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Short report

# Trehalose administration in C57BL/6N old mice affects healthspan improving motor learning and brain anti-oxidant defences in a sex-dependent fashion: a pilot study

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ABSTRACT

Aim of this study was to characterize the effects of oral trehalose administration (2%w/v) on healthspan in old mice. Trehalose was administered in drinking water for 1 month to male and female C57BL/6N mice aged 25-months. After behavioral phenotyping (grip strength, beam walking and rotarod tests), autophagy (LC3-II/actin) and oxidative stress were tested in the cerebral cortex and gastrocnemius muscle. The latter parameter was indirectly assessed by evaluating carbonyl groups added to proteins as a result of oxidative reactions, in addition to central levels of NRF2 protein, a transcription factor that regulates the expression of antioxidant enzymes. In comparison with sex-matched controls, trehalose-treated males performed better in motor planning and co-ordination tasks. This behavioral phenotype was associated with an activation of the ubiquitin-proteasome system, autophagy and antioxidant defences in cerebral cortex. Independently from trehalose administration, females were characterized by better motor performance and showed higher levels of ubiquitinated proteins and NRF2 in cerebral cortex, suggesting an up-regulation of basal antioxidant defences. In conclusion, trehalose was effective in counteracting some aspects of age-related decay, with specific effects in male and female subjects.

## 1. Introduction

Oxidative stress (OS) is a major component of aging that may cause molecular damage, cells and tissues dysfunction, compromising the health status of the organism (healthspan) (Berry and Cirulli, 2013; Fischer et al., 2016; Floyd and Hensley, 2002; Fuellen et al., 2018). Of notice, the mammalian brain shows poor antioxidant defences, high metabolic rate and reduced capability of cellular regeneration, being particularly vulnerable to OS insults (Floyd and Hensley, 2002). A wide range of approaches have been designed to reduce OS in order to increase lifespan and to promote healthspan. Among these, we can enlist caloric restriction (CR), namely a reduced caloric intake without malnutrition (Loeb and Northrop, 1917; Mattison et al., 2007; Most et al., 2017). Despite CR being an effective strategy to slow down the aging process, the feasibility of such intervention is tricky in a lifelong perspective, leading to reduced compliance in the general population, and possibly being unsafe for normal-weight, middle-aged and elder adults (Martens and Seals, 2016; Villareal et al., 2016).

Trehalose is an alpha-linked disaccharide of glucose synthesized by fungi, plants and invertebrates, but not by mammalian cells, potentially displaying CR-mimetic properties (Martens and Seals, 2016). Trehalose has been reported to function as a mechanistic target of the rapamycin (m-TOR)-independent inducer of autophagy (Mizunoe et al., 2018; Rodriguez-Navarro et al., 2010). In particular, trehalose-mediated inhibition of glucose transport might create a functionally "starved" state enhancing positive or bypassing aberrant energy-sensing signalling pathways that are dysregulated during the aging process (DeBosch et al., 2016; Martens and Seals, 2016).

A growing body of evidence in animal models suggests that administration of trehalose leads to increased lifespan and improved healthspan (Portbury et al., 2017; Sarkar et al., 2014; Tanaka et al., 2004). Despite these promising results, before this compound can be

*Abbreviations:* OS, oxidative stress; CR, caloric restriction; ROS, reactive oxygen species; NRF2, nuclear factor erythroid 2-related factor 2

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employed for human use, further studies detailing its mechanisms of action need to be carried-out. In this context, it is important to highlight the yet general scarcity of knowledge about sex-differences in response to food restriction or to CR-mimetic compounds that are emerging as promising anti-aging alternatives to food-restriction regimens (Anisimov et al., 2015; Piotrowska et al., 2016).

The aim of this pilot study was to explore the effects of oral trehalose administration on motor behavior, OS and autophagy in a murine model of physiological aging, taking into account sex differences and comparing central (brain) vs. peripheral effects. Thus, we selected 25-month-old C57BL/6N mice since this is one of the most commonly used strains for whom a growing body of data on the aging phenotype is starting to be available also with regard to sex differences. As an example, Baumann and co-workers, in a very recent and interesting paper, report that in this strain, at 26 months of age, the prevalence of frailty is 66% greater in females than males, males live 13% longer than females, females being characterized by greater physical activity in the voluntary wheel run, reduced endurance on the treadmill. Furthermore, when body composition was evaluated, females showed greater amount of fat percentage (Baumann et al., 2019). Since it has been recently suggested that trehalose might protect from OS by regulating the KEAP1-NRF2 and autophagy pathways (Mizunoe et al., 2018) we indirectly assessed levels of OS by evaluating carbonyl groups present into proteins as a result of oxidative reactions, in addition to central levels of the NRF2 protein, a transcription factor that regulates the expression of antioxidant enzymes. Moreover, levels of autophagy were assessed by evaluating levels of LC3-II, the main autophagosomal marker (Tanida et al., 2008).

