


# Effects of aging on proteasomal-ubiquitin system, oxidative stress balance and calcium homeostasis in middle-aged female rat colon

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## ABSTRACT

Aging is a multifactorial process, which is considered as a decline over time. It is increasingly clear that there is a gender difference in aging and in the prevalence of age-related diseases as well. We aimed to examine the effects of the aging process in the colonic tissue of female Wistar rats aged 10 weeks (young)

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and 13 months (middle-aged) at an early stage, according to three main symptoms associated with aging: a decrease in the efficacy of the proteasome and muscle function and an increase in oxidative stress. The aging process was found to cause a significant decrease in ubiquitin C-terminal hydrolase ligase (UCHL-1) and a significant increase in 3-nitrotyrosine (3-NT), total glutathione (GSH), calcium ( $\text{Ca}^{2+}$ ), calcitonin gene-related peptide (CGRP) and superoxide dismutase (SOD) activity in middle-aged animals. In summary, it is suggested that the reduced activity of the proteasomal degradation system may be the result of the diminished expression of the UCHL-1 enzyme and the decreased levels of ubiquitin; furthermore, we found some key targets which may help to better understand the fundamental aging process.

## KEYWORDS

aging, UCHL-1, proteasome, oxidative stress, calcium

## INTRODUCTION

Aging is a multifactorial phenomenon, which is considered as a general decline over time that occurs heterogeneously in numerous biochemical pathways and multiple organ systems [1, 2]. In addition, aging is regarded as a risk factor for several diseases, such as cancer [3], cardiovascular diseases [4], type II diabetes [5], and neurodegenerative disorders [6]. It is also suggested that a better understanding of the aging process may also be the key for the alleviation of age-associated pathologies [1].

As the largest surface exposed to external signals, the gastrointestinal (GI) tract plays an essential role in absorption, secretion and host defense [1, 7]. Aging has an impact on this organ system, which manifests particularly in constipation and delayed transit time in the elderly. A plethora of evidence suggests that key signs of the aging process, such as loss of neurons in the gut, increase in the amount of aggregated proteins, elevation in oxidative stress and decline of antioxidant pathways, are responsible for these impairments [8].

It is increasingly accepted that there is a gender difference in the aging process, and in the prevalence of age-related diseases [9] and gastrointestinal diseases as well. The female sex hormone estrogen appears to have a protective effect against several pathological conditions. In the postmenopausal phase of life this hormone declines in women, thus they lose this defensive line and tend to develop autoimmune or immune-mediated disorders [10]. A higher incidence of Irritable Bowel Syndrome (IBS) is also described in females, which suggests a role for sex hormones in autoimmune pathogenesis [11, 12].

Three main pathways, namely autophagy, lysosomal degradation, and the ubiquitin proteasomal degradation system (UPS) exist to eliminate damaged or misfolded proteins in the cell [13]. As a main component of UPS, the 26S proteasome is able to initiate degradation of the targeted (polyubiquitinated) damaged proteins. The 26S proteasome is a multi-protease complex, which is responsible for elimination of the majority of damaged proteins [14], and for the prevention of protein aggregation. For its appropriate function, several factors are necessary, such as ubiquitin. Furthermore, the proper function of the deubiquitinating (DUB) enzyme family also contributes to the efficiency of the 26S proteasome. Ubiquitin is a small, evolutionary conserved molecule, which has an essential role in targeting proteins for degradation [15, 16]. The DUB enzyme family is responsible for the availability of ubiquitin in the cell. As part of this



family, ubiquitin C-terminal hydrolase ligase-1 (UCHL-1), which is considered as a neuronal marker, is able to cleave ubiquitin monomers from the proteasomal targeting signal, the poly-ubiquitin chain [17]. This process enables recycling of ubiquitin, thus indirectly enhances the efficiency of the 26S proteasome. Age-related decrease in proteasomal activity has been described [18], but the background mechanism is unclear.

Reactive oxygen species (ROS), such as the hydroxyl radical ( $\text{OH}^-$ ), the superoxide anion ( $\text{O}^{2-}$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) are highly reactive substances [19], which are able to oxidize essential biomolecules (proteins, lipids, DNA) in the cell [20]. In addition, 3-nitro-tyrosine (3-NT), which is considered as an oxidative damage marker, is formed on L-tyrosine groups of proteins in correlation with the degree of oxidative stress. In order to reduce oxidative stress, a cellular defense system including several types of antioxidants, e.g. vitamins, enzymes, and antioxidant molecules emerged during evolution. Superoxide dismutase (SOD) is a principal antioxidant enzyme, which has an important part in the conversion of  $\text{O}^{2-}$  to  $\text{H}_2\text{O}_2$  [21]. Furthermore, glutathione (GSH), one of the key antioxidant molecules is a non-protein thiol, which plays an essential role as a modulator of cell proliferation and antioxidant defense [22]. It is suggested that through the aging process, oxidative mechanisms prevail and antioxidant pathways show a decline, thus the antioxidant system cannot keep pace with the damage.

