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EFFECTS OF GLOBAL WARMING ON BIODIVERSITY

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Abstract

The global warming in recent decades - especially due to anthropogenic activity - already has a negative impact on biodiversity and in the future is expected to become a more significant threat for the future decades. In the present paper are analyzed detrimental consequences of this phenomenon on natural vegetation and fauna but also on the „artificial" vegetation (agricultural crops) on Earth. Thus, higher temperatures coupled with changing rainfall regime, lead to the extinction of species of plants and animals the later migrating into areas where conditions are more or less close to their needs. The paper outline the state of forest fires, the melting of glaciers and glacial ice caps at the poles, affecting aquatic biodiversity. It analyzes the appearance of aridity of more tracts of land areas of the world and in Romania, the lack of water which affects the life of the planet in general as well those of the people by restricting the decrease of arable land and agricultural production.

Key words*:* biodiversity, fauna, global warming, natural and artificial vegetation, negative impact

Introduction

The global warming in recent decades already has a negative impact on biodiversity and in the future is expected to become a more significant threat for the future decades.

In recent decades there has been a change of climatic factors, an increase in annual temperatures, especially in summer, which extends in duration. This climate change is largely due to anthropogenic activity has a negative influence on the entire natural environment. The last 15 years (after 2000) were the warmest years after 1900, the temperatures rose by 0.9^{o}C.

In this paper we will refer only to the adverse effects on biodiversity products (both natural and "artificial").

Discussions

First, we present aspects of natural vegetation and fauna which is suffering due to temperature rises.

Rising temperatures coupled with changing rainfall leads to the disappearance of some species and their migration (the animal's case) in areas where conditions are more or less close to their needs. In this sense, are eloquent some examples:

In the mountain areas, species of plants and animals accustomed to colder and wetter habitats, migrate upward vertically. Many of them will disappear, the case of pika (*Ochotona princeps*) that live in the western mountains of North America and southwestern Canada, an endangered species included in the IUCIN Red List (Fig. 1, a) (Beever and Smith, 2011; Smith and Weston, 1990). It is accustomed to low temperatures at 2.800 m altitude and now is considered an endangered species. The species dies in 6 hours if is exposed to temperatures above 25.5^{o}C, being an important indicator of this global process (Hafner, 1993).

Another example is the small mountain vertebrate – the marsupial pygmy possum (*Burramys parvus*) living in the cracks mountains of N -E of Victoria and south of New South Wales, Australia, which is also an endangered species. The nocturnal Bogong moths (*Agrotis infusor*) are the vital source of food for pygmy possum when it wake up from hibernation. These moths migrate to the mountains every summer to escape from the heat of the lowlands. They will be forced to compete with other small mammals in the same habitat which is very hard and they are forced to migrate to other areas for other food sources. Here, in this new habitat, they are exposed to the danger of being eaten by cats and foxes and in this way their number decrease (Fig. 1, b) (Groves, 2005).

Fig. 1. Two endangered animal species: American pika (*Ochotona princeps*) and pygmy possum (*Burramys parvus*) (*Source*: Wildscreen Arkive, *photo* Linda Broome) (b) (a- from Bavaru and Bercu, 2014; b- Web 1).

The savannah areas, containing one of the richest qualitatively and quantitatively biodiversity, will have much to suffer from the increasing temperatures and by the reducing rainfall regime. Millions of animals will have nothing to eat from such an arid field and will disappear.

Another consequence of rising temperatures, especially in the warm season of the year, is the increasing occurrence of numerous fires in forests on Earth. In the US, who frequently face such fires, the researchers showed that an increase in summer temperatures by 1.6°C will double the number of fires, especially in the western of United States zones (California and Texas). Let us not forget that this summer in August in northern California fires have produced a disaster.

Russia, in summer 2010, was faced with very high temperatures, especially at the beginning of August, when the temperatures in Moscow and in European Siberia frequently reached 40°C. As a result there were numerous fires, some even near Moscow, burning forests and whole villages and were threatened two Russian military bases as well. The population of Moscow had to fight the wave of smoke for days, being required to wear protective masks (Bavaru and Bercu, 2014).

Every year warm period occurred in the last decade, both in southern Europe (Spain, Portugal, Italy, Greece etc.) took places a lot of devastating fires and in Australia or America.

In this summer - 2016 - in our country, such as in other countries in the Balkans, they have been numerous fires, both in the Eastern and Southern Carpathians and in Apuseni mountains as well, which destroyed hundreds of hectares of forest, generating a large losses of our country forest fund.

The specialists consider that in the future many wet areas of the world (South America, Central Africa or New Guinea) will become drier areas and species of trees in these forests (even trees species with wood essence of economic value) may disappear. The same will happen with other species of plants and animals.