## 2. Materials and methods

### 2.1. Experimental subjects and trehalose administration

Twenty-four male and twenty-two female mice aged 25-months of the C57BL/6N strain (Charles River) were housed under standard conditions at the animal facility of Istituto Superiore di Sanità (Berry et al., 2018). All subjects were housed 2–3/cage with a same-sex conspecific. Twelve males and eleven females were administered either with 2% trehalose w/v (Sigma Aldrich) or with regular tap water (12 males and 11 females) for 30 days. A fresh solution was prepared twice/week. Since we have data to show that 24-month-old C57BL/6N mice drink, on average, 5 ml of water each day (unpublished results) we calculated that each mouse received roughly 0.1 mg trehalose/*die*. The trehalose dose has been selected based on its efficacy, as reported in previous studies carried-out on different mouse models (particularly of neurodegenerative disorders) involving a dysregulation of the autophagic process (Kara et al., 2013; Schaeffer et al., 2012; Tanaka et al., 2004; Tanji et al., 2015). Before starting trehalose administration, body weight was registered and all subjects underwent a basal assessment of physical strength (grip strength) and motor coordination (beam walking). Body weight was recorded each week (by the time fresh solutions were prepared). At the end of treatment, mice were re-assessed for physical strength (grip strength) and motor coordination (beam walking and rotarod tests) and sacrificed immediately after. Brains (frontal cortex) and limb muscles (gastrocnemius) were dissected-out and frozen for further analyses (see Fig. 1 for an experimental timeline). All experimental procedures were conducted in conformity with the European Directive 2010/63/EU and the Italian legislation on animal experimentation, D.Lgs.vo 26/2014 and were approved by the Istituto Superiore di Sanità Ethical Committee (Organismo Preposto per il Benessere Animale - OPBA) and by the Italian Ministry of Health.

### 2.2. Behavioral phenotyping

#### 2.2.1. Grip strength

The grip strength is a main measure of muscular strength used for mice. Mice instinctively hold on to materials with a strong grip. This test is commonly used to assess gross changes in this parameter in different pathological conditions (e.g. animal models of Parkinson's disease and Huntington's chorea). The ability to remain clinging, with all four paws, to an inverted or tilted surface (a cage lid) was tested for a period of time up to 60 s (cut-off). A small surface was delimited to avoid the movement of subjects into the cage lid for a more reliable assessment of muscles strength (Deacon, 2013b).

#### 2.2.2. Beam walking

Fine changes in motor coordination and balance in response to trehalose administration were evaluated assessing the ability of mice to traverse two narrow beams of different diameter. The goal of this test is for the mouse to stay upright and walk across an elevated narrow beam. The motivation to perform the task is provided by a safe platform positioned at the end of the beam (Curzon et al., 2009). Beams were placed horizontally, 50 cm above the table. Mice were first trained to traverse a beam of 2 cm in diameter (1° BEAM) and 2 h later a beam of 1 cm in diameter (2° BEAM), to increase the task difficulty. Latency to traverse each beam, within the allotted time (cut-off 60 s), was recorded as well as the number of subjects that fell off the beams before and after treatment administration (Deacon, 2013a).

#### 2.2.3. Rotarod

Accelerated rotarod training can be regarded as a valid paradigm for motor skill learning over short (intra-session, minutes) and long time frames (inter-session, days) (Buitrago et al., 2004). Changes in motor learning, coordination and balance in response to trehalose administration were tested using an accelerating rotarod (Accelerating Rotarod, Ugo Basile, Gemonio, Italy) (Curzon et al., 2009). Mice with deficits in motor coordination or balance fall off the rotarod before the end of the 5-minute test session (Crawley, 1999). The test has been performed by placing a mouse on a rotating rod with a diameter of 3 cm and measuring the latency to fall (in seconds) through a switch placed on the floor below. Mice were given three trials each with a maximum time of 300 s (5 min), during which time the rotating rod underwent a linear acceleration from 4 to 40 rpm over 5 min. Animals were rested after a minimum of 10 min between trials to avoid fatigue (Paylor et al., 2006). Differently from the beam walking, this test of motor learning was performed only once (at the end of the treatment) to prevent a ceiling effect due to overtraining.

