxidative stress. According to Harman's "free-radical theory of ageing" ROS produce a pattern of cumulative damage of cellular macromolecules and the impairment of mitochondria function with age leads to a decrease in cellular energy production [17]. These changes result in a proceeding loss of cellular function which is regarded as a main reason for ageing [18,19]. In this regard ROS can be classified as toxic agents. On the other hand, ROS are necessary to maintain the cellular redox homeostasis, to control cell proliferation, and to trigger the activation of specific signalling pathways as intracellular messenger molecules [7,20].

The standardised extract of *Ginkgo biloba* leafs EGb761 is used as a popular dietary supplement to enhance blood

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circulation and cognitive functions and as a phytopharmacon to treat cerebral insufficiencies including Alzheimer's disease [21–23]. Among other pharmacological effects, EGb761 has antioxidant properties due to direct radical scavenging, increased expression of antioxidant enzymes and/or modulation effects on different signalling pathways [reviewed in 21,24,25]. In *Caenorhabditis elegans* EGb761 acts protectively on stress resistance and ROS accumulation and prolongs lifespan [22,23]. This nematodic organism is increasingly used as a model to study a variety of biological processes. Many key discoveries with relevance for mammals were made in *C. elegans* since there is a strong conservation in molecular and cellular processes between worms and mammals and *C. elegans* homologues have been identified for 60–80% of human genes [26,27]. *C. elegans* is a multicellular organism with a short lifespan, high reproductivity and transparent appearance that is easy to culture and to manipulate and therefore allows various experimental approaches. Assays can be carried out in microtiter plate formats either on solid or in liquid medium offering the possibility to perform experiments with high throughput [26,28]. In contrast to *in vitro* cellular models these characteristics enable the timesaving investigation of effects of toxicological and/or pharmacological substances in a multicellular organism and *C. elegans* a suited model for such studies.

In this paper we present assays that make use of these advantageous characteristics of *C. elegans* to examine the protective potential and the mode of action of EGb761. The impact of EGb761 on the survival of the worms under conditions of lethal thermal stress was examined with an assay using the fluorescent dye SYTOX Green® to determine the points of death in an automated and very precise way [29]. Since thermal stress leads to increased production of ROS we monitored the accumulation of ROS in individual living worms stressed by heat to investigate the effect of EGb761 on the ROS status of the worms. The expression of the stress-inducible glutathione *S*-transferase 4 (*gst*-4) and catalase genes was taken as a marker for oxidative stress. The influence of EGb761 on *gst*-4 expression in individual worms was examined in a reporter gene assay whereas the impact on catalase expression was explored by Northern blot analysis. Furthermore, we monitored whether EGb761 affects reproduction capacity and body size.

2. Material and methods

2.1. Materials

The standardised *G. biloba* extract EGb761 was provided as a gift by Schwabe Pharmaceuticals (Karlsruhe, Germany). All other chemicals were of analytical grade and were purchased from Sigma (Deisenhofen, Germany), Riedel de Haen (Seelze, Germany) or Merck (Darmstadt, Germany) unless stated otherwise.

2.2. Strains, culture and EGb761 treatment of C. elegans

The wild type *C. elegans* strain N2 (var. Bristol) was obtained from the *Caenorhabditis* Genetics Centre (University of Minnesota). The generation of the transgenic strain BL1 is reported elsewhere [30]. Both strains were routinely propagated at 20 °C on Nematode Growth Medium (NGM) plates with *Escherichia coli* strain OP50 as a food source as previously described [31]. Synchronisation of worm culture was achieved by treating gravid hermaphrodites with bleach (50% sodium hypochlorite [12% Cl]; 2.5 M sodium hydroxide) and recovering the hatched L1 larvae on NGM/OP50 plates [31]. If not stated otherwise, the treatment of worms with EGb761 was performed according to the following procedure. Three days after synchronisation the worms (L4—young adults) were transferred to liquid NGM [31] with 10 μl of a freshly grown OP50 culture containing 100 $\mu g\,ml^{-1}$ EGb761 (stock: 100 $mg\,ml^{-1}$ in DMSO) or an appropriate volume of DMSO as solvent control and incubated for 48 h at 20 °C.