We recall a single example spent in our country, in the summer of 2007, in Braila (Romania), the County where it hasn't rained for 147 days and summer temperatures have exceeded 30°C, often arriving and at 40°C (in the shade). In the Natural Park "Small Pond of Brăila", dried all trees and in the park began to appear, ruderal herbs characteristic of steppe grasses with no value (Bavaru and Bercu, 2014).

Fig 2. An image that shook the world: a dead polar bear washed ashore (a) (Source: World Supplement in News, January 12, 2016) A polar bear on an ice floe in Spitsbergen. (b); (Source: The Telegraph, June 12, 2015, photo: Rex Features) (a, b- Web 2).

The polar bears (*Ursus maritimus*), which are vulnerable species (Wiig et al., 2013), and the generally zone fauna of the cold areas of the globe), after the gradually melting of the polar ice caps and glaciers in the northern hemisphere, they are shrinking habitats, no longer able to procure food, only with great difficulty, are in danger of extinction (Fig. 2, a, b).

The rising temperatures will affect life – the biodiversity – from the oceans freshwater. A recent study conducted by US National Wildlife Federation (NFW) shows that in the future - in approx. 25-30 years - in the circumstances described above – the waters of rivers and streams that flow into the Pacific NW, they will no longer have trout and salmon, will not be able to organize farmers for these valuable species of fish because the water heating (Fig. 3 a, b). It envisages strong pressure on other cold water fish species in the NW area of the US and Canada.

Fig. 3. Atlantic Somonul (Salmo salar) (a) și brown trout păstrăvul (Salmo trutta)(b) (a- Web 3; b- Web 4).

Even greater problems are in the Earth waters of the seas and oceans. Biodiversity in these waters has already begun to be affected by a waters warming above normal (Bavaru et al. 2007). Arctic and Antarctica reflects the solar energy and maintain the constant temperature of the Earth. Unfortunately Arctic warmed twice as much as the rest. Especially in spring and summer polar ice caps are melting faster due to rising temperatures. As a result increase the level of seas and oceans (in the last year already increased by 22 cm). In 2100, if it continues this process of global warming, the seas and oceans will rise by 1 m, flooding and destroying numerous islands and towns on the shore (Maldives Marchal, islands and towns as Amsterdm, Rotterdam, New York etc.).

The ocean waters becoming warmer absorb more CO_2 from the atmosphere (also enriched increasingly more CO_2) dissolve it, making the waters more acidic, harmful to corals and other marine species - acidic pH does not favor life in general. Thus, the coral reefs of warm seas - extremely sensitive to environmental changes - began to suffer, becoming white - that is death. Temperature increases in the waters around them have favored the emergence of an epidemic that leads to coral death (disease green stripes, yellow, red) and a disease called "white syphilis" (Fig. 5).

The specialists in marine biology consider today that in the Pacific Ocean, the Great Barrier Reef zone (344 400 km2 in area, especially in the Port Douglas - Cape York Lizard Island). In the Indian Ocean as well almost 70 % of their coral reefs have been bleached and died!

These reefs are true marine biodiversity centers of the warm waters of the globe. It is estimated that 65% of marine fish species live and reproduce in this warm waters (Fig. 4). With the death of these reefs they will migrate, leaving an almost dead area. To will this adds the overfishing and other human impacts related (the coastal development and sedimentation processes), the case of Philippines reefs (Burke et al., 2001).

Concernng this subject, Dr. Russel, Reichelt Chairman and Chief Executive of the Great Barrier Reef Marine Park Authority (GBRMPA) said: „Collaborative efforts by a large number of institutions and tourism industry volunteers allow us to say with confidence that while bleaching caused by heat stress affected most of the Reef, the most severe mass bleaching and the greatest mortality has been restricted to north of Port Douglas".

Fig. 4. A healthy coral on the Great Barrier Reef
(photo Debra James/Shutterstock/WWF) (Web 5).

Since the beginning of the industrial revolution, the waters marine acidity increased by about 30% as it deems marine biologists. This acidification, according to the United Nations Environment Programme, makes the reef's skeletons limestone coral forming to be much more difficult.

Instead, in these special environmental conditions, feel good and proliferate, species of jellyfish and other Coelenterata species, worthless and even biological. In the Mediterranean Sea - for example - due to changes in the chemical content of water - populations of jellyfish, most of them poisonous, have developed much exaggerated, this large sea being, nearly suffocated by this poisonous creatures. They arrived this summer in the Black Sea, along the Turkish coast being threatened Bulgaria as well. In addition, the experts warn that this organisms are more poisonous than in the past. In the past three years, thousands of people who come on holiday in Spain arrived at the hospital after being touched by the jellyfishes.

Due to global warming, the Black Sea is becoming more like the Mediterranean Sea which, in its turn, begins to have large tropical characteristics.