### 2.3. Analyses on peripheral and central tissues

#### 2.3.1. Western blot

Brain and muscle tissues were lysed in lysis buffer containing 1% Triton X-100, 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4 and 75 U of aprotinin and allowed to stand for 20 min at 4 °C. The tissue suspension was disrupted by Dounce homogenization (ten strokes) and then centrifuged for 5 min at 1300 ×*g* to remove nuclei and large cellular debris. After evaluation of the protein concentration by Bradford dye reagent assay (Bio-Rad, 500-0006), the lysate was subjected to sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), protein loading: 30 µg/lane. Proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, 162-0177). Membranes were blocked with 5% defatted dried milk in TBS, containing 0.05% Tween-20 and probed with rabbit polyclonal anti-LC3 antibody (MBL Int Corporation, PD014), with rabbit monoclonal anti-Nuclear Factor Erythroid 2-related Factor 2 (NRF2, Abcam, ab62352), with mouse monoclonal anti-beta-actin (Sigma, A5316), with mouse monoclonal anti-Ubiquitin (Abcam, ab7254). Bound antibodies were visualized with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Sigma, A1949) or anti-mouse IgG (Sigma, A9044) and immunoreactivity assessed by chemiluminescence reaction, using the ECL western detection system (Amersham, RPN2106). Densitometric scanning analysis was

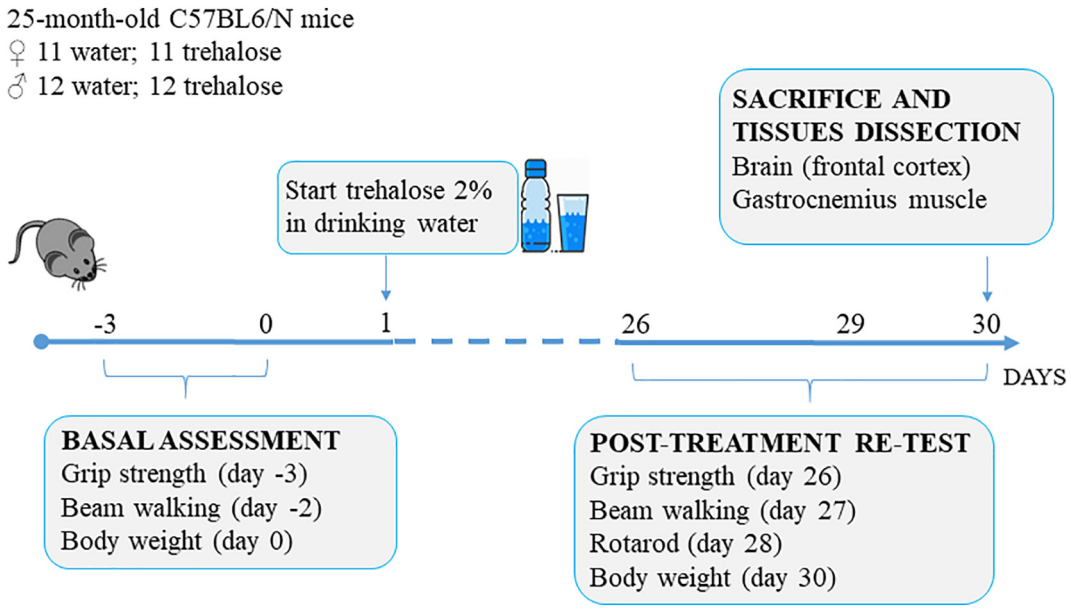


**Fig. 1.** Timeline of the experimental plan.

performed with Mac OS X (Apple Computer International), using NIH ImageJ 1.62 software.

#### 2.3.2. Protein carbonyl assay

Carbonyl groups present into proteins as a result of oxidative reactions were evaluated in brain tissue with Oxidized Protein Western blot detection (Abcam, ab178020; Cambridge, MA, USA). Twenty micrograms protein load was used in all groups; protein carbonyl groups were measured according to the manufacturer's instructions.

### 2.4. Statistical analysis

For those endpoints following a normal distribution (body weight, NRF2, carbonyl groups, ubiquitinated proteins and LC3-II) data were analysed using an exploratory 2-way-ANOVA with Treatment and Sex as between-subjects factors and repeated measure a within subject factor (body weight). Since a main effect of sex was found on all the parameters taken into account, to better characterize the effects of trehalose, male and female subjects were analysed separately by means of a one-way-ANOVA with only Treatment as a between subjects factor. In more detail: the effect on body weight was evaluated (separately in males and females) by looking at the difference between day 30 (last day before sacrifice) and day 0 and then transforming raw data in percentage change with respect to basal body weight (day 0). For ex-vivo parameters raw data were transformed to percentage change compared to the appropriate control (either male or female). Latencies to fall in the grip strength and rotarod were analysed with the non-parametric Mann and Whitney test (data were transformed to percentage change from basal value separately for males and females). For the beam walking, the McNemar's change test (a simple and frequently used test for binary matched-pairs data (Fagerland et al., 2013) was applied to evaluate changes in the motor performance from day 0 to day 30 of each single subject within each group of treatment. In more detail, a fall off the beam on day 0, but not on day 30, was considered as an improvement while a fall only on day 30 was considered as a worsening; subjects falling or not falling on both days were accounted within the "stable performance" group. Grubb's test (using 5% significance level critical values, (Grubbs, 1950)) was used to detect outliers in the case of data following a normal distribution. Statistical analysis was performed using Statview II (Abacus Concepts, CA) and GraphPad Prism 7. A significance level of 0.05 was chosen. See Supplementary Tables S1 and S2 for mean and SEM of all the parameters analysed by ANOVA and median values, minimum, maximum and interquartile rates for all parameters analysed by means of non-parametric analyses.