Maintaining sufficient  $\text{Ca}^{2+}$  homeostasis is crucial for the cells in several molecular pathways. Numerous functions of  $\text{Ca}^{2+}$  has been described, such as muscle function or initiation of apoptotic pathways [23]. The influx of  $\text{Ca}^{2+}$  from the extracellular space is mostly based on transient receptor potential channels (TRPs). Of these, one of the most important channels in the colon is transient receptor potential vanilloid 1 (TRPV1) [24]. TRPV1 is a non-selective cation channel, which modulates physiological functions in the gastrointestinal tract [1, 19]. In addition, the calcitonin gene-related peptide (CGRP) is a potent vasodilator and an important component in the maintenance of  $\text{Ca}^{2+}$  homeostasis [25, 26]. Furthermore, nitric oxide (NO) is essential in normal colonic motility [27]. In the gastrointestinal tract it is expressed by the endothelium and causes smooth muscle relaxation, thus contributes to colon relaxation and enhances colonic transit. It is produced by the nitric oxide synthase (NOS) enzyme family through the conversion of L-arginine to L-citrulline. Three main types of NOS have been described: the inducible (iNOS) and constitutive forms (cNOS). cNOS includes endothelial (eNOS) and neuronal (nNOS) isoforms [28]. Aging is associated with impairment of the NO system, and a dysregulation can be detected in both muscle function and  $\text{Ca}^{2+}$  homeostasis, but the relationship is not well established.

In our study, we aimed to examine the effects of the aging process in the female colonic tissue of middle-aged rats, according to three main indicators of aging: decline in proteasome activity, reduced muscle effectiveness and oxidative stress acceleration. The main objective was reflecting on this fundamental process of aging in a way to suggest some key targets for the amelioration of age-associated pathologies.

## MATERIALS AND METHODS

### Experimental Design

All manipulations were performed in accordance with the standards of the European Community guidelines on the care and use of laboratory animals, in addition to the approval by the Institutional Ethics Committee at the University of Szeged.



Female Wistar rats ( $n = 20$ ) were purchased from Toxicoop Ltd. (Dunakeszi, Hungary). Animals were acclimatized for 2 weeks in a room with constant temperature, under 12-h day/night cycles with food and water ad libitum. After the acclimatization period, at the age of 10 weeks (young) and 13 months (middle-aged), animals were sacrificed and 8 cm of the colon measured from the rectum was cut, immediately frozen in liquid nitrogen and then kept in  $-80^{\circ}\text{C}$  until biochemical measurements.

### **ELISA measurements of 26S proteasome, ubiquitin, 3-NT, TRPV1 and CGRP levels in the colon**

Homogenization of the colonic tissue was performed in ice-cold phosphate buffer (200 mM  $\text{Na}_2\text{HPO}_4$ ; 230 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.4). The samples were centrifuged at 3,000 rpm  $4^{\circ}\text{C}$  for 20 min. The levels of 26S proteasome, ubiquitin, 3-NT, TRPV1 and CGRP were determined by quantitative Enzyme Linked Immuno Sorbent Assay (Delisa and deLateur), using rat marker specific ELISA kits (GenAsia Biotech Co. and SunRed Biotechnology Company, Shanghai, China). The five parameters were measured according to the manufacturer's instructions and protocols, and optical densities (OD) were determined at  $\lambda = 450$  nm. Results are expressed in:  $\mu\text{g}/\text{mg}$  for 26S proteasome,  $\text{ng}/\mu\text{g}$  for ubiquitin,  $\text{pmol}/\mu\text{g}$  for 3-NT,  $\text{pg}/\mu\text{g}$  for TRPV1, and  $\text{ng}/\mu$  for CGRP.

### **Measurements of $\text{Ca}^{2+}$ levels in the colon**

A Colorimetric Calcium Detection Assay Kit (Abcam, ab102505) was used to determine calcium ( $\text{Ca}^{2+}$ ) levels in the colon. Samples were homogenized on ice using PBS + 0.1% NP-40 and centrifuged for 5 min at 10,000 rpm. Supernatants were collected, and measurements were performed according to the provided procedure. Optical densities (OD) were detected at  $\lambda = 575$  nm. Results are expressed in  $\text{ng}/\text{well}$ .

