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ORIGINAL ARTILCLE

Tocotrienol-Rich Fraction (TRF) Improves the Viability of Wild-Type *Saccharomyces cerevisiae* in the Initial Stationary Phase

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ABSTRAK

Minyak fraksi kaya tokotrienol (TRF) yang mengandungi isomer α, β, γ dan δ-tokotrienols dan sedikit α-tokoferol, telah dilaporkan mempunyai kesan anti-penuaan dalam kedua-dua organisma model manusia dan bukan manusia, tetapi masih lagi tidak dijelaskan dalam model yis. Ia juga dilaporkan mempunyai keupayaan untuk memanjangkan jangka hayat beberapa organisma. Kronologi jangka hayat adalah salah satu cara untuk mengukur penuaan dalam yis. Kesan TRF terhadap kebolehhidupan tiga jenis Saccharomyces cerevisiae (jenis liar, CTT1Δ dan GPx2Δ) telah dikaji. Analisis pertumbuhan fenotipik semua strain dilakukan selama 15 jam dengan mengukur penyerapan pada OD_{600nm} dan penghitungan sel. Dos TRF dioptimumkan dengan menentukan bilangan unit pembentukan koloni oleh jenis liar pada penghujung rawatan 24 jam dengan TRF (dari 0 µg/ml hingga 300 µg/ml). TRF pada 300 µg/mL menunjukkan hasil yang terbaik, dan dipilih sebagai dos untuk analisis selanjutnya. Rawatan sel dengan 300 µg/mL TRF meningkatkan daya tahan ketegangan jenis liar dalam fasa pegun awal, tetapi bukan pada jenis gen knockout. Penemuan ini menunjukkan bahawa TRF mempunyai potensi untuk memanjangkan kronologi jangka hayat S. cerevisiae, dan mungkin juga organisma lain.

Kata kunci: cerevisiae, penuaan, tokotrienol

ABSTRACT

Palm oil tocotrienol-rich fraction (TRF) containing majorly of α, β, γ and δ-

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tocotrienols and some α-tocopherols, was reported to have anti-ageing effects in both human and non-human model organisms, but still remains unexplored in the yeast model. It was reported to have the ability to extend the lifespan of several organisms. Chronological lifespan is one of the means to measure ageing in yeasts. The effect of TRF on the viability of three strains of *Saccharomyces cerevisiae (wild-type, CTT1Δ and GPx2Δ)* was studied. Phenotypic growth analysis of all strains was carried out for 15 hours by measuring the absorbance at OD_{600nm} and cell counting. The optimum dose of TRF was optimised by determining the number of colony-forming unit by the wild-type strain at the end of a 24-hour treatment with TRF (ranging from 0µg/ml to 300µg/ml). TRF at 300 µg/mL showed the best result, and selected as a working dose. Treatment of cells with 300 µg/mL of TRF improved the viability of the wild-type strain in the initial stationary phase, but not on the knockout strains. These finding suggests that TRF has a potential in prolonging the chronological lifespan of *S. cerevisiae*, and perhaps other organisms as well.

Keywords: ageing, *cerevisiae,* tocotrienol

INTRODUCTION

Free radicals and oxidative stress are identified as the prime factors in causing cellular damage and biological ageing, leading to the shortening of lifespan (Yan et al. 2017; Harman 1956). The free radicals are capable of disrupting the cell membrane structure and cause damage to almost all cellular constituents like proteins, lipids and nucleic acids; interfering with the normal functions of the cell. To prevent these damages, there are endogenous and exogenous antioxidants defence system that can eliminate the free radicals. Examples of endogenous antioxidants include the enzymes catalase and glutathione peroxidases which reduce hydrogen peroxide (Khor et al. 2017), while the exogenous ones include vitamins C (Aumailley et al. 2016) and vitamin E (Zadeh-ardabili et al. 2017).

The study of ageing and lifespan extension has been replicated in many different models: yeasts (Kwon et al. 2017), nematodes (Zhao et al. 2017), flies (Cao et al. 2017), rodents (Ke et al. 2017) and to some limited extent, humans (Qin et al. 2017). Baker's yeast *Saccharomyces cerevisiae* is a unicellular, eukaryotic organism that can reproduce rapidly, (Toret & Drubin 2007; Scarfone et al. 2015) the bud neck easily manipulated and cultured in controlled environment (Williams et al. 2016). It has a shorter lifespan of a few days (Kwon et al. 2017) in comparison with other model organisms, enabling a shorter duration of research. More importantly, the genes in *S. cerevisiae* are highly homologous to those in animals and human (Liu et al. 2017; Karathia et al. 2011; Inoue et al. 1999).