## 3. Results

### 3.1. Body weight and behavioral phenotyping

#### 3.1.1. Body weight

As expected male subjects showed higher body weight than females (sex main effect: $F(1,42) = 10.261$; $p = 0.026$; mean ± SEM: 28.5 ± 1.9 and 30.4 ± 2.2 respectively for females and males). When males and females were analysed separately, trehalose treatment was able to counteract the age-dependent decrease in body weight observed in females ($F(1,20) = 5.411$; $p = 0.0306$) but not in males ($F(1,22) = 0.678$; $p = 0.4192$), see Fig. 2a.

#### 3.1.2. Grip strength

No difference was found as a result of trehalose administration (females: $U(11,11) = 52.000$; $p = 0.5761$; males: $U(12,12) = 63.000$; $p = 0.6033$).

#### 3.1.3. Beam walking

Females showed a worsening in their performance over time when traversing the 1° BEAM (broad beam: $p = 0.0269$) but not the 2° (narrow beam: $p = 0.267$) while the opposite was observed in males (larger beam: $p = 1.000$; smaller beam: $p = 0.0159$).

No difference was found over time neither in control ($p = 0.1306$) nor in trehalose-treated subjects ($p = 0.2207$) on the 1° BEAM. By contrast, on the 2° BEAM - requiring more refined motor coordination abilities - trehalose-treated mice maintained a stable performance ($p = 0.1824$, see Fig. 2b) while control subjects showed a worsening in motor function over time ($p = 0.0389$).

#### 3.1.4. Rotarod

No difference was found as a result of treatment in trials 1 and 2 neither for females nor for males (females, trial 1: $U(11,11) = 55.000$; $p = 0.7178$; trial 2: $U(11,11) = 47.000$; $p = 0.3750$; males, trial 1: $U(12,12) = 68.000$; $p = 0.8173$; trial 2: $U(12,12) = 51.500$;

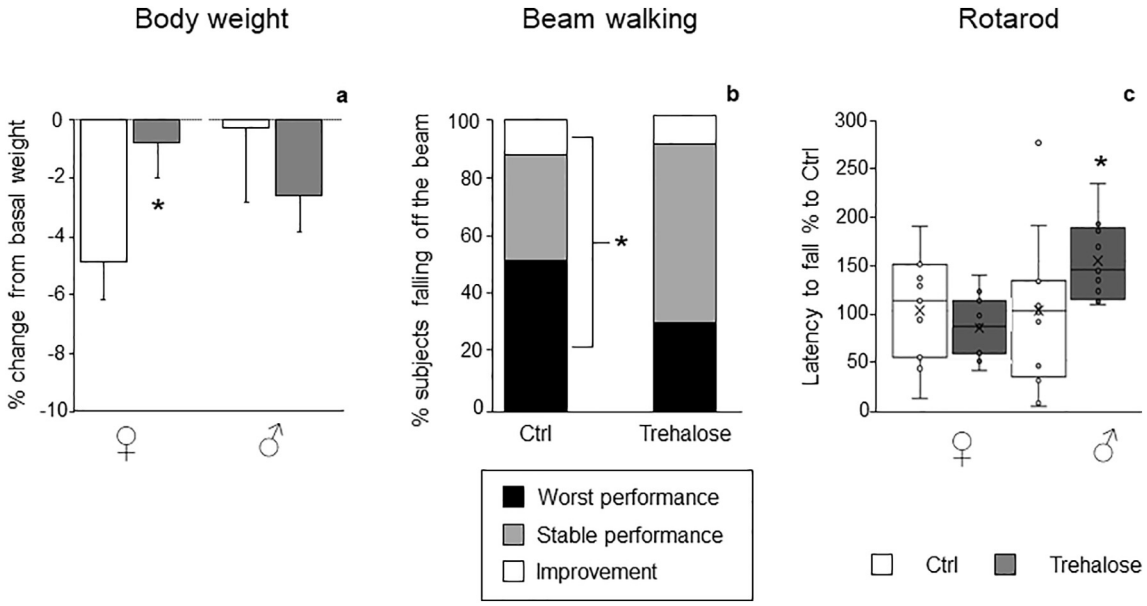


**Fig. 2.** Trehalose counteracted the age-dependent decrease in body weight observed in females (a), prevented the time-dependent decrease in motor function in all mice tested on the beam walking (narrow beam) (b) and improved motor learning in male subjects in the rotarod test (c). Data are presented as: mean + SEM (a); percentage of subjects falling off the beam (b); box plot (c). Observations outside these ranges in the box plot are represented with dots outside the boxes.*$p < 0.05$. Experimental subjects in each final group: F, control = 11, trehalose = 11; M, control = 12, trehalose = 12.

p = 0.2365). However, on trial 3, trehalose improved the motor performance of male subjects (males: U(12,12) = 30.500; p = 0.0165; females: U(11,11) = 48.500; p = 0.4304); Fig. 2c.