### **Measurement of SOD activity in the colon**

The homogenization method described under ELISA was also used for SOD activity measurements. SOD activity was measured with a specific kit (Abcam, ab65354). Enzyme activity measurement in the colon samples was done according to the manufacturer's instructions, and OD was determined at  $\lambda = 450$  nm. Results are expressed as  $\text{ng}/\text{mL}$ .

### **Determination of UCHL-1 and eNOS expression in the colon by Western blot**

After powdering the tissues in liquid nitrogen with a porcelain mortar and pestle, in the case of UCHL-1 the powder was suspended in RIPA buffer (50 mM Tris, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton x-100, 150 mM NaCl); for the detection of eNOS 1 mM  $\text{Na}_3\text{VO}_4$  was added to the RIPA buffer. The homogenates were centrifuged at 12,000 rpm for 15 min at  $4^{\circ}\text{C}$ . Protein concentration was determined by the Bradford assay with bovine serum albumin as a standard. After 5 minutes in denaturing conditions ( $100^{\circ}\text{C}$  water bath), the same amount of protein was loaded on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto nitrocellulose membrane (Amersham Protran, No. 10600016, Germany) (Transfer time: UCHL-1: 2.5 h; eNOS: overnight). Blots were incubated in 5% milk overnight, and were then probed with monoclonal anti-UCHL-1 (1:1000, Abcam, ab108986) and anti-eNOS (1:500,



Abcam, ab76198). Membranes were next incubated with secondary antibodies: UCHL-1: anti-rabbit conjugated with horseradish peroxidase (1:5000, Santa Cruz Biotechnology, sc-2370), eNOS: anti-mouse conjugated with horseradish peroxidase (1:1000, Dako, P 0161). Signals were developed by an enhanced chemiluminescence system (Pierce ECL #32209, Thermo Fisher Scientific, Rockford, USA). Bands were analyzed by the Quantity One software (Bio-Rad). Results are expressed as intensity  $\times$  mm<sup>2</sup>.

### Measurement of GSH levels

Colon samples were homogenized in a solution containing 0.25 M sucrose, 20 mM Tris, and 1 mM dithiothreitol (DTT) and centrifuged at 15,000 g for 30 min at 4 °C. The supernatants were incubated in 0.1 M CaCl<sub>2</sub>, 0.25 M sucrose, 20 mM Tris, and 1 mM DTT at 0 °C for 30 min and then centrifuged at 21,450 g for 60 min at 4 °C. The cytosolic fraction was used for further analyses. A diluent buffer containing 125 mM Na phosphate and 6.0 mM EDTA was used for diluting stock solutions of glutathione (GSH), glutathione reductase, 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), and  $\beta$ -nicotinamide adenine dinucleotide phosphate ( $\beta$ -NADPH). All reagents were purchased from Sigma-Aldrich. 40  $\mu$ L of blank, standard, or colon sample and an equal volume of DTNB stock solution, then  $\beta$ -NADPH (140  $\mu$ L) were added to each well. After 5 min incubation at 25 °C, 10  $\mu$ L glutathione reductase was added to each well to initiate the reaction. After 10 minutes the absorbance was measured at 405 nm in a microplate reader. Results are expressed as nmol/mg protein.

### NOS activity measurement

For determining the activity of NOS, we used a previously described method [29] with minor modifications. The quantification is based on the conversion of [<sup>14</sup>C]-radiolabelled L-arginine to citrulline. A segment of colon was homogenized (Ultra-turrax T25; 13,500/s; twice for 30 s) in ice-cold 10 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES, Sigma-Aldrich), 32 mM sucrose (Sigma-Aldrich), 1 mM dithiothreitol (DTT, Sigma-Aldrich), 0.1 mM EDTA, 10  $\mu$ g/mL soybean trypsin inhibitor (Sigma-Aldrich), 10  $\mu$ g/mL leupeptin (Sigma-Aldrich) and 2  $\mu$ g/mL aprotinin (Sigma-Aldrich); pH 7.4. Homogenates were then centrifuged for 30 min at 20,000 g at 4 °C. Samples (40  $\mu$ L) were incubated for 10 min at 37 °C in 100  $\mu$ L of assay buffer [50 mM KH<sub>2</sub>PO<sub>4</sub>, 1.0 mM MgCl<sub>2</sub>, 50 mM L-valine, 0.2 mM CaCl<sub>2</sub>, 1.0 mM DTT, 1.0 mM L-citrulline, 15.5 nM L-arginine, 30  $\mu$ M flavin adenine dinucleotide, 30  $\mu$ M flavin mononucleotide, 30 M tetrahydro-L-biopterin dihydrochloride, 450  $\mu$ M  $\beta$ -NADPH, 12 pM [<sup>14</sup>C]-L-arginine monohydrochloride (all from Sigma-Aldrich). The reaction was terminated by the addition of 0.5 mL of 1:1 (v/v) suspension of ice-cold DOWEX (Na<sup>+</sup>-form) in distilled water. The mixture was resuspended with the addition of 850  $\mu$ L of ice-cold distilled water. The supernatant (970  $\mu$ L) was removed and radioactivity was measured by scintillation counting. Calcium dependence of NOS activity was determined by the addition of 10  $\mu$ L of ethylene glycol-bis ( $\beta$ -aminoethyl ether) tetraacetic acid (EGTA; 1 mM, Sigma-Aldrich). NOS activity was confirmed by inhibition with 10  $\mu$ L of N-nitro-L-arginine-methylester (LNNA; 3.7 mM, Sigma-Aldrich). Constitutive NOS activity was calculated from the difference between citrulline formation that was inhibited by EGTA and the total activity. As the nature of the constitutive isoform (eNOS or nNOS) was not determined, this activity is referred to as cNOS. NOS activity is expressed as pmol/min/mg protein.