2.3. Thermotolerance assay

The survival of the worms at the lethal temperature 37 °C was determined in a semi-automated assay according to Gill et al. [29] with some modifications. After EGb761 treatment for 48 h the wild type N2 worms were washed in M9-Gel (M9 buffer/0.001% gelatine; [31]) and than individually transferred into 1 μl M9-Gel to the wells of a 384-well microtiter plate (Greiner Bio-One, Frickenhausen, Germany, #788096) containing 9 μl M9-Gel with 1×10^7 OP50 bacteria ml^{-1}. Subsequent to the complete transfer of the worms 10 μl 2 μM SYTOX® Green Nucleic Acid Stain (Molecular Probes Inc., Leiden, The Netherlands) in M9 buffer was added to the wells and the plate was sealed using BackSeal-96/384 Black (Perkin-Elmer, Wellesley, USA, #6005189) to avoid evaporation. The fluorescent dye SYTOX® Green Nucleic Acid Stain is not membrane-permeable and can only enter cells with compromised plasma membranes. After binding to DNA it exerts bright fluorescence so that the fluorescence intensity can be used as a marker for cellular damage and thus for the viability of the worms [29]. The thermotolerance assay was performed in a Wallace Victor^2 1420 multilabel counter (Perkin-Elmer, Wellesley, USA) preheated to 37 °C. The fluorescence from each well was measured every 30 min through the transparent bottom of the microtiter plate for a minimum of 12 h, with a 0.2 s integration time for each well. The excitation wavelength was set to 485 nm and the emission wavelength was set to 535 nm. Individual fluorescence curves were calculated and the cut off value for every single well was determined by multiplying the average fluorescence of the first four measurements by a factor of 3. The time when the measured fluorescence exceeded the cut off value for the well was defined as the point of death of the corresponding worm. The factor 3 in the calculation of the cut off value was shown to be suited by Gill et al. [29] and we verified it by touch-provoked determination of points of death in an experiment performed under identical conditions (data not shown). The individual times of death were used to assess survival curves as well as median and maximum lifespan.

2.4. Assessment of lifespan

Three days after synchronisation wild type N2 worms were transferred to liquid NGM with OP50 bacteria containing 100 μg ml^{-1} EGb761 or an appropriate volume of DMSO as solvent control and kept at 20 °C. Survival of the worms was observed daily by touch-provoked movement [32]. Worms that reacted to a mechanical stimulus were scored as alive and transferred into fresh medium to avoid confounding of generation and bacterial contamination.

2.5. TEAC assay

The TROLOX equivalent antioxidative capacity (TEAC) was measured spectrophotometrically analysing the decolourisation of a stable radical cation 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) in comparison to the synthetic Vitamin E analogue TROLOX which was used as an antioxidant standard. Absorption was measured 3 min after mixing DMSO, TROLOX or EGb761 with the ABTS solution.

2.6. Measurement of reactive oxygen species (ROS)

After EGb761 treatment for 48 h wild type N2 worms were washed in PBST (1× PBS/0.1% Tween20) and than individually transferred into 1 μl PBST to the wells of a 384-well microtiter plate (Greiner Bio-One, Frickenhausen, Germany, #788096) containing 9 μl PBS. Subsequent to the complete transfer of the worms 10 μl 100 μM 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes Inc., Leiden, The Netherlands) in PBS was added to the wells and the plate was sealed using BackSeal-96/384 Black (Perkin-Elmer, Monza, Italy, #6005189) to avoid evaporation. H_2DCF-DA is a membrane-permeable substance which can enter the cells of the worm and is intracellularly converted to H_2DCFs. This nonfluorescent probe can be oxidised by ROS to yield the fluorescent dye DCF. The changes of fluorescence in single worms with time indicate the individual accumulation of ROS. The measurement was performed in a Wallace Victor^2 1420 multilabel counter (Perkin-Elmer, Monza, Italy) preheated to 37 °C. The fluorescence from each well was measured every 20 min through the transparent bottom of the microtiter plate for a minimum of 12 h, with a 1 s integration time for each well (excitation wavelength, 485 nm; emission wavelength, 535 nm).

2.7. GST-4 reporter gene assay with the transgenic C. elegans strain BL1

After EGb761 treatment for 48 h transgenic BL1 worms [30] were incubated for another 24 h in liquid NGM containing either 100 μg ml^{-1} EGb761 or 100 μg ml^{-1} EGb761 and 20 μM juglone. The intracellular redox cycler juglone enters the cells, is reduced with NAD(P)H by a diaphorase and reduces oxygen to superoxide anion so that it generates an intracellular oxidative stress. The control worms (DMSO) were treated either in an appropriate volume of DMSO alone or in combination with 20 μM juglone. Worms of the transgenic *C. elegans* strain BL1 contain a construct consisting of the promoter and coding region of *C. elegans gst*-4 (glutathione *S*-transferase; Accession No. NM069447) translationally fused to GFP as an extrachromosomal array [30]. Since *gst*-4 is inducible by ROS [30] the intensity of appearing fluorescence was taken as a marker for oxidative stress in the living worms. After the treatment living worms were placed on microscope slides, capped with coverslips and epifluorescence images were collected from a Axiolab fluorescence microscope (Zeiss, Göttingen, Germany) using a 100× magnification with a CoolSnap CF Digital Monochrome Camera (Intas, Göttingen, Germany) equipped with Image ProPlus software (version 4.5, MediaCybernetics, Silver Spring, MD, USA). The fluorescence in the area of the pharynx was densitometrically determined.