### 3.2. Analyses on peripheral and central tissues

In some cases, following the extraction procedure, tissue was not sufficient to carry-out all the ex-vivo assessments on all the experimental subjects; in the specific case of carbonyl groups 1 ctrl male and 1 trehalose-treated male were found to be outliers and were excluded from the analysis; likewise 1 ctrl male and 1 trehalose-treated female were excluded from the analysis when assessing ubiquitinated proteins in the frontal cortex. Thus, the final number of the samples available for each sex and treatment and for each tissue was between 8 and 12 (see Supplementary Tables S1 and S2).

#### 3.2.1. Analysis of oxidative markers in frontal cortex

When OS markers were assessed in the frontal cortex, a main effect of sex was found with females showing elevated levels of carbonyl groups (F(1,33) = 14.322, p = 0.0006, mean ± SEM: 3.093 ± 0.617 and 0.307 ± 0.042 respectively for females and males). Data expressed as % change to control and separately analysed in males and females showed no difference as a result of treatment neither in females nor in males (females: F(1,19) = 0.949; p = 0.3423; males: F(1,14) = $9.34^{10-6}$; p = 0.9976, see Fig. 3a). A main effect of sex was found for NRF2 with females being characterized by higher protein levels (F(1,38) = 9.940; p = 0.0032, mean ± SEM: 0.452 ± 0.0089 and 0.151 ± 0.042 respectively for females and males, data not shown). Data expressed as % change to control and separately analysed for males and females, showed that males were characterized by increased NRF2 levels as a result of treatment (F(1,20) = 4.604, p = 0.0444), this was not observed in females (F(1,18) = 0.539; p = 0.4721), see Fig. 3b.

#### 3.2.2. Autophagy (LC3II/actin)

A main effect of sex was found with male subjects showing greater autophagic rate when compared to females in the frontal cortex (F(1,40) = 8.391; p = 0.0061, mean ± SEM: 0.179 ± 0.018 and 0.314 ± 0.049 respectively for females and males). Data expressed as % change to control, and separately analysed for males and females, revealed that trehalose increased LC3-II/actin ratio levels only in males (males: F(1,20) = 7.006; p = 0.0155; females: F(1,20) = 1.428; p = 0.2460, Fig. 3c). No effect of treatment was observed in the gastrocnemius muscle (males: F(1,19) = 0.381; p = 0.544; females: F(1,19) = 1.046; p = 0.3192, Fig. 4a).

#### 3.2.3. Ubiquitination (ubiquitin/actin)

A significant sex x treatment interaction (F(1,39) 5.870; p = 0.0201, see also Supplementary Table S1) was found in the frontal cortex. Data expressed as % change to control and separately analysed for males and females revealed that trehalose-treated males showed a strong trend towards increased levels of ubiquitinated proteins (F(1,20) = 4.272; p = 0.0519) while treatment administration did not affect this parameter in females (F(1,19) = 2.602; p = 0.1232); see Fig. 3d. Trehalose did not affect ubiquitinated protein levels in the gastrocnemius muscle (males: F(1,18) = 0.144; p = 0.7083; females: F(1,20) = 0.016; p = 0.9010); see Fig. 4b.

## 4. Discussion

Results from this study show that one month of oral trehalose administration leads to positive sex-dependent effects in senescent mice. In more detail, trehalose prevented the age-related decrease in body weight in old females, while improving motor function in males, this latter effect being accompanied by increased levels of NRF2 and of autophagy in the frontal cortex. These effects were specific for the brain and were not observed in muscles.