## Protein determination

For the determination of protein concentration, we used the Bradford method with bovine serum albumin (BSA) as a standard. 20  $\mu\text{L}$  was taken from the diluted sample (25 $\times$  or 50 $\times$  with distilled water) and mixed with 980  $\mu\text{L}$  distilled water. A 200  $\mu\text{L}$  volume of Bradford reagent was added to each sample. After mixing and 10 min of incubation, samples were measured by spectrophotometry at 595 nm. Protein concentration is expressed as mg protein/mL.

## Statistical analysis

All data are presented as mean  $\pm$  SEM. Statistical comparisons were performed using Student's two-tailed unpaired t-test. Differences were considered significant when  $*P < 0.05$ ;  $**P < 0.01$ .

## RESULTS

### Aging leads to a decrease in UCHL-1 expression in female middle-aged vs. young rat colon

Upon analyzing the expression of UCHL-1 by Western blot, we observed a significant decrease in the middle-aged group as compared to the young. The reduction was almost by half in the middle-aged group, from  $575.07 \pm 60.49$  to  $304.65 \pm 38.48$  intensity  $\times$   $\text{mm}^2$  (Fig. 1A).

### Effects of aging on ubiquitin level in middle-aged rat colon

As a possible marker of proteasome activity, ubiquitin levels were measured via a specific ELISA kit. We found decreased ubiquitin levels in the colon of the middle-aged group, but this was not significant compared to the young group (Fig. 1B).

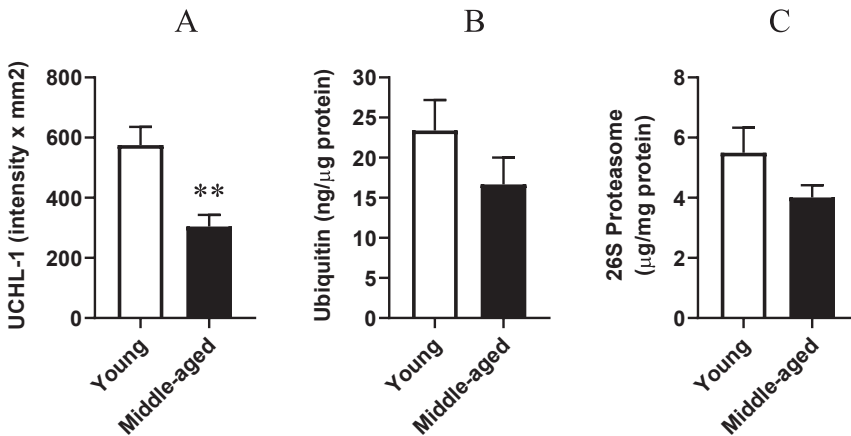


Fig. 1. Effect of aging on the expression of ubiquitin C-terminal hydrolase ligase-1 (UCHL-1) (panel A) and on ubiquitin (panel B) and 26S proteasome levels (panel C) in middle-aged rats. Data are shown as mean  $\pm$  S.E.M; ( $n = 5-7$  animals/group)



## Effects of the aging process on 26S proteasome level in middle-aged rats

To identify the changes in the 26S proteasome we used an ELISA method, and we found that aging caused a marked decrease in 26S proteasome levels in middle-aged rats compared to young females, but the difference between the groups was not statistically significant (Fig. 1C).

