Cellular autophagy – treatment target for the aging process

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Abstract

Autophagy is a protein degradation system, a catabolic process as a response to stress or as a natural way to destroy and recycle useless or deteriorated components. The term is derived from a Greek word meaning "autodigestion" and the process is present in all eukaryote cells. The process was first observed by K. Porter and T. Ashford in 1962 in the liver of a rat after administering glucagon and a year later, in 1963, was named "autophagy" by the Belgian biochemist C. De Duve (Klionsky et al., 2008).

Cellular organelles are enclosed in double membrane vesicles called autophagosomes and are subsequently presented to the lysosomes in order to be broken down.

Autophagy is a basic function in the organisms of mammals and is influenced by certain factors such as oxidative stress, hunger and hypoxia.

We **hypothesized** that pharmacological stimulation of autophagy leads to partial regeneration of aged tissues and thus stimulates healthy aging from a cognitive, as well as a functional point of view.

Our **objectives** were: testing cognitive function and spatial memory (Water-Maze), testing motor function (Rotor-Rod), observing the survival period, studying brain autophagy processes at tissue and cellular level in the brain and the liver through genomic, proteomic and immunohistochemical methods.

Although a research subject of great interest, until now studies have never been conducted on aged rats, but only on Drosophilla and yeasts, which do not reflect the mechanisms of mammal autophagy.

The doctoral thesis is comprised of two parts and seven chapters. Part one consists of general data about the current state of knowledge in the field of aging and autophagy mechanisms, whereas the second part of the thesis includes personal contributions to the study of autophagy pharmacological manipulation by using two groups (treatment and control) of randomly selected old Sprague-Dawley rats (18 – 24 months old) and describes the techniques and protocols used, as well as the study's results.

Significance of the studied topic

Aging, a normal and inevitable process, has an important effect on the body and on the mind. Cardiovascular diseases affects other organs including the brain. Depression and stroke have a higher incidence in the elderly. As life expectancy increases, the is an ever growing interest in preventing these diseases and multiple studies are looking for the answer to a healthy aging.

The world's population is aging at an alarming rate – in the year 2000, approximatively 10% of the entire population was over 60 years old. It is estimated that in the year 2050, this percentage will increase to approximatively 21%.

Cellular autophagy is a process of degradation, of cellular protection, which is present in all eukaryote cells. Recently, several studies have focused on various aspects of cellular autophagy. This process plays an important role in cytoprotection and in maintaining cellular homeostasis by preventing accumulation of toxic proteins and by eliminating pathogens. Studies have linked autophagy to multiples conditions such as cancer, myopathies, neurodegenerative disorders, diabetes or inflammatory diseases.

Mechanisms of autophagy

Cellular autophagy is the process through which cellular organelles are broken down by lysosomes and the resulting substances are reused (Klionsky et al. 2011).

The first step of the process is represented by the formation of the phagophore at the pre-autophagosomal site. The second step is the elongation of the phagophore and the third step includes the autophagosome maturation and the assimilation of the cellular content. The last step involves the fusion between the mature autophagosomes and the lysosomes, which leads to the formation of a unique compartment called an autolysosome (Eskelinen et al. 2009; Ravikumar et al. 2010). In mammals, an equivalent of the pre-autophagosomal site, present in yeasts, has yet to be discovered (Klionsky et al. 2007). The substrate is enclosed in double membrane vesicles, called autophagosomes. This substrate consists of damaged organelles, degraded proteins and invasive pathogenic agents.

In yeasts, certain proteins (atg) have been found to have a regulatory function. In mammals, there similar proteins (ATG), that have the same functions (Feng et al.2014; Yang et al. 2010). mTORC1 and AMPK modulate autophagy in case of hunger. In the presence of nutrients and growth factors, the complex formed by ULK1, ATG13, ATG101 and FIP200 is down regulated by MTOR, resulting in the suppression of autophagy (Jung et al.2010; Mizushima et al.2010, Chan et al.2012; Hosokawa et al.2009; Wong et al.2013).

On the other hand, hunger inhibits mTORC1, activates AMPK și ULK1 and as a result autophagy is stimulated and initiated (Jung et al.2010;Mihaylova et al.2011;Kim et al.2011).