2.8. Northern blot analysis

After EGb761 treatment for 48 h total RNA was isolated from wild type N2 worms using TRIzol® reagent (Invitrogen, Karlsruhe, Germany). Gel electrophoresis and transfer of the total RNA to Hybond N nylon membrane (Amersham Pharmacia Biotech, Little Chalfont, England) was performed as described elsewhere [33]. The hybridisation probes were generated by RT-PCR (Promega, Mannheim, Germany) according to the instructions of the manufacturer using primer pairs directed against *ctl*-1 (Accession No. U55384) and *ctl*-2 (Accession No. X82175) as well as against *gpd*-1 (Accession No. X52674) and *gpd*-4 (Accession No. X52673) as internal control (5′ctl-1/2: 5′-gcc aaa cga tcc atc gga ta-3′, 3′ctl-1/2: 5′-gtc atg ggt gac ctc gaa g-3′, 5′gpd-1/4: 5′-ggt ttc gga aga atc gga cg-3′, 3′gpd-1/4: 5′-gcg atg tat ccg atg agg tc-3′). Since *C. elegans* have more than one gene coding for catalases and glyceraldehyde-3-phosphate dehydrogenases with a high level of nucleotide sequence identity it was not possible to create gene specific primers. The PCR products were separated by gel electrophoresis, eluted from the gel [33] and labelled using the Random Primed Labeling Kit (Boehringer, Mannheim, Germany) and α-P^{32}-dCTP (Hartmann Analytic, Braunschweig, Germany). Nucleotides not incorporated were removed by using Micro Spin G-50 columns (Amersham Pharmacia Biotech, Little Chalfont, England). Hybridisation and washing was performed according to standard procedures [33]. Subsequent to a 24 h exposure signal intensity was determined with Typhoon 8600 variable mode imager (Amersham Pharmacia Biotech, Little Chalfont, England) and analysed densitometrically.

2.9. Body size of C. elegans

After EGb761 treatment for 48 h the size of wild type N2 worms was determined by microscopy (Axiolab fluorescence microscope, Zeiss, Göttingen, Germany) with an ocular scale.

2.10. Reproduction of C. elegans

Immediately after synchronisation developing larvae of the wild type strain N2 were incubated with 100 μg ml^{-1} EGb761 or an appropriate volume DMSO (as solvent control) in liquid

NGM at 20 °C in the presence of OP50 bacteria. Three days after synchronisation the worms were individually transferred to wells of microtiter plates containing EGb761 or DMSO in liquid NGM with OP50 bacteria. On a daily basis the worms were moved to a new well containing fresh medium and 2 days after this transfer the offspring in the original well was counted. This procedure was continued until the parental worms were dead or stopped producing progeny.

2.11. Statistical analysis

The significance of differences in the EGb761 treated populations and the controls was determined either by unpaired *t*-tests for the comparison of mean values or by Log Rank tests using SPSS 12.0.1 software (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $p < 0.05$ or $p < 0.01$ (see figures).

3. Results

3.1. Effect of EGb761 on resistance against lethal thermal stress and on the lifespan of C. elegans

To study the protective properties of EGb761 we heated the worms to 37 °C to generate a thermal stress that is lethal for the worms within a few hours and determined the points of death with an assay first described by Gill et al. [29]. This assay revealed that worms pre-treated with EGb761 were more resistant against the lethal thermal stress than untreated control worms. The survival curves calculated from the points of death of individual worms presented in Fig. 1 show that almost all worms of both populations survived the thermal stress for the first 5 h. At all later time points the percentage of worms alive was clearly higher in the EGb761 treated population demonstrating a higher resistance against the thermal stress. The EGb761 treatment prolonged the mean, median and maximal lifespans by approximately 12%, 8% and 19%, respectively (Table 1).

EGb761 did not only confer an increased resistance against an acute stress but also prolonged the lifespan of worms cultured under normal growth conditions. After 17 days of adulthood

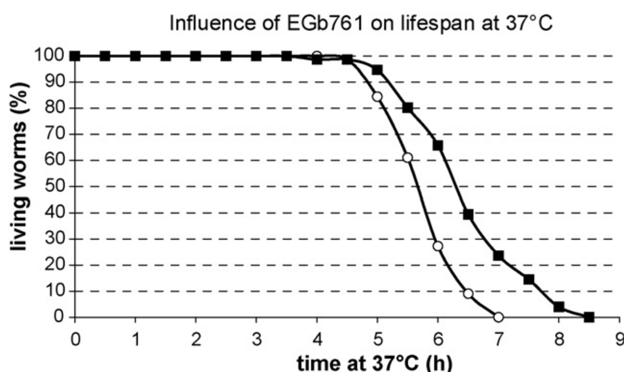

Fig. 1. The effect of EGb761 on the lifespan of *Caenorhabditis elegans* at 37 °C. Three days after hatching age-synchronised *C. elegans* worms were incubated for 48 h with 100 μg ml^{-1} EGb761 (black squares) or an adequate volume of DMSO (white circles) in liquid culture medium. The point of death at the lethal temperature of 37 °C for 80 individual worms of both treatment populations was determined in three independent experiments by the increase of fluorescence generated by a fluorescent dye (SYTOX® Green) in a 384-well plate. The survival curves represent the percentage of worms that were considered to be alive at the given time points. Statistical significance ($p < 0.01$) of the difference between the curves was demonstrated by Log Rank test.

until the end of the experiment the percentage of living worms of the population permanently incubated with EGb761 was higher than that of the control population (Fig. 2). The treatment increased the mean lifespan by roughly 10%, the median lifespan by 16% and the maximal lifespan by about 17% (Table 2).