## 3-NT levels were increased due to aging in middle-aged vs. young rat colon

We measured 3-NT levels, a well characterized oxidative stress marker in the colon with a specific ELISA kit. We observed a significant increase in 3-NT levels in the middle-aged ( $0.069 \pm 0.007$  pmol/ $\mu$ g protein) compared to the young group ( $0.049 \pm 0.005$  pmol/ $\mu$ g protein) (Fig. 2A).

## Aging caused an elevation in SOD enzyme activity in middle-aged female rat colon

The antioxidant superoxide dismutase enzyme is especially important in eliminating reactive oxygen species. In our study SOD activity was measured by ELISA method. We found a significant increase in SOD activity in middle-aged rats with more than two-fold higher values ( $291.54 \pm 45.76$  inhibition rate %) than in young rats ( $124.38 \pm 15.29$  inhibition rate %) (Fig. 2B).

## Total levels of GSH were elevated in the colon of middle-aged rats

GSH is a highly conserved thiol in eukaryotes which contributes to antioxidant defense. As shown in Fig. 2C, GSH concentration was significantly increased in the middle-aged ( $72.59 \pm 6.65$  nmol/mg protein) compared to the young group ( $50.96 \pm 4.64$  nmol/mg protein).

## Calcium levels were increased in middle-aged vs. young rats

Calcium content was measured by a specific ELISA. Analysis of the data revealed a significant increase in the calcium content of the middle-aged ( $1814.74 \pm 179.08$  ng/well) compared to the young group ( $1203.49 \pm 112.47$  ng/well) (Fig. 3A).

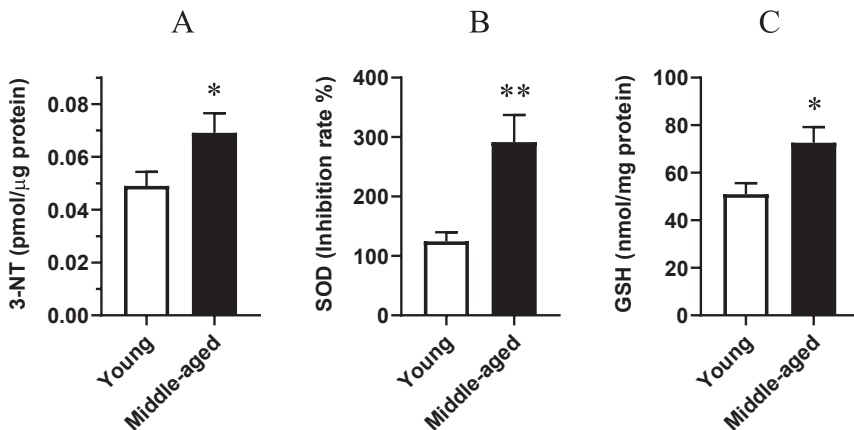


Fig. 2. Effect of aging on the level of 3-nitrotyrosine (3-NT) (panel A), activity of superoxide dismutase (SOD) (panel B) and levels of glutathione (GSH) in middle-aged rats (panel C). Data are shown as mean  $\pm$  S.E.M; ( $n = 6-7$  animals/group)



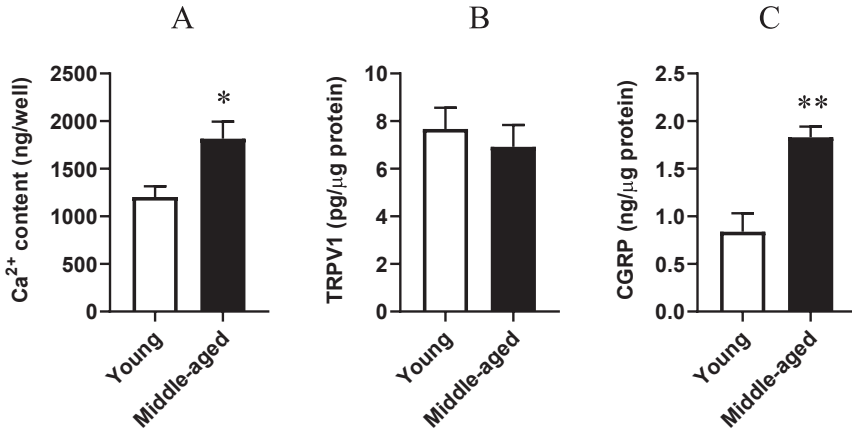


Fig. 3. Changes in the calcium (panel A), Transient Receptor Potential Vanilloid 1 (TRPV1) (panel B) and Calcitonin Gene Related Peptide (CGRP) levels of middle-aged and young colon (panel C). Data are shown as mean ± S.E.M; (n = 5-6 animals/group)

### Effects of aging on the TRPV1 channel in middle-aged rats

The level of the nonselective cation channel TRPV1 was measured via a specific ELISA kit in our study. We found that TRPV1 was not changed significantly by the aging process, as there was no difference between the groups (Fig. 3B).