S. cerevisiae lacking in antioxidants have many detrimental effects, among them is shorter lifespan (de Sá et al.

2013; Demir & Koc 2010). Previous studies have demonstrated a few means to prolong the lifespan of *S. cerevisiae*. Among these are calorie restriction, introduce changes in mitochondrial metabolism (Barros et al. 2004), and regulating proteins involved in the ageing mechanism such as TOR (Powers et al. 2006; Pan et al. 2011) and sirtuin signaling pathways (Howitz et al. 2003; Anderson et al. 2003) Extension of lifespan via intervention of antioxidants and other compounds have also been proven (Nakaya et al. 2012; de Sá et al. 2013). However, the exact mechanism of their effect has not been elucidated yet.

Vitamin E has eight isomers consisting of α-, β-, γ-, δ-tocopherols (Matthäus & Musazcan Özcan 2015; Fu et al. 2017) and α-, β-, γ-, δ-tocotrienols (Lim et al. 2014; Black et al. 2000). Palm oil tocotrienol-rich fraction (TRF) contains major composition of α, β, γ, õ-tocotrienols and minor composition of α-tocopherols (Lim et al. 2013; Makpol et al. 2011). The most prominent benefit of TRF as anti-oxidant is the effect in slowing down ageing process. Based on a human study (Chin et al. 2011), a continuous supplementation of TRF for 6 months showed a reduction of protein carbonyl, an oxidative stress indicator, in a target group of more than 50 years. When oxidative stress indicator is reduced, an organism will face less oxidative stress and would have a longer lifespan (Aumailley et al. 2016). At the cellular level, human diploid fibroblast treated with TRF showed a reduction in senescence characteristics, which could be attributed to the anti-oxidant effect of TRF (Makpol et al. 2013).

Ageing process of yeast can be observed in two aspects: chronological ageing and replicative lifespan. Replicative lifespan is defined as the total number of daughter cells generated by a mother cell (Sarnoski et al. 2017). Chronological ageing refers to the lifespan of *S. cerevisiae* which is measured by monitoring the mean and maximum survival times of populations of post-mitotic yeast cells that have stop replicating and continue to live the chronological lifespan (Kwon et al. 2017). It is established that there is a link between stress resistance and longevity with evidence for the conservation of the pathways that regulate lifespan in phylogenetically distant eukaryotes (Fabrizio & Longo 2003; Mirisola et al. 2014).

In this study, the effect of TRF on the viability of three *S. cerevisiae* strains in the initial stationary phase was determined. The three strains were the wild-type, *CTT1Δ* and *GPx2Δ.* The *CTT1Δ* strain lacked the gene encoding cytoplasmic catalase (Martins & English 2014); while the *GPx2Δ* strain lacked the gene encoding mitochondrial glutathione peroxidase (Tanaka et al. 2005).

MATERIALS AND METHODS

YEAST STRAINS AND MEDIA (YEAST STRAINS AND CULTURE CONDITIONS)

S. cerevisiae strains used in this study were listed in Table 1. Yeast extract peptone dextrose (YPD) liquid medium

(Difco, USA) contained 1% yeast extract, 2% peptone and 2% glucose, while YPD agar medium (Difco, USA) had similar composition but with additional 1.5% agar.

YEAST GROWTH MEASUREMENT

One of the objectives of this study was to determine if TRF has the preventive effect of an antioxidant, thereby extending the lifespan of an organism. Keeping that in mind, the growth for all strains were measured to compare variations in growth patterns, and to determine the initiation of the stationary phase. Overnight culture was prepared by inoculating single colonies picked from those grown on YPD agar plate, into YPD broth agitated at 220 rpm in a shaking incubator (30°C) (Jung et al. 2015). Biological triplicates were prepared from three different colonies. The overnight culture was transferred into 10 ml YPD broth to an initial density of 1-2 x10^6 cells/mL or OD_{600nm} ≈ 0.2 (Fabrizio & Longo 2003). The culture was incubated at 30°C, at 220 rpm in shaking incubator (IKA KS 4000i contro). Absorbance of culture was measured by Shimadzu 2450 UV-visible Spectrometer at 600nm wavelength (Murakami et al. 2008), and appropriately diluted with Trypan Blue before counted manually by hemocytometer. The whole process was repeated every hour for a total of 15 hours. The cell concentration was calculated from hemocytometer cell count using the formula as follows:

$$\text{Cell concentration} = \frac{\text{Average cell count of the small box x Dilution factor}}{\text{Volume of a small square (mL)}}$$