3.2. Effect of EGb761 on ROS accumulation and oxidative stress in C. elegans

Since the toxicity of thermal stress as well as the process of ageing is associated with damage caused by oxidative stress [7,17–19] we investigated the antioxidative capacity of EGb761 and the effect of EGb761 on ROS accumulation in individual worms under conditions of thermal stress (37 °C). The antioxidative capacity of 10 μg ml^{-1} EGb761 is comparable with 10 μM of the strong antioxidant TROLOX which is a synthetic Vitamin E analogue and with the concentration of EGb761 used for the experiments in this study of 100 μg ml^{-1} all radicals were immediately scavenged by EGb761 (Fig. 3). Thermal stress

Table 1
Influence of EGb761 on life span at 37 °C

Trial	Treatment	Adult life span, 37 °C (h)			*n*	*p* vs. control (Log Rank)
		Mean, S.E.	Median, S.E.	Maximal, S.E.M.		
I	Control	5.88, 0.15	5.50, 0.20	7.00	16	
	EGb761	7.33, 0.21	7.50, 0.26	8.50	16	0.0000
II	Control	6.00, 0.10	6.00, 0.07	7.00	32	
	EGb761	6.34, 0.18	6.50, 0.26	8.00	32	0.0325
III	Control	5.83, 0.12	5.50, 0.20	7.00	32	
	EGb761	6.48, 0.14	6.50, 0.09	8.50	32	0.0110
Combined	Control	5.90, 0.07	6.00, 0.08	7.00, 0.00	80	
	EGb761	6.60, 0.11	6.50, 0.11	8.33, 0.29*	80	0.0000

For experimental details see Fig. 1. S.E., standard error; S.E.M., standard error of the mean; *, $p < 0.05$ (unpaired *t*-test, means of the maximal adult life spans).

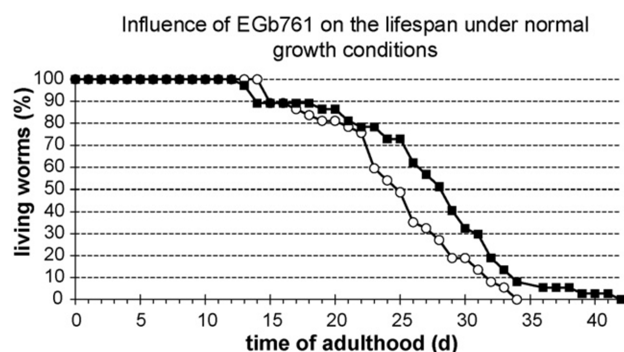

Fig. 2. The effect of EGb761 on the lifespan of *C. elegans* under normal growth conditions. Three days after hatching 40 age-synchronised worms were incubated in liquid medium either containing 100 μg ml^{-1} EGb761 (black squares) or an appropriate volume of DMSO (white circles) in four independent experiments. The survival of the worms was monitored daily and worms that did not show any movement in response to a mechanical stimulus were considered to be dead. During the reproductive phase the worms were regularly transferred to new plates to avoid mixing of different generations. The survival curves represent the percentage of worms that were alive at the given time points. Statistical significance ($p<0.05$) of the difference between the curves was demonstrated by Log Rank test.

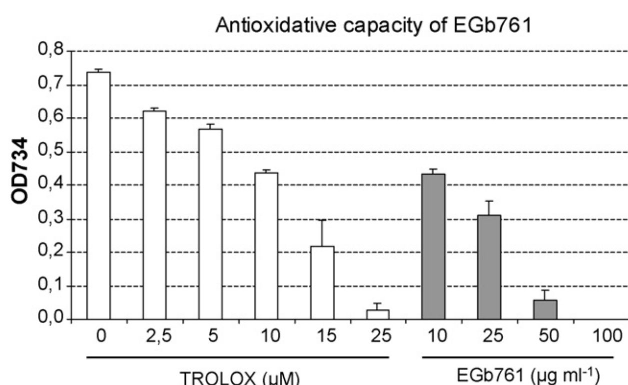

Fig. 3. The antioxidative capacity of EGb761. The TROLOX equivalent antioxidative capacity (TEAC) of different concentrations of EGb761 was measured photometrically at 734 nm 3 min after mixing with ABTS-radical solution in six independent experiments. The synthetic Vitamin E analogue TROLOX was used as an antioxidant standard.