The terrible poisonous jellyfish, Portuguese galley (*Physalia physalis*) which can kill a man just by touch, specific to the warm waters washing the Portugal shores (Fig. 6, a), was carried by winds to nearby beaches of Great Britain. Thousands of Portuguese galley invaded the beaches of France. A series of expansive beaches of the Côte d'Azur of France, the Great Barrier Reef of Australia and Waikkal and Virginia, United States areas, were closed because of the threat posed by the jellyfish invasion.

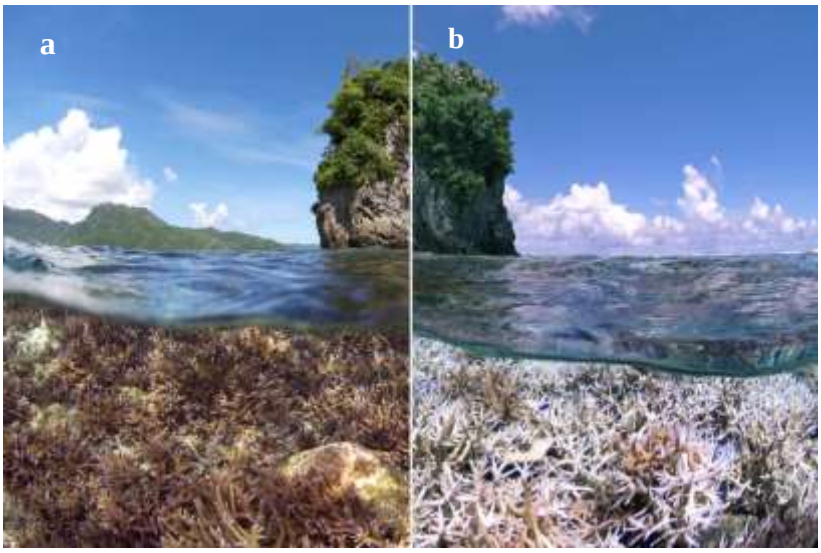

Fig. 5. Before and after image of the bleaching in American Samoa.
The first image (a) was taken in December 2014. The second image (b) was taken in February 2015 (Photo: XL Catlin Seaview Survey, Chaisson, 2015) (Web 5).

According to biologist professor Nikolai Marfenin from Moscow State University, because of the unprecedented heat, the freshwater jellyfish *Craspedacusta sowerbyi* (lapsus), it has greatly multiplied, reaching in the Moscow River, which cross through the Russian capital (Fig . 6, b).

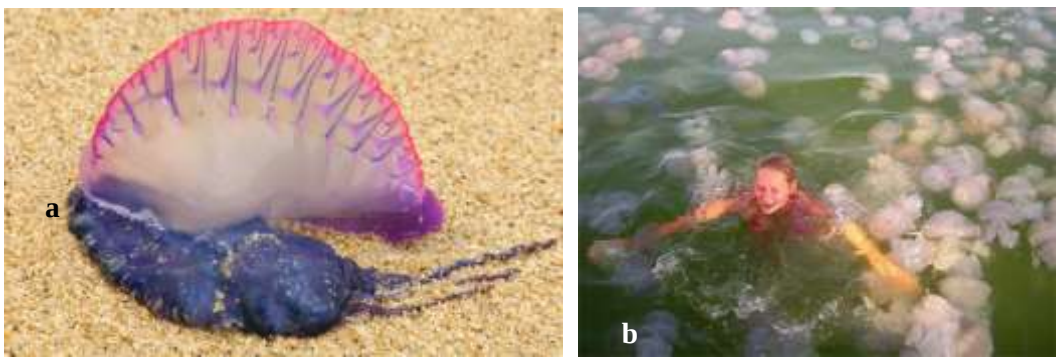

Fig. 6. The terrible poisonous jellyfish, Portuguese galley (Physalia physalis) (a).
The freshwater jellyfish Craspedacusta sowerbyi (b)
(a- Web 6; b- from Bavaru and Bercu, 2014).

According to the most recent Intergovernmental Panel on Climate Change (IPCC) report in 2016, the sea level is predicted to rise by 26–98 cm by 2100, due to the thermal expansion of the oceans and the melting of polar ice-caps and ice sheets. The warming of the marine waters coupled with the effects of storm surges, which are expected to be of a greater magnitude in a warmer world and damage lands (Fig. 7, a), this increase in sea level could threaten many coastal ecosystems such as the mangrove ecosystems from S -E Asia (Indonesia, Vietnam, Philippines, etc.) but also those of Australia (Kakadu National Park, the largest terrestrial national park of the Nothen Territory of this continent) (Fig. 7, b). These areas are also preferred habitat of many species of fish and even other marine organisms, especially for reproduction. These ecosystems (mangrove forests) will be swallowed by mangroves and water - if the sea levels will rise (Web 8).