DETERMINATION OF VIABILITY BY COLONY-FORMING UNIT (CFU)

Cultures were diluted in sterile water with 10^5 dilution factor to achieve a cell density of 1 to 5 x 10^3 cells/mL. One hundred microliters of the diluted sample was spread onto YPD agar plates, incubated at 30°C, and colonies formed were counted at the end of 48 hours (Longo et al. 2012; Fabrizio & Longo 2003).

Chronological lifespan of the strains were determined by measuring the survival of non-dividing stationary phase cells (Mesquita et al. 2010). All cultures were presumed to be 100% viable at 13th hour when the cells were in stationary phase, with subsequent CFU measurements normalized to the 13th hour CFUs. Averages and standard

Table 1: *Saccharomyces cerevisiae* strains used in this study.

Yeast strain	Genotype
ATCC 201390 (wild-type)	MATa/MATalpha his3delta1/his3delta1 leu2delta0/leu2delta0 lys2delta0/+ met15delta0/+ ura3delta0/ura3delta0
ATCC 4034718 (*CTT1Δ*)	ΔCTT1
ATCC 4023384 (*GPx2Δ*)	MATa/MATalpha his3delta1/his3delta1 leu2delta0/leu2delta0 lys2delta0/+ met15delta0/+ ura3delta0/ura3delta0 ybr244w::KanMX4

deviations for at least three biological replicates were calculated for each experiment (Murakami et al. 2008).

PREPARATION OF TRF

Stock solution of TRF was freshly prepared in the dark room by dissolving 1 g of Gold Tri E 70 (Sime Darby Bioganic Sdn. Bhd., Malaysia) in 1 mL of 100% ethanol (1:1) and kept at -20°C for not more than one month (Lim et al. 2013). The palm-derived TRF consisted of 80% tocotrienol (26.76% α-tocotrienol, 4.29% β-tocotrienol, 32.60% γ-tocotrienol, and 15.53% δ-tocotrienol) and 20% α-tocopherol. Subsequently, there were 159.5 mg α-tocopherol, 205.1 mg α-tocotrienol, 32.9 mg β-tocotrienol, 249.8 mg γ-tocotrienol, and 119 mg δ-tocotrienol in every gram of TRF. TRF was activated by incubating 10 µL stock TRF (1 g/1 mL) with 990 µL 100% ethanol overnight at 30°C (Khee et al. 2014).

DOSE DETERMINATION OF TRF

The effects of various concentrations of TRF on *S. cerevisiae* viability were studied to determine the optimum dose on the wild type. Overnight culture of wild type was firstly prepared and then diluted in a new medium to achieve $OD_{600nm} \approx 0.2$. The cells were then incubated for 13 hours to achieve stationary phase and ready to be treated with different concentration of TRF. The final TRF concentration used were 0, 50, 100, 150, 200, 250 and 300 µg/mL (Lim et al. 2013). CFU was counted at time 0 hour and 24 hours of treatment. The CFU results at 24 hours were normalised to 0 hour. The optimum dose (300 µg/mL) was then applied to all strains of the yeast.

TREATMENT WITH TRF

Overnight culture was prepared as described previously, and then diluted in a new YPD medium to achieve $OD_{600nm} \approx 0.2$. The cells were then incubated for 13 hours to achieve stationary phase and treated with (300 µg/mL) of TRF.

STATISTICAL ANALYSIS

Each experiment was carried out in biological triplicates. Results were presented as mean ± standard deviation (SD). Comparison between control and TRF treated groups were made by Student's t-test (two-tailed). ANOVA test was used for comparing different TRF doses and TRF treatment on wild type and deletion strains, followed by Tukey's honestly significant difference (HSD) post hoc test. $P<0.05$ was considered to be statistically significant.