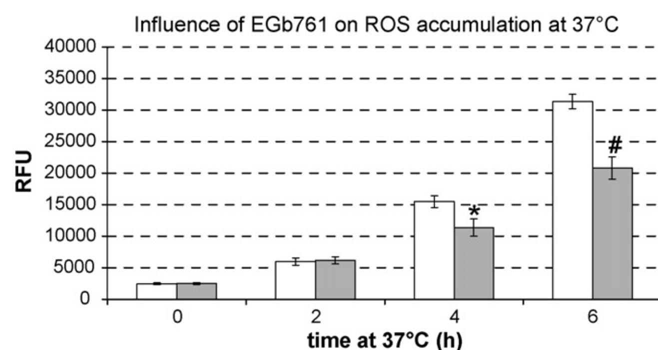

Fig. 4. The effect of EGb761 on ROS accumulation at 37 °C. Three days after hatching age-synchronised *C. elegans* worms were incubated for 48 h with 100 μg ml^{-1} EGb761 (grey columns) and an adequate volume of DMSO (white columns) in liquid culture medium. Individual worms of each population were transferred into the wells of a 384-well plate and 50 μM H_2DCF-DA (final concentration) was added. The measurement was carried out at 37 °C. The results are presented as means ± S.E.M. of relative fluorescence units (RFU) of 22–28 individual worms. Statistical significance ($^{*}p<0.05$; $^{\#}p<0.01$) was demonstrated by the *t*-test.

resulted in a time-dependent increase of fluorescence intensity and thus of the amount of ROS in both *C. elegans* populations but in the EGb761 pre-treated worms the fluorescence rose to a lower extent (Fig. 4). EGb761 treatment reduced the fluorescence intensity after 4 and 6 h of thermal stress by 27% and 34%, respectively.

In another approach to investigate the effect of EGb761 on oxidative stress we performed reporter gene assays using transgenic *C. elegans* strain BL1 [30]. Since the reporter construct in these worms consists of *gst*-4 that is inducible by oxidative stress [30] and GFP the fluorescence intensity was used as a marker for the level of oxidative stress. To induce ROS production and intracellular oxidative stress the redox cycler juglone was administered to the worms. In Fig. 5A microscopical pictures of worms with and without juglone challenge are presented and Fig. 5B shows the dramatic increase of fluorescence in the pharynx area of worms treated with juglone. The pre-treatment with EGb761 resulted in a reduction of fluorescence intensity in worms kept under normal growth conditions as well as in worms

Table 2
Influence of EGb761 on adult life span under normal growth conditions

Trial	Treatment	Adult life span, 20 °C (day)			n	p vs. control (Log Rank)
		Mean, S.E.	Median, S.E.	Maximal, S.E.M.		
I	Control	23.0, 1.7	25.0, 2.1	29.0	10	
	EGb761	27.3, 2.1	29.0, 3.1	35.0	10	0.0484
II	Control	25.6, 2.2	23.0, 2.3	34.0	10	
	EGb761	29.9, 1.4	30.0, 1.5	38.0	10	0.5022
III	Control	26.5, 1.4	26.0, 1.1	32.0	10	
	EGb761	27.1, 2.7	28.0, 2.4	41.0	10	0.2818
IV	Control	25.4, 1.2	25.0, 0.9	31.0	10	
	EGb761	26.1, 2.1	26.0, 2.6	34.0	10	0.2415
Combined	Control	25.2, 0.9	25.0, 0.7	31.5, 1.0	40	
	EGb761	27.6, 1.1	29.0, 1.0	37.0, 1.3*	40	0.0373

For experimental details see Fig. 2. S.E., standard error; S.E.M., standard error of the mean; *, $p<0.05$ (unpaired *t*-test, means of the maximal adult life spans).