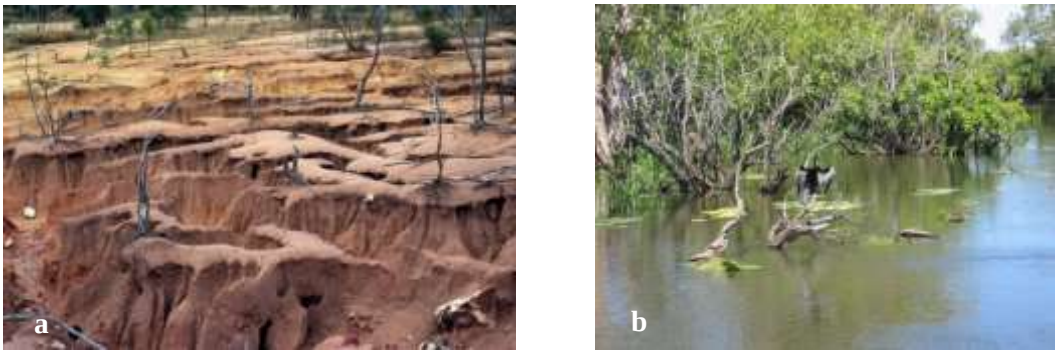

Fig. 7. Changes in rainfall patterns can damage land, plants and animals. (Image source: Willem van Aken/CSIRO Science Image) (a). Mangroves and wetlands in Kakadu National Park are some of the areas under threat from rising sea level. Source: NOVA, image: Paul Morrison/Flickr) (a, b- Web 7).

Finally, we mention a study by IUCN, stating that given that climate change will continue in the future, in less than 100 years , a quarter of species of plants and animals on Earth will disappear . Dr. Ahmed Djoghaf, executive scretar of UN Convention on Biological Diversity, said recently that "the erosion of biodiversity is continuing at an unprecedented rate in history, extinction rates of species could be 1000 times higher than those recorded by the natural over time." The vertebrate species populations have already been reduced by about 33% between 1970 – 2006 according to information published by UN.

Many scientists believe that the current extinction of biodiversity is second in size after the first one that took place in late Permian (about 250 million years ago) (Bavaru and Bercu, 2014).

An ongoing project called - "The Economics of Ecosystems and out biodiversity (EEB)" - to quantify the financial value of services that nature brings them to us (air and water purification, wildlife ecotourism, food source, etc.). For example only annual fund forest destruction amounts to 2000-5000 billion dollars - well above the current banking crisis costs (Brown, 2011).

II. Secondly we present some aspects of artificial vegetation effects of global warming of agricultural production, especially grain production.

The specialists in the field say that any temperature increase of one degree above optimum temperatures of crop plants leads to a high 10% decline in grain production. What is the explanation?

First it is affected photosynthesis process. The leaves begin to turn and photosynthesis is suffering. Add to this increase in temperature the lack of the usually water and we will better understand why field crops are threatened. Experiments conducted in USA, Japan, India, Taiwan, etc. on the major world crops (wheat, corn, rice, soybean, etc.) have shown that sometimes yield crops by more than 10 %. For soybeans and corn, American experts have estimated that production decreases by 17%. If temperatures would rise by 2°C what climatologists say it is possible in the future), then wheat production (and others) would be reduced between 37 % and 58 % and these figures remain close even if the crops are irrigated (the experiment was done in India).

It is considered by professionals that plants die if temperatures pass over 40°C in the shade, when photosynthesis stops and the plants wither and die. It is already affected when the temperature passes 35°C in the shade, practically is capped.

Secondly, there is also a reduction of pollination, the plants remain sterile, cannot bear fruit. Reproductive parts of the flower begin to dry. Pollen cannot germinate without having the necessary humidity. Experiences made in US and Philippines showed that the most affected the large crops plants are maize and rice. At 38°C the pollinated plants percent is almost 0 and 40°C throughout the crop is destroyed.

A calculation in the EU - by specialists in agriculture - shows that plant pollination, especially by insect is in an evident decline in Europe, and this would cause damage of 15 billion euros per year. But globally, that would be the case? We can see from the foregoing that this increase in temperature can affect the food security of the world. Until 50-60 years ago, agriculture has undergone few

major climate changes, climate being more or less stable. So the warming process makes us think of the bad and very bad things (Brown, 2012).