### Aging led to an elevation in CGRP levels in middle-aged female rats

We measured CGRP levels in the colon with ELISA method and found that aging resulted in a significant elevation in middle-aged rats (1.83 ± 0.11 ng/μg protein) compared to the young group (0.84 ± 0.19 ng/μg protein) (Fig. 3C).

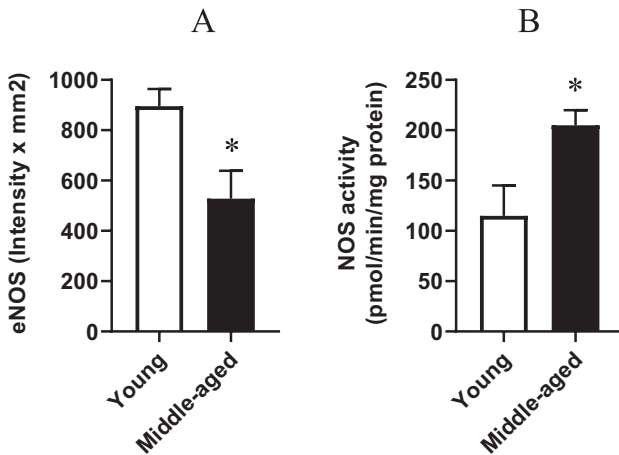


Fig. 4. Effects of aging on the expression of eNOS (panel A) and on the activity of cNOS in middle-aged rats (panel B). Data are shown as mean ± S.E.M; (n = 5-6 animals/group)





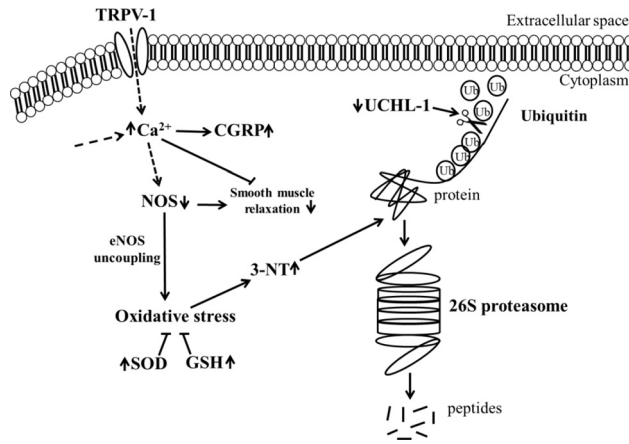


Fig. 5. Summary of the measured parameters. TRPV1: Transient Receptor Potential Vanilloid 1, CGRP: Calcitonin Gene Related Peptide, UCHL-1: Ubiquitin C-terminal hydrolase ligase-1, 3-NT: 3-nitrotyrosine, SOD: superoxide dismutase, GSH: glutathione, NOS: nitric oxide synthase

### Aging caused a decrease in the expression of eNOS, while increased the activity of cNOS in middle-aged rats

NO is a gasotransmitter which causes relaxation of smooth muscles. It is produced by nitric oxide synthases, mainly eNOS. Therefore, we measured the expression of the eNOS enzyme (panel A) via ELISA, and the activity of cNOS (panel B) in the colon. We found a significant decrease in eNOS expression and a significant increase in the activity of cNOS due to aging in middle-aged rats ( $204.8 \pm 15.011$  pmol/min/mg protein) compared to the young group ( $114.8 \pm 30.203$  pmol/min/mg protein) (Fig. 4).

## DISCUSSION

In our study we investigated the effects of aging on female rat colon at a relatively early aging stage (middle-aged) through examining several key elements, which may responsible for the formation of aging symptoms in the elderly: the proteasomal degradation system, the oxidative stress/antioxidant pathways and factors related to muscle function (Fig. 5). Our results suggest that the aging-related decrease in proteasome activity is already observable at a relatively early stage of the aging process, in middle-aged rats. We suggest that this activity loss perhaps occurs due to the diminished expression of UCHL-1 and the mitigated levels of ubiquitin, rather than the reduction of the proteasome level itself, in which some oxidative stress parameters and  $Ca^{2+}$  homeostasis play a role.