RESULTS

All cultures had similar growth patterns. To determine whether the knockouts strains exhibit any different phenotype than the wild-type, the growth pattern of the strains were observed. All three strains showed similar growth patterns (Figure 1A), conforming to the normal phases of yeast growth; lag phase (0-2nd hour), log phase (2nd-12th hour) and stationary phase (12th hour

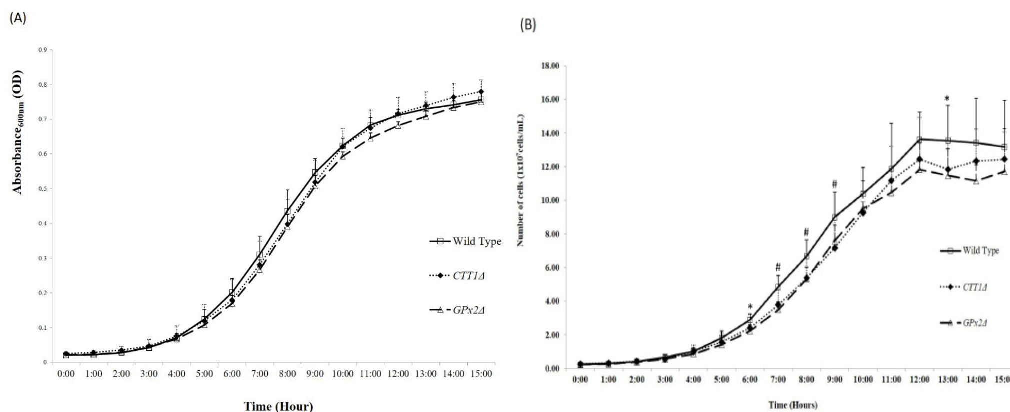

Figure 1: Growth curves of three different strains of *S. cerevisiae* (wild-type, *CTT1Δ* and *GPx2Δ* based on (A) absorbance and (B) haemocytometer cell count. The three different strains of were sampled and analysed every hour for 15 hours. Data are presented as mean ± SD, n = 3. #Wild-type shows significantly higher cell counts than both *CTT1Δ* and *GPx2Δ* strains ($p<0.05$). *Wild type shows significantly higher cell counts than *GPx2Δ* strain ($p<0.05$).

onwards) (Salari & Salari 2017). Figure 1B confirmed the growth pattern by haemocytometer cell counting, where the wild type showed the highest significant cell concentration readings after six to nine hours (Figure 1B, $p<0.05$), compared to the other two strains. Between six to nine and 13 hours post culture, the wild-type strain has a higher number of cells ($p<0.05$) than the *GPx2Δ* strain. The number of cells of the *CTT1Δ* strain is significantly lower ($p<0.05$) than that of the wild-type between six to nine hours post culture. Although, the graphs showed that the cultures are beginning the stationary phase at the 12th hour (Figure 1B), the TRF treatment started at the 13th hour, to ensure that all strain cultures have truly entered the stationary phase.

Optimal dose of TRF was 300 µg/mL. As there is no available report of *S. cerevisiae* treated with TRF, it was critical to determine the effect of TRF on the viability of *S. cerevisiae*, as well as determining the optimal working dose for this experiment. Figure 2 demonstrated wild-type *S. cerevisiae* viability after incubation with various concentration (0-300 µg/mL) of TRF for 24 hours. The viability was normalised to the viability of cells prior to treatment. Generally, there was increased viability of cells as the TRF dose was increased. Both doses of 250 µg/mL and 300 µg/mL showed significant increase in viability, as compared to the control group ($p<0.05$). Since the higher viability was achieved by 300 µg/ml of TRF, this dose was selected as the working dose.

Increased viability of the wild-type strain after treatment with TRF. Stationary phase yeast cells of 13th hour culture were treated with 300 µg/mL of TRF for 24 hours. Shown in Figure 3, only the wild-type showed significant increase in viability compared to the control group ($p<0.05$).