But not only large crops are affected by these climate changes and especially the rising temperatures. For example, another case occurred in India in cultures of tea. The tea cultures from the Indian province of Assam produce around 55% of India's tea production and about 1/3 of the world production of tea. In the winter of 2009-2010, the temperatures in the province have not decreased, in the winter season, below 9°C. As a result, the tea leaves remained on branches, they have not fallen. In the recent years, the average temperatures in this province have increased by 2°C and rainfalls decreased by 1/5. April and May are more rainy seasons, favoring the emergence of different pests (especially fungi). These crops are organic (ecologic crops) and cannot be sprayed (the main part of production goes to export, being severely controlled). As a result the production decreases, both quantitatively and qualitatively, modifying especially aroma. In 2010 tea production fell to 460,000 t - the lowest production in recent years. Mridul Hazarika, Director of the Tea Culture Research Association of India, appreciates that all these shortcomings are due to climate change and especially to temperature increases and changes in rainfall regime in the province of Assam.

The most regions of the world affected by temperature increases are the subtropical and tropical, where hunger produces already many damages. In our country as well, the extremely hot summers, with little rainfall in recent years and especially this summer, led to a process of aridity, even desertification in some south areas.

As a result, specialists in agriculture recommends that in the future in these areas to be cultivated varieties of wheat and corn resistant to drought. Moreover, there are few specialists recommended for cultivation of sorghum south, a resistance to drought Poaceae species, rich in proteins and active substances, well above corn’s (currently grown in Africa for its qualities).

Conclusions

Understanding the complex interaction between the impact of climate change and how the environment responds to these natural changes is now essential not only for nature conservation, but also for preserving the benefits that nature provides us, as livelihoods. Through the common efforts of all profile organizations, scientists and countries governments but also everyone else's we can to save the nature and us, humans as well.

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ASPECTS REGARDING ORGANOGENESIS INITATION IN *Sedum telephium ssp.maximum L.* CALLUS UNDER BLUE FLUORESCENT LIGHT

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Abstract

Environmental factors may influence organogenesis on phytoinoculi metabolism. *Sedum telephium ssp. maximum L.* callus, subcultivated for 30 days on basic *Murashige-Skoog* (1962) (MS) medium proved to be a plant material with particular reactive capacities, depending on the nature of growth regulators in medium and on the light wavelength used to illuminate phytoinoculi. Our experiments showed that a certain cytokinine in the medium induced accumulation of a red pigment in the vacuole sap of meristematic cells in root apices, but not in caliptral cells or cells from the growth area. *Sedum* callus culture under blue fluorescent light cultivation synthesized the red pigment in root meristems on basic MS medium supplemented with 2,5 mg/l 2,4-dichlorphenoxyacetic acid and with 1,5 mg/l benzilaminopurine. Concerning organogenesis, rhizogenesis was stimulated by blue light. We highlight the detection of secondary metabolic processes in root meristematic and callus cells.

Keywords: Sedum, organogenesis, callus, blue fluorescent light

Introduction

In plant biotechnology, vitrocultures usually require exposure of phytoinoculi to artificial sources of light, represented, in most cases, by white fluorescent tubes. Light is required for both photosynthesis, as well as for cell

differentiation alterations, morphogenesis initiation and regulation, photoperiod and phototropism dependent organ growth etc. According to Herman (2013), the light wavelength may exert certain influences on phytoinoculi, as the light intensity and duration do. Zhu Xingui and Guo Yong (1998) (according to Herman, 1999) showed that blue light led to a 10 fold accumulation of anthocyanins in Hibiscus sabdariffa cell suspensions, compared to red or orange light.

Muleo and colab. (2001) (according to Herman, 2002), experimenting with plum shoot apices explants, observed that blue light led to a more intense bud differentiation compared to red light and consider that light quality may increase micropropagation efficiency in vitrocultures obtained from plum apices or buds.

More recently, Liu et al. (2011) and Lin et al (2011) showed that in *Oncidium* and *Dendrobium* protocorms cultivated under blue light, cell differentiation was accompanied by increases in biomass, protein and assimilating pigments synthesis and enzymes activities, therefore leading to a higher rate of shoot genesis. Combining red and blue monochromatic light further stimulated those processes, as well as a accumulation of dry biomass in these protocorms (according to Herman, 2011 a and b).

However, Park et al. (2013) (according to Herman, 2013 b), *Panax ginseng* root vitroculture exposure to blue light increased synthesis of secondary metabolism compounds, such as α-tocopherol and β-amyrine and certain phenolic compounds in cells. Meanwhile, they stated that ,,blue light could be a useful source for the production of ginseng roots-cultured in vitro-with higer nutricional value and antioxidant activities".

Alvarenga et al. (2015 –according to Herman, 2015) observed that blue light is the best for *in vitro* cultivation of *Achillea millefolium* vitrocultures. Blue LED light stimulated, in Picea and Robinia cotiledonary epidermal cells, accumulation of electron dense particles in vacuole sap or in the tonoplast (Cachiță și colab., 2015).