Trehalose was able to reduce the sharp decrease in body weight in female subjects just before sacrifice. The weight loss observed in females has been previously reported by Fahlström and colleagues in C57BL/6 females older than 20 months (Fahlstrom et al., 2011) and might suggest a general worsening due to the progression of aging that is more apparent in this sex. Indeed, as reported by Kunstyr and Leuenberger, 25 months of age for C57BL/6 mice is a commonly accepted landmark for lifespan since at this age mortality rate is about 50% (Kunstyr and Leuenberger, 1975). Moreover, with regard to sex-differences, although environmental conditions might greatly affect this parameter, a consistent number of evidence suggest that, at least for

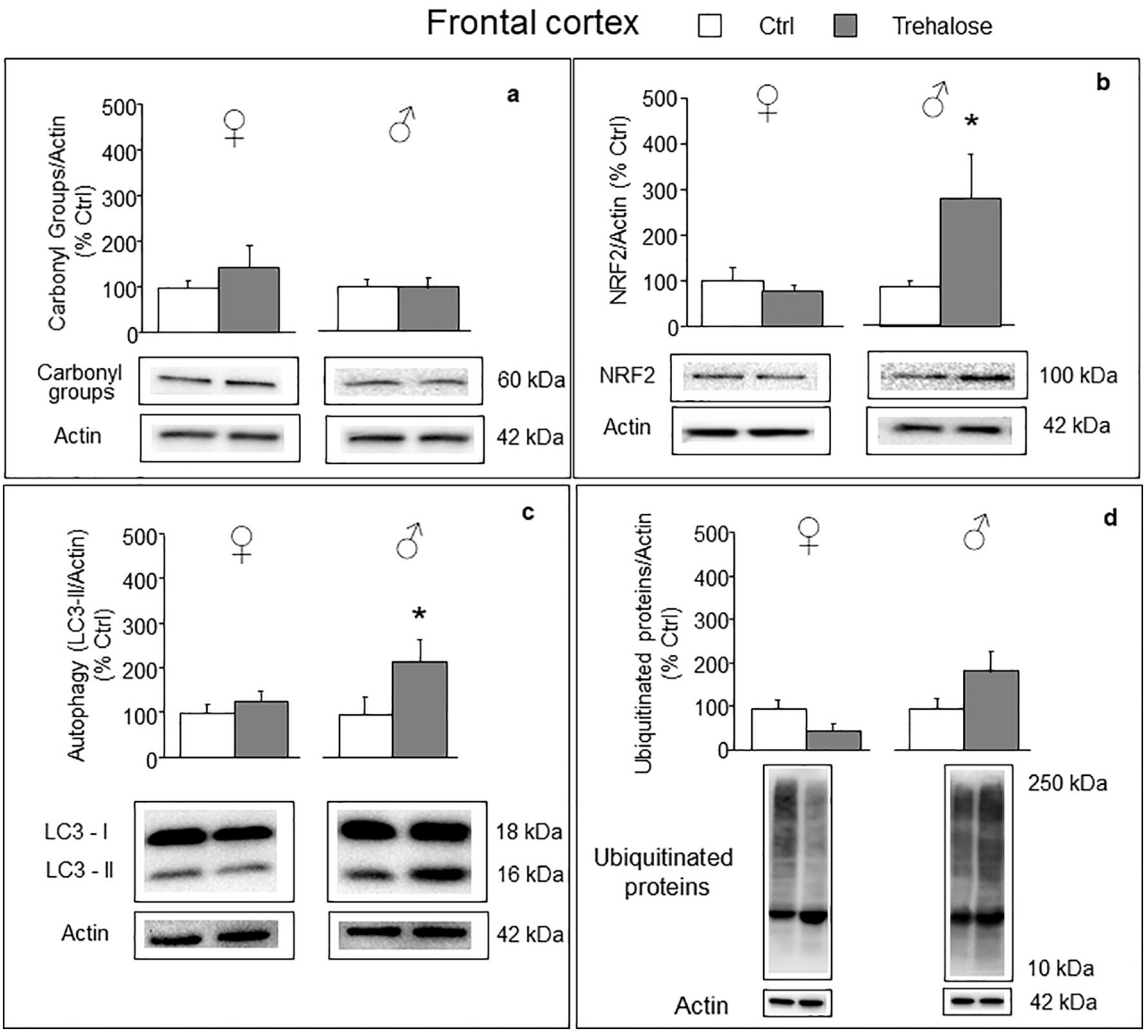


**Fig. 3.** Quantification of carbonyl groups was not affected by trehalose treatment (a). Trehalose increased NRF2 levels in males (b). Trehalose increased the autophagic rate in the brain of male subjects (c). A strong trend was observed in males towards increased ubiquitinated proteins upon trehalose treatment (d). Densitometric analysis data are shown as mean + SEM *$p < 0.05$. Protein levels of LC3-II, ubiquitinated proteins and NRF2 protein levels, carbonyl groups and actin were analysed by western blot. Actin served as loading control. Representative blots are shown. Subjects for each group: autophagy (LC3-II): F, control = 11; trehalose = 11; M, control = 11; trehalose = 11; ubiquitinated proteins: F, control = 11; trehalose = 10; M, control = 10; trehalose = 12; Nrf2: F, control = 9; trehalose = 11; M, control = 12; trehalose = 12; carbonyl groups: F, control = 10; trehalose = 11; M, control = 8; trehalose = 8.

this strain, females have a shorter lifespan than males (see (Austad and Fischer, 2016) and references therein for a complete review). To this regard, the protective effect of trehalose on body weight might suggest that 25–26 months of age may represent a critical window of opportunity for healthspan-promoting treatment administration.