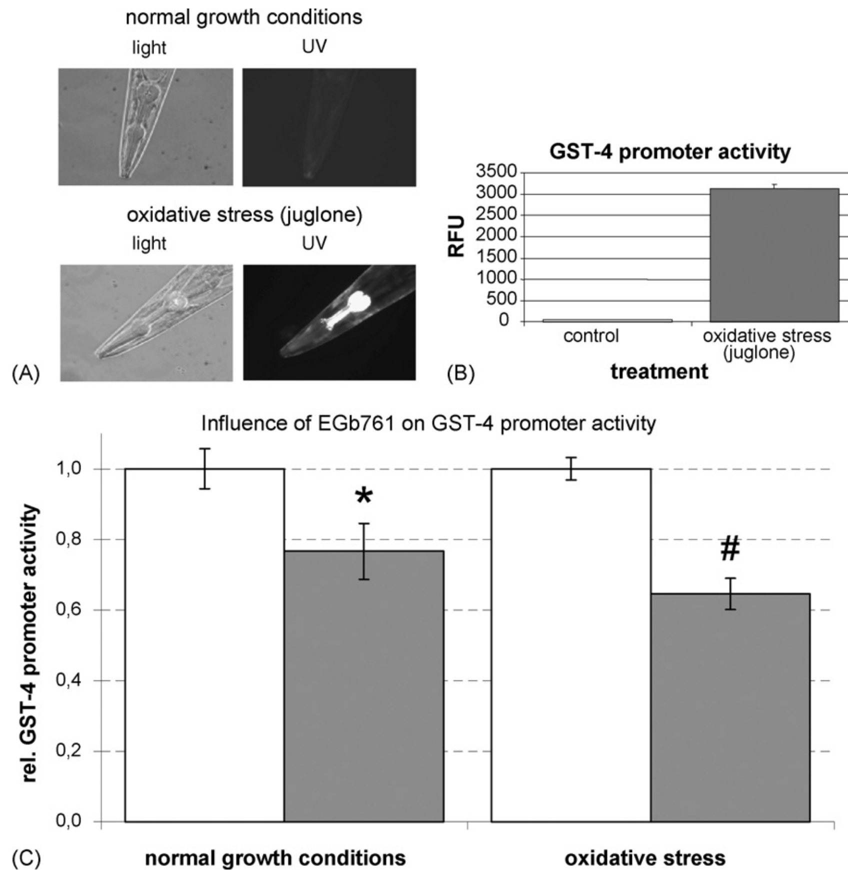

Fig. 5. The effect of EGb761 on the activity of the GST-4 promoter. (A) Examples of light and UV microscopic pictures of worms cultured under normal growth conditions and challenged with 20 μM juglone for 16 h to generate oxidative stress. (B) Densitometrical analysis of the fluorescence in the pharynx of control worms and worms challenged with 20 μM juglone for 16 h. (C) Three days after hatching age-synchronised worms were incubated for 48 h with 100 μg ml^{-1} EGb761 (grey columns) or an adequate volume of DMSO (white columns) in liquid culture medium. Half of the two populations were additionally challenged with 20 μM juglone for 16 h to generate oxidative stress. The fluorescence intensity in the pharynx of 15–20 worms of each group in two independent experiments was determined by densitometrical analysis. The absolute fluorescence intensity under normal growth conditions was: control, 47 ± 3 RFU; EGb761, 36 ± 4 RFU (RFU, relative fluorescence units), and under conditions of oxidative stress: control, 3136 ± 101 RFU; EGb761, 2023 ± 141 RFU. The results are presented as means ± S.E.M. of the relations to the control (set to 1). Statistical significance ($^{*}p < 0.05$; $^{\#}p < 0.01$) was demonstrated by the *t*-test.

exposed to intracellular oxidative stress to 77% and 65% of the control fluorescence, respectively (Fig. 5C). This means that the activity of the *gst*-4 promoter was decreased by EGb761 suggesting that EGb761 had lowered the level of oxidative stress in *C. elegans*.

3.3. Effect of EGb761 on catalase transcripts in C. elegans

Catalase detoxifies the reactive oxygen species H_2O_2 and therefore it acts as an antioxidant enzyme [reviewed in 34] and it is inducible by oxidative stress in *C. elegans* as well as in mammals [35–37]. In *C. elegans* at least two catalases exist, CTL-1 localised in the cytosol and CTL-2 localised in the peroxisomes, revealing a high degree of sequence identity [38].

Because of this sequence identity and the very similar length of the two transcripts it was not possible to design transcript specific hybridisation probes for a Northern blot analysis and to distinguish between the hybridisation signals of the catalase transcripts. The Northern blot analysis revealed a reduction of steady state catalase transcripts in the EGb761 treated worms by 32% compared to the control (Fig. 6).

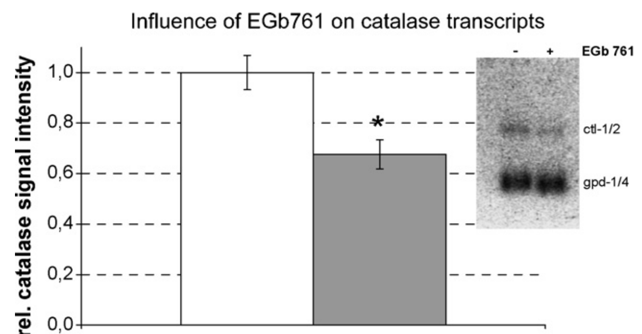

Fig. 6. Influence of EGb761 on catalase transcripts. Total RNA from 5-day-old worms pre-treated for 48 h with EGb761 (grey column) and from DMSO treated control worms (white column) was used to perform Northern blot analysis with probes directed against the transcripts of *ctl*-1/2 and *gpd*-1/4 as an internal standard. An typical hybridisation is shown in the insert. The intensity of the *ctl*-1/2 signal was normalised by relating it to the intensity of the *gpd*-1/4 signal and the normalised *ctl*-1/2 signal intensity from the EGb761 treated worms was set in relation to the signal intensity of the control (set to 1). The results are presented as means ± S.E.M. of the normalised relations from seven independent experiments. Statistical significance ($^{*}p < 0.05$) was demonstrated by the *t*-test.