The Beclin1 complex, which includes Beclin 1, p150, Atg 14L, Ambral, Rubicon, is involved in the formation of the autophagosomes and in the increase of phosphatidylinositol-3-phosphate (PI3P) production in the presence of autophagy stimulating factors (He et al.2010; Itakura et al.2008). In the elongation process, two ubiquitin-like systems are involved: Atg5-Atg12si LC3-Atg8 (Nagatogawa et al.2013).

Oxidative stress appears when reactive oxygen species surpass the cellular reserve of antioxidants and the cells undergo various modifications. Multiple studies confirm that oxidative stress stimulates autophagy, which leads to the recovery of functionality and cellular protection. (Chen et al.2007; Chen et al. 2008).

Other studies suggest that there is a connection between autophagy stimulation and the increase and decrease in surrounding oxygen pressure (Tanaka et al.2012, Lee et al.2011).

Some authors consider that cellular autophagy can be involved in acute respiratory insufficiency and sepsis, two major causes of morbidity and mortality in the entire world (Ryter et al. 2014). Acute respiratory insufficiency can be determined by mechanical ventilation and hyperoxygenation. Tanaka and collaborators have discovered that exposure to hyperoxia leads to an increase in autophagy marker values, both histologically, as well as biochemically, which will lead to the accumulation of LC3B-II and will start the autophagy process (Tanaka et al., 2012).

Chen and collaborators have researched autophagy markers in lung tissue of patients suffering from Chronic Obstructive Pulmonary Disease. They discovered that several markers were increased, such as LC3-II and other proteins from the ATG group. Furthermore, they found that there is an increase in autophagosome formation as a result of exposure to cigarette smoke. (Chen et al 2008).

The presence of certain genetic deficits in the molecular process of autophagy are involved in multiple diseases, for exemple: Atg 7 in the case of Klebsiella pneumoniae or Atg 9 in the case of Legionella pneumophila (Tung et al.2010; Ye et al.2014).

Other authors claim that autophagy is involved in neuroprotection in model an animals affected by Huntington's disease or Alzheimer (Menzies et al.2011), while some studies confirm its importance in Parkison's disease (Nixon et al.2012).

The replication of hepatitis C virus is conditioned by cellular autophagy, therefore by inhibiting this process, viral replication could also by inhibited, leading the way to a new therapeutic target (Ke et al.2014;Sir et al.2008). Vescovo and collaborators have performed hepatic biopsies in patients infected with hepatitis C and have noticed a reverse correlation between autophagy activation and hepatic steatosis concluding that autophagy can remove excess (Vescovo et al.2012;Ke et al.2014).

Material and methods.

Model animals and experiment design

The study is conducted on animal model, 60 male Sprague Dawley rats (500 – 900 g) aged 18-20 months at the beginning of the study. They were kept in standard laboratory conditions, in a facility dedicated to animal research in the University of Medicine and Pharmacy of Craiova and were approved by the Ethics Board.

Randomization

Rats were randomized into two groups - control and treatment, depending on their performances in preliminary tests.

Administering treatment

A substance that stimulates autophagy (Spt100) was administered in the drinking water of laboratory animals, 25 mg/kg/day in 0.2% Trehalose for the treatment group, while the control group received only the solution of 0.2% Trehalose for the first experiment. For the second experiment, 25 mg/kg/day of SPT 100 were administered, without trehalose, while the control group received only water and for the third experiment only a dose of 5 mg/kg/day was used.

Behavioral tests

The behavioral tests that were conducted were: Rotor rod – Rotating pole (for the evaluation of motor function), Water-maze (for the evaluation of cognitive function), Forced-swim (for the evaluation of motor function, of the desire to survive), Latency – curiosity (for evaluating the interest for the environment), Elevated plus maze (for the evaluation of anxiety). The tests were performed at 14-day intervals.

Evaluation of vestibular-motor function through the rotarod test (rotating pole)

This test evaluates vestibular and fine motor functions in animal - models. Each rat was tested for its capacity to cross a rotating pole, for which the rotating speed can be modified.

The device is composed of a 160 cm long cylinder with a 12 cm diameter, covered with a semi rough surface to ensure that the rats do not fall off. The cylinder can spin and its speed can be adjusted to values between 0 and 6 rotations per minute.

The rat was placed inside a cylinder and we recorded the time needed to cross the cylinder and to reach its distal end where a group of rats inside a cage is waiting, in order to stimulate his

crossing. Their behavior during the crossing of the rotating pole was quantified and used to assess their neurological state.