Trehalose treatment prevented the time-dependent decrease in motor function in all mice tested on the narrow beam reducing the number of subjects falling-off the beam over time. Moreover, we observed a specific and sex-dependent improvement in motor learning after trehalose administration as assessed in the rotarod test. In general, improvements in the rotarod test are not only the result of enhanced locomotor abilities or fitness, that may be trained in the running wheel, but rather rely on specific changes in motor strategies that are needed in order to master this motor test. In fact, differently from other motor tasks, mice must learn to associate sensory states (proprioceptive, vestibular, position in space and on the rod) with the appropriate motor response to facilitate remaining on the accelerating rotating rod rather than falling (Beeler et al., 2010; Buitrago et al., 2004). The difference between simple motor performance and motor learning is clearly apparent in animal models of Parkinson's disease. For example Beeler and co-workers found that PITx3-deficient mice do not display motor deficits unless they are assessed in more complex tasks that require learning of new motor skills, such as the rotarod test (Beeler et al., 2010). Our results show that trehalose treatment improved motor learning in males only (trial 3). Some evidence suggests that females perform better than males in motor coordination/motor balance tests. As an example, Oliveira and colleagues found that female C57BL6 mice outperformed males both in the rotarod as well as in the beam walking test, showing a shorter latency to cross the beam and a higher latency to fall off the accelerated rod (Oliveira et al., 2015). Moreover, Jung and Metzger showed that female rats were characterized by a better performance compared to males on the rotarod, upon an oxidative insult, suggesting that the cerebellum, that is involved in motor learning, might be a preferred target for these regulations (Jung and Metzger, 2016). Although we did not analyse this specific brain area, we can hypothesize that trehalose might specifically improve motor learning abilities, rather than muscle-related motor function, in the sex showing the worst performance, possibly through mechanisms involving central modulation of OS status. This piece of data is partially supported by the observed increase in the levels of NRF2 and autophagy in the frontal cortex (but not in limb muscle) of trehalose-treated male mice. Our observations are in line with very recent evidence by Mizunoe and