Aging is associated with a general functional decline due to an imbalance in cell homeostasis. This manifests in a shift towards oxidative mechanisms and a decline in the efficiency of the proteasome, thus the aging organism will be less resistant to stress conditions and to several diseases [30]. It is demonstrated that the aging process affects the GI tract, which is supported by



the following signs and symptoms: delayed transit time, constipation, neuronal loss, and general low-grade chronic inflammation, but the underlying mechanisms are not well elucidated [31, 32]. It is well-known that due to the presence of different gonadal hormones, there are pre-disposition differences between males and females in the aging process. Estrogen hormone appears to play a protective role in several organs. In the stage of menopause, which means low estrogen levels, females go through a physiological decline due to the loss of the beneficial effects of estrogen [33].

Conflicting data exist on the relationship between human and rat aging in the literature, but it is widely accepted that rats undergo an accelerated aging process. Several studies suggest that depending on the life phase of the animal, a period of time can be calculated (14–19 days), which correlates to human age. In average, a 17-day period for a rat seems to be equal to 1 human year. Based on these, in our study the age of the rats included in the young group was 10 weeks, which equals human young age (~12 human years), and the age of those in the middle-aged group, 13 months, which equals ~40 human years [34].

The proteasome degradation pathway as one of the most important protein degradation pathways is in the focus of intensive research [35]. The proteasome consists of several protease enzymes, thus this multi-protein complex is able to cleave polyubiquitinated damaged proteins [36]. Numerous research publications reported that the efficiency of the proteasome shows a decay through the aging process, but the exact mechanism is not fully understood [37]. It is suggested that the promotion of this fundamental degradation pathway may be the key to decelerate aging-associated diseases [38]. To further examine this activity loss, ubiquitin and 26S proteasome levels were measured in the colon, along with the expression of the UCHL-1 enzyme. UCHL-1 is a deubiquitinating enzyme family member, contributing to proteasome effectiveness [39]. Regarding the proteasome levels there was no statistical difference between the middle-aged and young groups in our study. Giannini et al. used aged rat brain to determine the 20S and 26S proteasome activities and levels, and they found no statistical difference between the 3-week-old young and the 24-month-old aged animals [40]. Our results are consistent with theirs in case of the 26S proteasome in 13-month-old rats, thus it seems that the level of the 26S proteasome is not impaired by the aging process even at an earlier phase of aging, in middle-aged rats. Interestingly, however, Altun et al. found a significant increase in the 26S proteasome level and activity in the skeletal muscles of 30-month-old Sprague-Dawley rats, compared to 4-month-old adult ones [41]. This discrepancy with our results measured in the colon suggests that the proteasome system's insufficiency is not specific for aging and it may depend on the type of the tissue as well.

Our results also showed that the expression of ubiquitin and UCHL-1 enzymes undergo a decline, which is consistent with the findings of Coulombe and colleagues [39]. They investigated the effects of aging on the enteric nervous system in 3-month-old UCHL-1 knockout mice, and found that the loss of the UCHL-1 enzyme may induce age-related changes in the brain. Zhang et al. found the same changes in UCHL-1 expression in a mouse model of Alzheimer's disease, and proposed that the overexpression of this enzyme could be a therapeutic strategy to reduce the plaque formation and to treat this serious age-associated health issue [42]. Based on our above-mentioned results we suggest that the widely reported decrease in proteasome activity might occur due to the reduction in the expression of the UCHL-1 enzyme and thus ubiquitin levels, and is less affected by the proteasome level itself in the aging process.



Oxidative stress is considered to be an imbalance between oxidative mechanisms and antioxidant defense systems. The oxidative stress theory of aging claims that oxidative stress is the main factor in the induction of the aging process [43]. To clarify the effects of aging in the colonic tissue, 3-NT was measured as an oxidative stress marker, and a significant increase was found in middle-aged animals. Our results are in accordance with the oxidative stress theory along with several previous reports [44, 45]. From these, Thangaswamy et al. compared the 3-NT levels measured in the mesenteric lymphatic vessels of aged (24 months) and adult (9 months) Fischer-344 rats, and found an elevated 3-NT production in the aged group. On the other hand, Cakatay et al. [46] examined 3-NT levels in Wistar rat brains of three main age stages: young (5 months), adult (13 months) and old (24 months), and they found a significant reduction in the amount of 3-NT. Interestingly, they found a significant change only in the comparison between young and old animals, and not between young and adult. This finding goes against our results, because we found an elevated 3-NT production already at an earlier aging stage (13 months) in the colon. To investigate the impact of aging on the antioxidant defense, the activity of the SOD enzyme and total GSH level were measured. In the case of the SOD enzyme a significant enhancement was found in its activity. In contrast with our findings, in the same previously mentioned study Thangaswamy et al. [45] showed a reduction in the activity of SOD through the aging process. This discrepancy is presumably due to the different tissue type or to the fact that Thangaswamy et al. investigated a subsequent phase of the aging process (24 months). In addition, Vucevic et al. investigated the activity of SOD enzyme as well, but in three different parts of the GI tract, namely the stomach, the pancreas and the liver of 18-month-old Wistar rats. They also found a decline in SOD activity through the aging process in all the tissues examined, compared to the 3-month-old adult group [47]. The significant elevation in GSH antioxidant molecule levels in the middle-aged colon, which we observed, is in contrast with the outcomes of both Sandhu and Kaur [48] and Liu [49], who found a reduction in GSH levels in aging male rat brain. We presume that the difference between these findings are due to the differences in tissue type and gender. In contrast to these, but accordance with us, Hoensch et al. [50] found in human colon mucosa that there are gender- and age-related differences in GSH-related enzymes such as GSTP1 and GST. They showed that in over 50-year-old female individuals there is a detectable elevation in the GSH system, which suggests a reduced vulnerability against oxidative stress for females through the aging process.