Two types of results were recorded: the time needed to cross and the score given by the observer depending on the behavior of the animal model during the crossing according to the following rules:

0 – the rat falls immediately after being placed on the cylinder;

1 – the rats stays on the pole, but does not advance, maintaining though his position;

2 – the rat advances, but falls before reaching the distal end of the cylinder;

3 – the rat successfully crosses, having some difficulty in maintaining his equilibrium;

4 – the rats crosses without any faults the cylinder, having symmetrical movements of its limbs.

For this test, the rats have to be trained at least 10 days before the treatment, initially without spinning and afterwards slowly increasing the rotation speed until it reaches 6 rotations per minute.

Morris Water Maze

The water maze test, described by Morris, was used to evaluate memory and spatial learning. One week before the beginning of treatment, the elderly rats were trained to find a submerged platform in a large pool (180 cm in diameter) at a maximum depth of 20 cm. The pool is dived into 4 quadrans (North, South, East, West). Various stimuli were placed in each of the quadrants. In order to determine their special learning capacity, each animal was subjected to 4 daily trials over a period of seven days.

Forced swim

The test is conducted in a 20 cm diameter cylinder

1. The cylinder is filled 3/4 with water. Ideally, the rat should not touch the base of the cylinder neither with his claws, nor with his tail. It is important that he does not swin freely or float in the cylinder.

2. Water temperature should be between 22-26 degrees Celsius.

3. A video camera is placed so that the entire cylinder can be recorded.

4. The rat is placed in the water starting with his hind legs.

5. The rat is removed from the water when it can no longer hold his nose above the water surface. There is a possibility that during the test, the rats will try to find an escape at the base of the cylinder. If they do not surface in 3-4 seconds or do not swim, they will be removed from the cylinder.

6. The rat is taken out of the cylinder no more than 15 minutes after it was submerged.

7. During the experiment we measured the total time in seconds, the rat's resistance to effort and the determination to survive (maintaining the capacity to breathe).

8. The activity time in the cylinder is measured (in seconds) – the period of time during which the rat is moving, swimming or climbing, is not resting against the walls of the cylinder (the determination to survive).

9. The time that the rat stays still without moving is measured (in seconds) – the time during which the rat gives up trying to escape which suggests depression.

Tests for the evaluation of anxiety. Elevated plus maze.

We used a four-armed labyrinth that was suspended 70 cm above the floor: two arms were closed and dark and the other two open. The rat is placed at the end of one of the open arms and is filmed for 5 minutes. We measured the time spent by the rat in the open arms (the desire to explore, the lack of anxiety) and the time spent in the closed arms (anxiety).

At the end of the experiment the animal model was killed, tissue samples were taken and genomics, proteomics and immunohistochemistry studies were conducted.

Results

ROTATING POLE (ROTOR ROD)

Score: Before treatment, the rats cross the pole at a speed of 3 rotations per minute with certain difficulties related to age and receive a score of 3. When the difficulty is increased (6 rotations per minute), the aged rats received a score of 2. Initially, no differences were noticed between the the two groups of animals. After starting treatment with SP 100 (25 mg/kg/day), their test performances decreased progressively with age for both study groups over a 14-day period. Consequently, treated rats began to register better results that the control rats and the improvement became statistically significant for both speeds at week 28. The treatment significantly influenced the test scores (Two-way Anova: $F=5.57$, $p=0.02$, for 3 rpm and $F=3.64$, $p=0.06$ for 6 rpm), which improved significantly over time ($F=2.62$, $p=0.0016$ for 3 rpm and $F=2.10$, $p=0.01$ for 6 rpm) resulting in a statistically significant treatment-time interaction ($F=21.17$, $p<0.0001$ for 3 rpm and $F=9.61$, $p<0.0001$ for 6 rpm).

Time: Pretreatment rats crossed the cylinder in approximatively 15 seconds at 3 rotations per minute. When difficulty was increased (6 rotations per minute), the aged rats crossed the cylinder in approximatively 22-26 seconds. No significant differences were observed between the performances of the control animals and the treated animals. After administering SPT100 (25 mg/kg/day) in their drinking water, the time needed to cross the pole at 3 rpm increased to 40 seconds for the control group and remained 22 seconds for the treatment group. At 6 rotations per minute, the rats in the control group crossed the rotating pole in 38 seconds, while the performance of treated animals did not decrease significantly, except during the last days of treatment. We noticed a significant improvement in the time required to cross the rotating pole starting with week 18, for both speeds, but which was transitory for the 6 rpm difficulty. The treatment significantly influenced the time necessary to cross the rotating pole ($F=6.36$, $p=0.017$ for 3 rpm, $F=4.75$, $p=0.037$ for 6 rpm) and the crossing period significantly improved over time ($F= 4.69$, $p<0.001$ for 3rpm, $F=2.44$, $p=0.0035$ for 6 rpm), resulting in a significant treatment-time interaction ($F=19.66$, $p<0.0001$ for 3 rpm and $F=6.14$, $p<0.0001$ for 6 rpm).