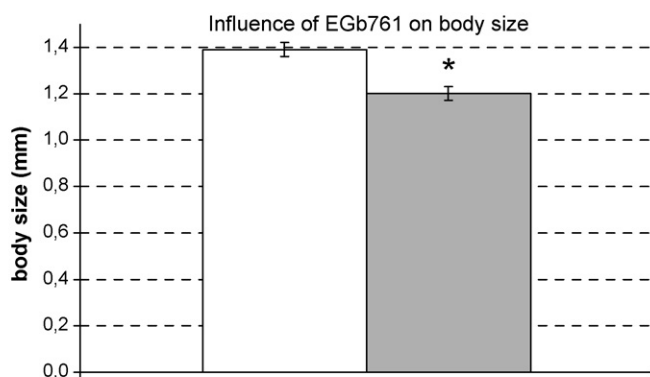

Fig. 7. The effect of EGb761 on the body size of *C. elegans*. Three days after hatching 32 age-synchronised *C. elegans* worms were incubated for 48 h either with 100 μg ml^{-1} EGb761 (grey column) or an adequate volume of DMSO (white column) in liquid culture medium in three independent experiments. Body size was determined by using an ocular scale during microscopy. The results are presented as the means ± S.E.M. of the body sizes. Statistical significance ($^{*}p < 0.05$) was demonstrated by the *t*-test.

3.4. Effect of EGb761 on body size and egg laying capacity of C. elegans

During the experiments we got the impression that the EGb761 treated worms were smaller in size than the control worms. Therefore we measured the body size of worms incubated with EGb761 in comparison to control worms. EGb761 treatment led to a reduction of the worm's body size of approximately 17% (Fig. 7).

We could not find any effect of EGb761 on the number of offspring per day, on the beginning of reproduction period and on the total number of offspring (data not shown) so that we conclude that EGb761 does not influence the reproduction of *C. elegans*.

4. Discussion

The standardised extract EGb761 from the leaves of *G. biloba* is used as a dietary supplement to prevent atherosclerosis and cognitive dysfunctions and as a phytopharmacon for the therapy of dementia. In our study the treatment of the worms with EGb761 increased the mean, median and maximal survival of wild type worms challenged with a lethal thermal stress (Fig. 1, Table 1) and therefore it acts as protectively by enhancing the thermal resistance of *C. elegans*. The EGb761 treatment did not only increase the resistance against an acute stress but also prolonged the mean, median and maximal lifespan of the worms (Fig. 2, Table 2). These results are consistent with data from a previous study by Wu et al. [22].

The use of the fluorescent dye SYTOX Green® for the determination of the points of death of individual worms has some advantages compared to the "classical" way to distinguish living from dead worms by touch-provoked movement. Because of the automated fluorescence measurement the determination of the points of death it is (i) more precise since the observation/measurement intervals can be chosen to be shorter, (ii) less time and work consuming and (iii) not prone to variation in the rating by different experimentators. The possibility to perform the assay in a 384-well format provide the opportunity to achieve a higher throughput. Nevertheless it needs to be stated that the output of this assay were "virtual" points of death because they were determined by the rise of the fluorescence above a certain cut off value defined for every single well. Nevertheless, the increase of fluorescence was taken as a marker for the cumulative damage of the worms and since the aim of the assay was to obtain comparative information about the damage caused by thermal stress the results of the experiments reliably demonstrated the protective effect of EGb761. Furthermore, a comparison of the classical method to determine the points of death by mechanical stimuli and the fluorescence based way revealed very similar results in the survival rate of the worms (data not shown). The convenience of this method was also verified in the study initially describing this assay [29].

In *C. elegans* there is a good correlation between stress resistance and lifespan since most genetic mutants with a longevity phenotype have increased resistance to a variety of acute stressors like oxidative stress [39,40] and heat [32]. For this reason acute stressors have been successfully used as surrogates to identify new gerontogenes in genetic screens [41–43] and it was discussed whether such a surrogate assay might also helpful in identifying compounds that affect lifespan [28]. In this study we found such a correlation between stress resistance and lifespan since EGb761 conferred an increased resistance in a surrogate assay using heat as well as an extension of lifespan. In our hands heat was a suited surrogate since the worms died within a few hours under conditions of an acute lethal thermal stress and the bacteria acting as a source of food were not affected by the temperature. The thermal stress caused an increase of ROS accumulation in the worms (Fig. 4) and therefore it is likely that the death of the worms was at least partially due to oxidative stress. A direct application of oxidative stress during the measurement is not suitable for this assay as long as bacteria are included. Most substances that mediate oxidative stress, like redox cyclers, are also toxic for the bacteria so that the fluorescence exerted from the damaged bacteria and from the damaged worms is not distinguishable. Furthermore, many redox cyclers reveal autofluorescence that interferes with the measurement and the determination of the points of death.

From our point of view this fluorescence-based assay is a fast and convenient method to investigate effects of toxic or beneficial compounds on the resistance of *C. elegans* using heat as surrogate and it is presumably also suited to predict effects of compounds on the lifespan of worms.