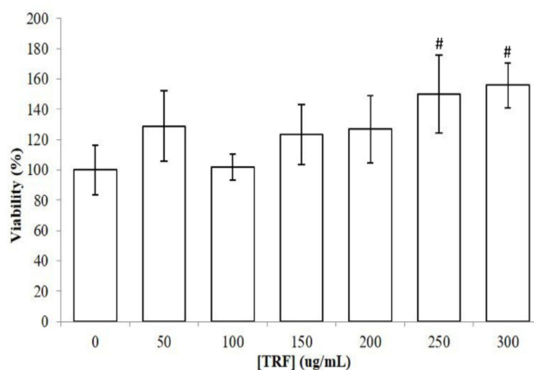

Figure 2: Treatment of the wild-type strain with various doses of TRF for 24 hours. The viability of S. cerevisiae increased steadily with the increment of the dose until 300 µg/mL. Data are presented as mean ± SD, n = 3. #The viability of S. cerevisiae is significantly higher than that of the control (0 µg/mL) ($p<0.05$).

DISCUSSION

Conventionally, the concentration of yeast cells in a suspension is assayed by the means of spectrophotometry. The absorbance of yeast culture is measured at 600 or 660 nm, and repeated measurement of the same culture over the course of a series of time, enable researchers to monitor the growth of yeast cells (Barber & Lands 1973; Murakami et al. 2008; Salari & Salari 2017). However, this method allows measurement of absorbance of any solute and particles, without discriminating the live cells from the dead (turbidimetrically). Besides spectrophotometry, there are several methods available, that can assay the growth and viability of microbial cells. In this research, direct cell count (Figure 1B) and observation of CFU were used. Dilution of the cells prior to counting with haemocytometer allows only the

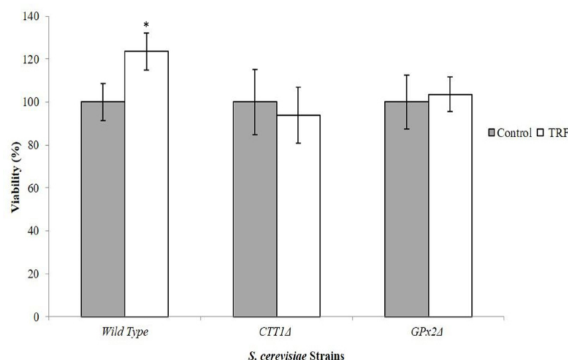

Figure 3: All the three strains were treated with 300 ug/mL of TRF for 24 hours, and viability was compared to their own control group. Data are presented as mean ± SD, n = 3. *Significant increase compared to the control ($p<0.05$).

non-viable cells to take-up the Trypan Blue, leaving the viable cells colourless (Tran et al. 2011). Due to this method of differentiation, the values in the cell counting method does not exactly match the absorbance of the growth.

ROS deactivation is crucial to ensure the survival of all aerobic life forms. When an overproduction of free radicals and ROS exceeds the capacity of antioxidant mechanism of the cell, the cell will experience oxidative stress. High level of ROS becomes toxic to cells, causing severe damage to cellular components such as proteins, DNA and lipids (Dezest et al. 2017).

CTT1 and *GPx2* encode for cytoplasmic catalase (Martins & English 2014) and mitochondrial glutathione peroxidase in yeasts (Tanaka et al. 2005), respectively. Catalase and glutathione peroxidase are amongst the antioxidant enzymes to inactivate the ROS in cells. Catalase prevents cell damage by catalyzing the reduction of hydrogen peroxide to water and oxygen (Zeller & Klug 2004), while glutathione peroxidase catalyzes the reduction of hydrogen peroxide and other hydroperoxides to water or the corresponding alcohols, respectively, using glutathione as the reductant (Wang et al. 2017). Hence, cells lacking antioxidant enzymes are expected to have shorter lifespan and slower reproduction (de Sá et al. 2013; Demir & Koc 2010), as exhibited in Figure 1. In this study, the reduction of yeast cell count in *CTT1Δ* and *GPx2Δ* strains as compared to the wild type may suggest that *CTT1Δ* and *GPx2Δ* strains suffer a higher oxidative stress when approaching the stationary phase (Longo et al. 1996; Trancíková et al. 2004). However, previous studies have also shown that catalase (*ctt1*)-knockout *S. cerevisiae,* were able to survive without the enzyme, not even for H_2O_2 resistance (Okada et al. 2014; Martins & English 2014). Other studies showed that catalase is only needed when the cells are exposed to stressors like H_2O_2, heat-shock and ethanol (Du & Takagi 2007). The same effect was observed in glutathione peroxidase-knockout cells too, where the strain survived under normal condition, and even under peroxide (Inoue et al. 1999) and H_2O_2 assault (Okada et al. 2014). These abilities of the cells to survive could not be observed in our study model, since the cells were monitored at the initial stage of the stationary phase.