Considering that in the last decade the number of articles focused towards the reaction of phytoinoculi as a function of the illumination type, our study is aimed at the same issues, using *Sedum telephium* explants as a model.

As such, in studies performed by our group (Ardelean şi colab., 2013), using *Sedum telephium* ssp. *maximum* explants under 16 h/day white fluorescent light, rhizo- and caulogenesis was observed (Fig 1). The apices of roots regenerated in primary cultures on Murashige-Skoog (1962) with α-naphtilacetic acid (ANA) and kinetin (KIN) supplementation 1,5 mg/l, were red colored (phenomenon described the first time in the literature by us). Researches pointed out that only in root apical, meristematic cells, and not in caliptral ones, red

pigmentation may be met, depending on the growth regulators used (Fig. 1 B și D). The pigmentation phenomenon was not observed in roots regenerated form explants on basic Murashige-Skoog (1962) (MS) (Fig. 1 A and C) without growth regulators while it was present, however less intense, in Sedum inoculi where growth regulators such as indolilbutiric acid (AIB) 1,5 mg/l was used; the phenomenon was much more intense in explants grown with α-naphtilacetic acid (ANA) or kinetine (KIN) 1,5 mg/l supplementation. Increased pigmentation was observed in plantlets grown with blue fluorescent light, 16 h/day photoperiod.

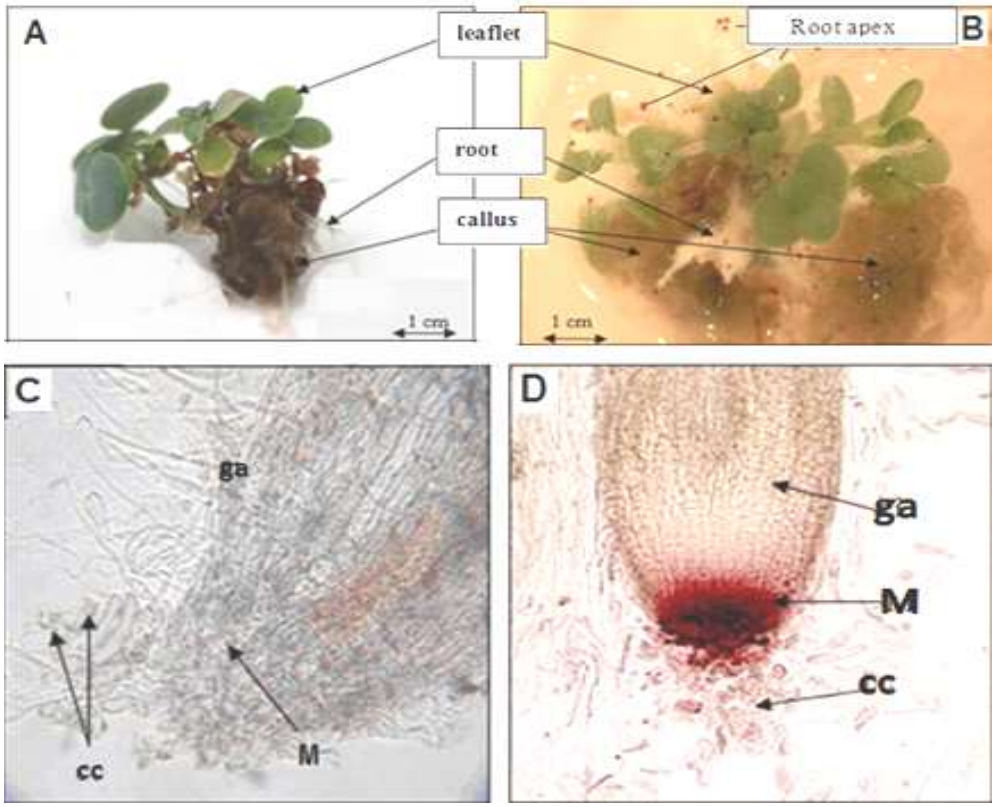

Fig. 1. Images of in vitro cultures of *Sedum telephium* ssp.maximum L. plants (after Ardelean et al., 2013) (Fig. 1 A and B), grown for 30 days in Murashige-Skoog basal medium (1962) with the addition of NAA 1.5 mg / l (A), or NAA and BAP 1.5 mg / l (B); (C, D) root apices - C representing the detail of a apex from a vitro cultures performed on MS medium, but with the addition of NAA 1 mg / l (position a), D – red colored root apex from a culture performed on MS medium with suplementation of ANA plus BAP 1 mg / l (abbreviation: cc – caliptral cells; M meristematic zone, ga- growth area stretch).

Material and methods

For examination of morphogenesis in calluses, macro and microscopically observations were performed, in superficial and profound callus layers, depending on the type of illumination.