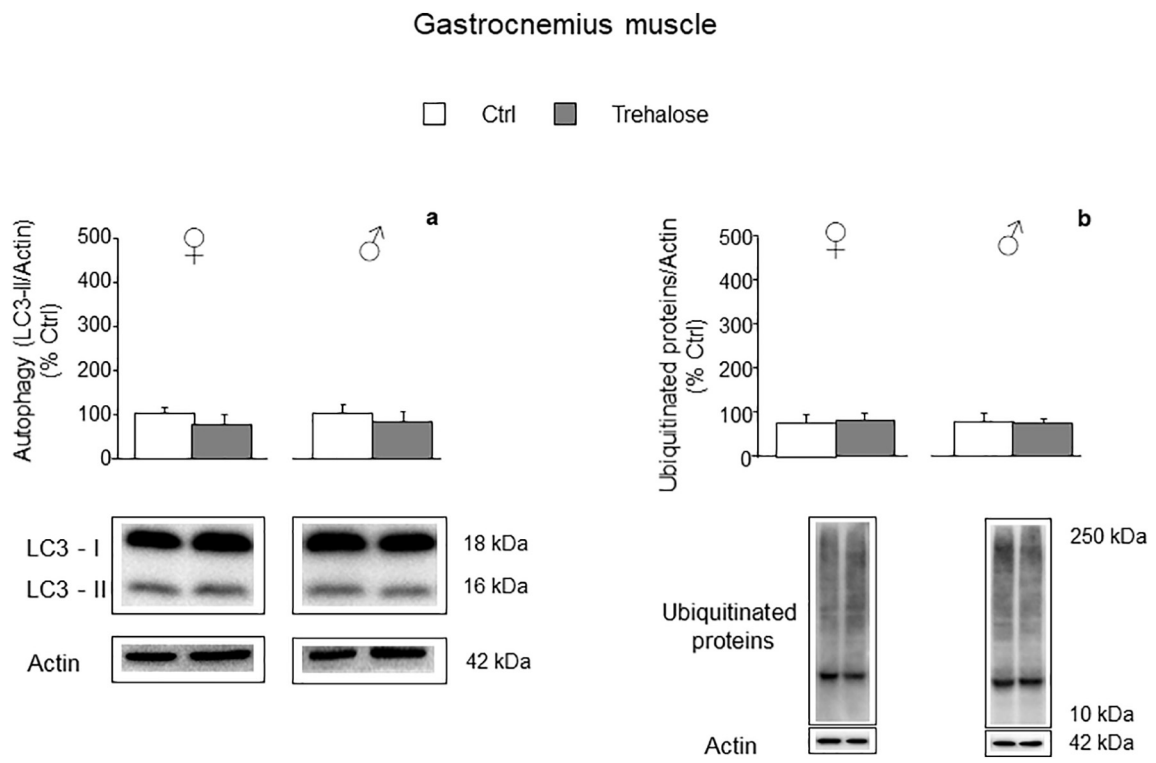


**Fig. 4.** Trehalose treatment did not affect neither the autophagic rate (a) nor levels of ubiquitinated proteins (b) in the gastrocnemius muscle. Data from densitometric analysis are shown as mean + SEM Protein levels of LC3-II, ubiquitinated protein and actin (control for sample loading) were analysed by western blot. Representative blots are shown. Subjects for each group: autophagy (LC3-II): F, control = 11; trehalose = 10; M, control = 10; trehalose = 11; ubiquitinated proteins: F, control = 11; trehalose = 11; M, control = 9; trehalose = 11.

colleagues suggesting that trehalose, acting as an antioxidant, protects against OS by regulating the KEAP1–NRF2 and autophagy pathways (Mizunoe et al., 2018). In more detail, trehalose might be able to promote the nuclear translocation and transcriptional activity of NRF2 - that triggers the transcription of downstream target genes encoding for antioxidant enzymes - as well as the activation of the autophagic machinery. As far as autophagy is concerned, this is an essential cellular function that regulates cellular homeostasis by degrading organelles and proteins. Worth noticing, disruption of autophagy has been associated with multiple pathological conditions ranging from cancer and metabolic disorders to neuro-inflammation and neurodegeneration (Nedelsky et al., 2008), all issues that may negatively impact on the quality of life during aging.

There are data to indicate that the brain of female mice is characterized by higher antioxidant capacity throughout life, including aging (Berry et al., 2012; Guevara et al., 2009; Sobocanec et al., 2003). Indeed, we found that female mice showed increased NRF2 levels, when compared to males. Thus, once again it is possible to hypothesize that the trehalose-dependent increase in NRF2 protein levels occurs in the sex that is more vulnerable (i.e. males). These results are in line with previous data from our group showing that females prenatally exposed to the antioxidant *N*-acetyl-cysteine, in association with a high-fat diet, are more resistant to changes in glutathione levels in the central nervous system compared to males, while the opposite holds true in the periphery (Berry et al., 2018).

Although there is a growing interest in understanding the mechanisms underlying sex differences in healthspan, these are still far from a complete comprehension and the role played by sex hormones throughout life (from early life to senescence) should not be neglected (Austad and Fischer, 2016). To this regard, it is well known that estrogens hold anti-oxidant and anti-inflammatory properties and we could speculate that this might explain, at list in part, the observed increased basal levels of NRF2 in the brain of old females. This result is also in line with our previous data showing preserved neurogenesis only in the brain of old p66Shc−/− mice an animal model of reduced OS (Berry et al., 2012).

As far as autophagy is concerned, both chromosomal make up as well as sex hormones may affect this important intracellular remodelling system throughout life, both in central and peripheral tissues. Within the brain, sex steroids are able to affect the process of autophagy through insulin and neurotrophic signalling, or through steroid receptors on mitochondria (Congdon and Sigurdsson, 2018; Tao et al., 2018). Thus, although we did not observe differences in basal levels of autophagy between males and females, but only in males as a result of treatment administration, we cannot exclude that different doses of trehalose might be required to modulate this system in a sex-dependent fashion. Further studies should address this issue.

## 5. Concluding remarks

Taken together, our data, although preliminary, suggest that oral trehalose administration leads to improved healthspan in old mice in a sex-dependent fashion with no overt side effects. Mechanisms potentially involved in the beneficial effects of trehalose seem to rely upon both the activation of the NRF2 pathway - and the consequent improvement in antioxidant defences - as well as to a fine modulation of autophagy; these mechanisms might be toned-down differentially by the aging process in male and female subjects. Further studies will need to carefully address sex differences identified in response to trehalose administration taking also into account the mechanisms underlying the crosstalk between peripheral and central tissues. Moreover, it would be worth assessing whether the observed improvement in healthspan, after a short-term trehalose administration (started at senescence), might also increase longevity.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exger.2019.110755.

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## Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

## Contribution statement

A.B. and M.M. designed all the experiments, carried out the statistical analyses and wrote the paper; F.C. designed all the experiments and wrote the paper; V.B. carried out all the behavioral experiments; R.V. and L.G. performed autophagy and ubiquitination analyses; P.M. and C.M. carried-out the analysis of oxidative markers and wrote the paper; C.F., M.L. and F.C. carried-out the statistical analysis and contributed to paper writing.

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