In the elderly population, a decrease in the colonic motility and a reduction in transit time are evident, but the exact mechanisms of these are not fully understood [51]. To test this notion, key elements related to muscle function and  $\text{Ca}^{2+}$  homeostasis were measured. Calcium is one of the most important ions for the normal function of muscle fibers, and it is also crucial for the initiation of apoptotic pathways [52]. In our study,  $\text{Ca}^{2+}$  content was significantly increased in middle-aged rat colon. Oh et al. [53] found the same changes in  $\text{Ca}^{2+}$  levels in the hippocampus of male Fischer 344 X Brown Norway rats in 29–31-month-old versus 2–4-month-old groups. Their evidence, along with ours, suggests that aging causes elevation in  $\text{Ca}^{2+}$  levels, which occurs already in an earlier phase, way before the actual old age (>20 months). We also measured the non-selective cation channel TRPV1, to find out whether it plays a role in the observed increase in  $\text{Ca}^{2+}$  levels, but TRPV1 levels showed no statistical differences between young and middle-aged colon. Keating et al. [54] investigated the mechanosensory function and TRPV1 mRNA expression in C57BL/6 mouse GI tract at the age of 3, 12 and 24 months. They found a significant loss of TRPV1 function in the colon of



24-month-old rats, but the expression of TRPV1 was not affected at the age of 12 months, which is consistent with our findings. This suggests that TRPV1 has a role in aging, but it seems maybe only in the later phase of the process. CGRP was also measured with respect to  $\text{Ca}^{2+}$  homeostasis, and in our study it was increased significantly in the middle-aged rats. Carrier and Connat [55] found a significant difference regarding gender in CGRP in hepatic portal veins. They compared 19-day-old and 22-month-old Wistar SIV rats and they found that aging has apparently no effect on CGRP innervation in female portal veins, but has an effect in males. In contrast with our findings, Kang et al. [56] showed a decrease in CGRP, and suggested a role for this protein in the susceptibility of GI to mucosal damage. Furthermore, as  $\text{Ca}^{2+}$  is essentially important in apoptosis [57], elevation of  $\text{Ca}^{2+}$  levels in the colonic cells led to cell death and neurodegeneration. This causes a reduction of muscle innervation and age-related symptoms in the colonic tissue, which occurs already in an early phase of the aging process, in middle-aged rats.

Nitric oxide has several important physiological functions, such as vasodilation, inhibition of leukocyte adhesion, thrombocyte aggregation and smooth muscle cell proliferation [58]. By measuring the expression of eNOS and the activity of cNOS, we found that the expression showed a decline, but the activity of the enzyme increased in middle-aged rats. Takahashi et al. [59] observed the same reduction in the expression of the nNOS enzyme in the colon of 22-month-old Fisher rats, suggesting that the decelerated colonic transit is a consequence of the expression change measured.

## CONCLUSIONS

In summary, our study suggests that the observable reduction in proteasomal efficiency, which already occurs in a relatively early phase of aging may actually be due to the lower expression of the UCHL-1 enzyme and the decreased availability of ubiquitin. Furthermore, we found some key components, which might help shed light on the molecular mechanisms of this fundamental age-related deterioration.

*Conflicts of interest:* The authors declare that there is no conflict of interest.

*Author contributions:* All authors contributed to the study conception and design. Material preparation was performed by SzT and AA, data were collected by SzD, data analysis was performed by ÁCs, CsT, ZsM. The first draft of the manuscript was written by NA and ZsM, the manuscript was reviewed by KK, CsV, DB and AP. All authors read and approved the final manuscript.

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