Microscopic examinations of the callus or the tips of the roots structure were performed either on live tissues or on tissues that have been fixed prior to their use to obtain semi-fine sections using an ultramicrotome. For examination using the optical microscope, the thick sections must be around 500 nm (0.5 pm). For callus section preparation the tissue samples are first pre-fixed in 2.7% glutaraldehyde prepared in 0.1M, pH 7.4, phosphate buffer solution, followed by 4 successive washings with the same buffer. The tissues are then post-fixed using a l-2% osmium tetroxide solution made in 0.1M, pH 7.4, phosphate buffer then washed 2-3 times with the same buffer. The samples are then dehydrated by placing them in baths with increased acetone concentration. After that, the samples are passed through 2-3 baths containing propylene oxide solution. The tissue samples thus prepared were placed in baths of Epon 812 (epoxy resin) mixed with anhydrous acetone with increased resin concentration, the final bath containing only the Epon. The samples impregnated with Epon were embedded in gelatin capsules. The capsules were then finish filled with resin and kept at 50 – 60^0C, for 48 - 72 hours to cure the resin which will become hard and transparent.

Once the samples are cured, the hardened resin can be modeled under a stereo microscope using a new razor blade to obtain a small ,,pyramid trunk" at one end. For ultrafine sections, the sides of the pyramid trunk should be around 0.1 - 0.2 mm.

The sections were obtained using a Leica UC 6 ultramicrotome equipped with a Diatone diamond knife. The semi-fine sections were stained with Epoxy tissue stain, a special epoxy stain. The sections for electron microscopy were contrasted in the first stage with uranyl acetate solution, followed by contrasting with lead citrate solution, technique which is currently used in electron microscopy laboratories, around the world (Cachiță and Crăciun, l99l; Hayat, 2000). The semithin sections were examined by us using a Olympus BX 5l optical microscope equipped with a CCD camera.

Results and discussions

As can be seen in figure 2, the *Sedum* callus subcultured for 30 days under white fluorescent light (Fig. 2 A), maintained the green color, while callus exposed to blue light became dark red (Fig. 2C). In addition, the callus lighting with blue fluorescent light presented caulogenesis processes and rootedness.

From a microscopic point of view, the green callus presented cells with large vacuoles and cytoplasm which contained many chloroplasts (Fig. 2 B), whereas the samples exposed to fluorescent light blue, parenchyma of callus held cells with a color almost black. Their vacuoles presented numerous particles, solitary, or conglomerates; in other areas, callus tissue presented numerous meristematic nodules (Fig. 2 D and Fig. 4 A and 5 A) with small cells and large nuclei.