Chronological lifespan refers to the duration where the non-dividing, stationary phase cells maintain their viability. The stationary phase marks the ongoing depletion of source of glucose (Vasicova et al. 2015). Cells will begin to switch from fermentative metabolism to a respiratory metabolism (Williams et al. 2016). This change comes with increased ROS production (Trancíková et al. 2004; Watanabe et al. 2014). Results in Figure 3 indicates that TRF increases the viability of only the wild type strain in the initiation of stationary phase. This leads to the assumption that the presence of TRF cannot overcome the shortcomings of a knockout cell, lacking in catalase or glutathione peroxidase. Based on this observation, we postulated that TRF needs either catalase or glutathione peroxidase 2, or the presence of both

enzymes, in order to increase the chronological lifespan at the initial stage. There is a lack of data as the effects of vitamin E on *S. cerevisiae* has not been widely explored, let alone the effects of vitamin E or TRF on its lifespan during the stationary phase. On the other hand, one research reported on the ability of α-tocopherol reducing the replicative lifespan of *S. cerevisiae* (Lam et al. 2010).

Nevertheless, over the years, TRF was documented to exhibit many benefits. In one of the more recent studies, TRF was able to restore actin in cells of murine pre-implantation embryos, after been exposed to oxidative stress triggered by nicotine (Hamirah et al. 2017). It also has healing properties; such as protecting astrocytes against glutamate toxicity (Abedi et al. 2017), and reducing UV-induced skin redness (Yap 2018). Furthermore, TRF restored the lifespan of oxidative stress-induced *Caenorhabditis elegans*, even with increased DNA oxidation (Aan et al. 2013). TRF also inhibits melanogenesis in melanocyte cell culture, by reducing the expression of *TYRP2* gene, resulting in reduced melanin content and tyrosinase activity (Suzana et al. 2009).

The benefits of TRF are attributed to the constituents, the various isoforms of vitamin E. Tocotrienols on their own, possess numerous health benefits. Although there are no documented findings of the effect of tocotrienol on *S. cerevisiae,* tocotrienols have been tested on many other organisms. A recent study reported how γ-tocotrienol counter radiation injury by instigating metabolic shifts (Cheema et al. 2017). Another study reported δ-tocotrienol causes cell cycle arrest in G1 and G2/M phase simultaneously, leading to an anti-prostate cancer activity (Sato et al. 2017). Furthermore, tocotrienol prolonged the lifespan of *C. elegans*, and increased resistance to infections (Kashima et al. 2012)

In *S. cerevisiae,* the α-tocopherol, another constituent of TRF; can prevent lipid peroxidation, owing to the hydroxyl group in domain I of the structure of tocopherol, as well as its lipophilic characteristic (Bitew et al. 2010). α-Tocopherol can also reverse detrimental effects caused by edelfosine (Bitew et al. 2010) and endosulfan (Sohn et al. 2004). A study by Raspor et al., (Raspor et al. 2005) demonstrate that treatment with Trolox, (α-tocopherol analogue) increased the cell viability, decreased intracellular ROS formation and suppressed DNA oxidation in *Saccharomyces cerevisiae.* In a human myoblast cell culture model, TRF treatment reduced the amount of intracellular ROS, reduced lipid peroxidation and decreased the number of cells undergoing late apoptotic events in senescent cells (Khor et al. 2017).

Due to the limit of the duration of this particular research, it would be more advantageous to test the effect of TRF for longer hours in future. We conclude that the TRF is able to improve the viability of *S. cerevisiae,* but cannot replace the functions of absent antioxidant enzymes, to improve the chronological lifespan of *S. cerevisiae* at the initial stationary phase.

CONCLUSION

The data showed that TRF increased the chronological lifespan of *S. cerevisiae*, at the stationary phase. The ability of TRF to increase the lifespan of an organism could be applied in other organisms as well.

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