EGb761 is known to have antioxidative properties [reviewed in 24] and it revealed strong radical scavenging activity at the concentration used in this study (Fig. 3). The challenge of the worms by thermal stress led to an increase in ROS accumulation with time (Fig. 4) so that we examined the impact of EGb761 on the redox state in the worms. The ROS accumulation was significantly reduced after 4 and even more after 6 h of thermal stress in worms treated with EGb761 (Fig. 4). A 4 h period of thermal stress was survived by almost all worms of both populations but after 6 h of thermal stress the survival rate of control worms (<30%) dramatically differed from the survival rate of EGb761

treated worms (>65%, Fig. 1). This temporal correlation of the increase in the survival rate and the reduction of ROS accumulation indicates that the protective impact of EGb761 was at least in part due to its antioxidative properties. This result is in line with other studies that showed an attenuation of ROS accumulation by EGb761 in wild type and transgenic worms [23,44,45]. In contrast to these studies that used pooled and lysed worms we applied the fluorescent probe used for the ROS measurement to individual worms in a microtiter plate. This procedure opened the possibility to measure the kinetic of ROS accumulation in single living worms under thermal stress.

Since GST-4 is known to be induced by oxidative stress [30] we used the GST-4 reporter gene construct of the transgenic *C. elegans* strain BL1 [30] as a marker for the extent of oxidative stress in living worms. The promoter activity of the *C. elegans* GST-4 was decreased by EGb761 under basal growth conditions as well as under conditions of induced oxidative stress (Fig. 5). We interpret the reduction of GST-4 promoter activity as an attenuation of oxidative stress in the worms conferred by EGb761. In mammalian cells EGb761 can upregulate the expression of antioxidant enzymes such as heme oxygenase-1, Mn-superoxide dismutase and the regulatory subunit of γ-glutamyl-cysteinyl synthetase [reviewed in 21] but no reference to the effect on the antioxidant enzyme catalase was found in literature. We demonstrated that the catalase transcription was reduced in response to EGb761 treatment (Fig. 6). In *C. elegans* (at least) two catalases exist that were indistinguishable from each other by Northern blot analysis because of the high degree of sequence identity and the similarity in transcript size [38]. Both catalases are inducible by oxidative stress [46] so that the decrease in the total amount of catalase transcripts is regarded as another indication for the attenuation of oxidative stress by EGb761. Strayer et al. [23] reported that the transcription of a stress inducible small heat shock protein of *C. elegans* (*hsp*-16.2) was also suppressed by EGb761 and the authors concluded that this was due to decrease of cellular stress.

According to our data, EGb761 treatment led to a reduction of ROS accumulation in *C. elegans* and presumably to a decrease of oxidative stress, too. It can be assumed that this antioxidative property of EGb761 is at least partially responsible for the enhancement of resistance against thermal stress and the extension of lifespan. The two stress-inducible genes examined in this study were downregulated indicating a direct antioxidant effect of EGb761 by radical scavenger properties although we cannot rule out some indirect effects on signal transduction pathways.

The different effects of EGb761 on antioxidative enzyme expression in mammalian systems [21] and in *C. elegans* may be a consequence of cellular metabolism, applied dose or cell properties.

Since the fertility and the time needed to produce the first offspring was not altered by EGb761 the reduction in body size observed in response to EGb761 treatment (Fig. 7) is probably not due to toxicity or caloric restriction but may be explained by the interaction of EGb761 with signalling processes that influence the worm's body size. A reduction in activity of proteins (RNAi) involved in the protein import to the inner mitochondrial membrane (TIN-9.1, TIN-9.2 and TIN-10; *C. elegans* homologs of human Tim9) and the maintenance of proper function of electron transport chain proteins (FRH-1; *C. elegans* homolog of human frataxin) resulted in smaller body size [47,48]. Mutations of certain genes involved in the insulin/insulin-like growth factor pathway also lead to reduced body volume (*daf*-2, *age*-1 and *aap*-1; [49]). All these body size decreasing influences are associated with reduction of reproductive capacity, an effect not observed after treatment of *C. elegans* with EGb761. The TGFβ signalling pathway is also know to regulate body size and some components of this pathway have been shown to affect the size of the worms without an reported effect on the reproductivity (DBL-1, SMA-2, SMA-3, SMA-4, RNT-1; [50–53]). Therefore, it may be possible that EGb761 interferes with one or more of these proteins to cause the decrease in body size.

5. Conclusion

In our study we used different approaches to investigate the impact of EGb761 on resistance to thermal stress, on ROS accumulation and on gene expression in individual living worms. The protective effect of EGb761 demonstrated in a semi-automated fluorescence-based assay by increase in resistance to lethal thermal stress was verified by prolongation of lifespan. EGb761 revealed strong radical scavenging activity, attenuated the accumulation of ROS and decreased the expression of the stress-inducible GST-4 as shown in a reporter gene approach. These results and the reduction of transcription of the also stress-inducible catalase genes indicates that the beneficial impact of EGb761 is at least partially due to its antioxidative capacity.

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