WATER MAZE

During the 2-week training period, the rats learned to identify and climb the hidden platform and their performance improved over this time. For both groups, the path taken to the platform became shorter and shorter with each training sessions. Before the beginning of treatment, the time needed to find the platform was 24 seconds for both groups. After the beginning of treatment with SPT100 (25 mg/kg/day) administered in their drinking water, the time needed to identify the platform increased abruptly up until week 28 for both groups. In the treated group a transitory improvement was observed that was statistically significant ($F=8.93$, $p=0.0066$).

WEIGHT VARIANCE DURING TREATMENT

Before the beginning of treatment, at 18 months of age, the rats weighed, on average 721 g. After the beginning of treatment, the aged rats progressively lost weight, but animals in the treatment group were considerably heavier than those belonging to the control group ($p<0.0001$). Despite this, the treatment – time interaction was not significant. Before being killed, the control group had an average weight of 454 g, while the treated animals had an average weight of 532 g.

LIQUID CONSUMPTION DURING THE TREATMENT PERIOD

Before the beginning of treatment, at the age of 18 months, the volume of water ingested was, on average, 35 ml/animal/day. After the beginning of treatment the volume decreased over time to 22 ml/animal/day for the control group and 26.6 ml/animal/day for the treatment group ($p=0.68$ according to t-test). The treatment had no significant influence on water intake.

A significant difference was observed when measuring liquid consumption in the first experiment (25 mg/kg/day concentration with trehalose), during which the treatment group drank

more liquids that the control group (t test, p=0.0081). There were no differences in food ingestion.

TEST FOR THE EVALUATION OF ANXIETY (ELEVATED PLUS MAZE)

The elevated plus maze test is a behavioral test frequently used for evaluating anxiety in rodents. Animals in the control group showed a reduced interested in exploring their environment, spending more time in the dark arms, which is an indicator of anxiety. The treatment had a positive effect on animals, the difference between the groups being significant (F=4.81, p=0.03).

LATENCY TEST – CURIOSITY

Aging is associated with a decrease in curiosity. The latency in exploring the cylinder was recorded for a period of 5 minutes for the elderly animals and subsequently analyzed in a double-blind manner using a slow motion camera. Regarding the latency time, during the treatment period, significant differences were observed (F=5.26, p=0.0007) between the control group and the treatment group.

FORCED SWIM TEST

Force and endurance diminishes with aging. We measured the desire to survive of the animals by calculating the time spent swimming or climbing (the acitivity time). No significant differences were observed between the two groups regarding treatment – time interaction, but time had a significant effect ($p<0.0001$).

SURVIVAL CURVES

No significant differences were observed regarding survival periods between the control and treatment group.

GENE EXPRESSION ANALYSIS THROUGH REAL TIME – PCR

Several studies have suggested that SPT100 might have an anti-inflammatory effect. Consequently, we hypothesized that long-term treatment with SPT100 could reduce gene expression of various inflammation markers, including: C11, CR3, Tgfb, Cxcl10, CXCR4, Fcgr3a and Stat1. Astroglia activity (marker GFAP) was also tested. Through Real time – PCR, we discovered significant decreases in the expression of inflammation and astroglial markers in the mRNA transcription code for C11, CR3, CXCR4 and GFAP after treatment.

Conclusions

Seen that the elderly represent a significant percentage of the world population, cellular autophagy is regarded as a possible solution for a healthy aging process.

The current paper is a preliminary study, which shows that stimulation of autophagy ensures cytoprotection during the aging process.

Treatment with SPT100, a substance that enhances autophagy, improved the cognitive and motor functions of the aged rats.

This substance has also promising anti-inflammatory and anti-apoptotic effects on the central nervous system.

These results may offer a basis for further studies regarding the protective mechanisms of autophagy stimulation on the aging process.