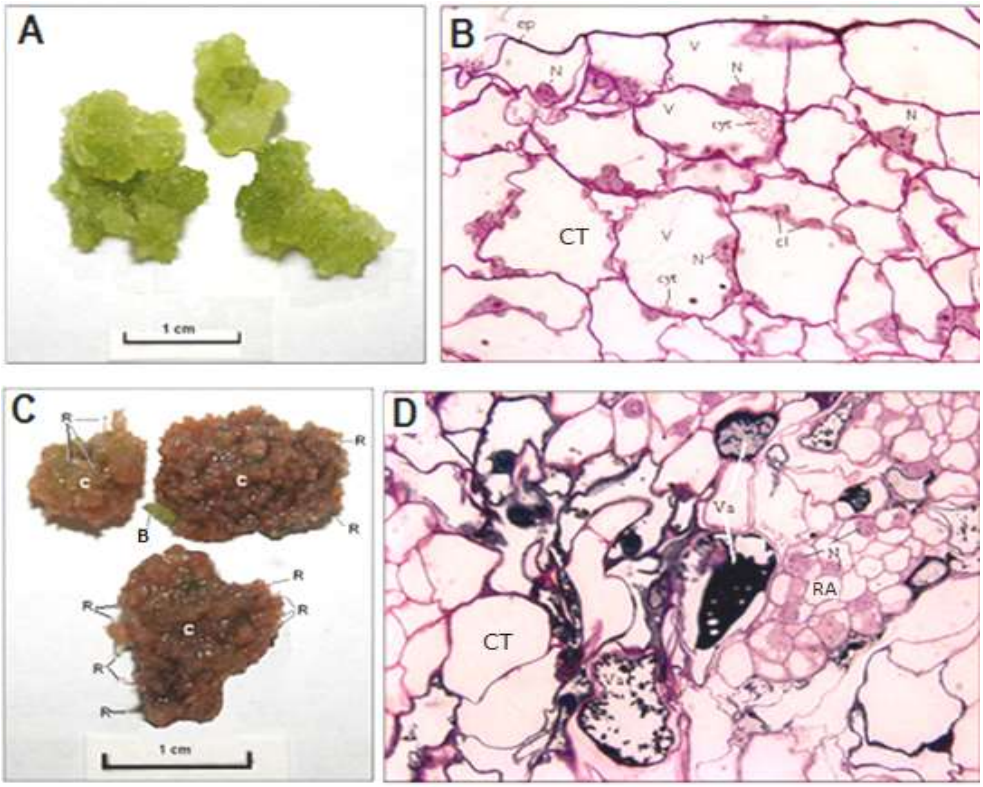

Fig. 2. The macroscopic (position A and C) and microscopic aspects (optical microscopic - 100x) of the Sedum callus grown 60 days on basic Murashige-Skoog medium (1962), with 2 4-dichlorophenoxyacetic and benzylaminopurine at concentrations of 1.5 mg / l and illuminated with white fluorescent tubes – positon A and B - or blue - positions C and D (abbreviations: cyt - cytoplasm; cl - chloroplasts ; ep - epidermis; N - nucleus; V – vacuole; Va - vacuole with anthocyanins; B – bud, R - roots; C – callus, Va - vacuole with anthocyanins, CT- callus tissue).

Also, in such cells chloroplasts were absent. Noteworthy is that on the surface of calluses colored in red white roots, a few millimeters long, were present, with red apices as a result of red pigment present in vacuolar juice of meristematic cells (Fig. 3A). This phenomenon is similar to that observed in root apices formed on the colony seedling which were obtained from in vitro cultivated apexes on MS medium suplimented with 1.5 mg /l ANA and 1.5 mg/l KIN (compare images in Fig. 1B and D in the Fig. 2 C).

Therefore, it can be said that under subculture *Sedum* callus conditions where KIN was replaced by BAP, especially when lighting was done with fluorescent tubes emitting blue light, rootedness was stimulated and more frequently caulogenesis was present (Fig. 2C). The color callus became red, a similar process to that highlighted in vacuoles of meristematic cells located in the root apices.

In native samples of *Sedum* roots (Fig. 3) meristematic cells presented a naturally pigment, evenly spread in the vacuolar juice of these cells. The application of a hydroxide solution of Na over this preparations produced a turning color of cells from red to purple, what could constitute proof that this is a anthocyanin pigment type and metabolism of these cells is not only the first type, but secondary.

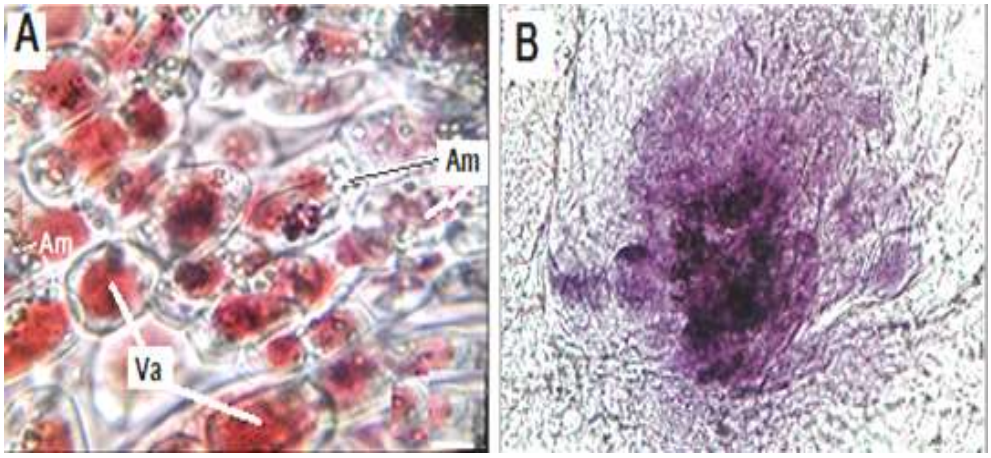

Fig. 3. Cytological aspects observed with an optical microscope (ob. 40) in the apical root regenerated from seedling or from tissue callus of *Sedum telephium spp. L. maximum* plants grown for 60 days on Murashige -Skoog (1962) culture medium with 1.5 mg / l NAA and 1.5 mg / l KIN. It can been observed the change of color of cells from red (position A) to purple (position B) by treatment with sodium hydroxide solution (abbreviations: Am- amyloplasts; Va - vacuoles containing red pigment).

Anthocyanins, a particular group of compounds, are one of the more than 6,000 members of the flavonoid family of polyphenol phytochemicals found in various plant foods. In addition to anthocyanins, the flavonoid group includes flavanols, flavones, flavanones, flavan-3-ols, and isoflavones. Anthocyanin pigments have been used in folk medicine for generations, but only recently the specific pharmacological properties of these compounds have been isolated and studied (Roy and colab., 2009).

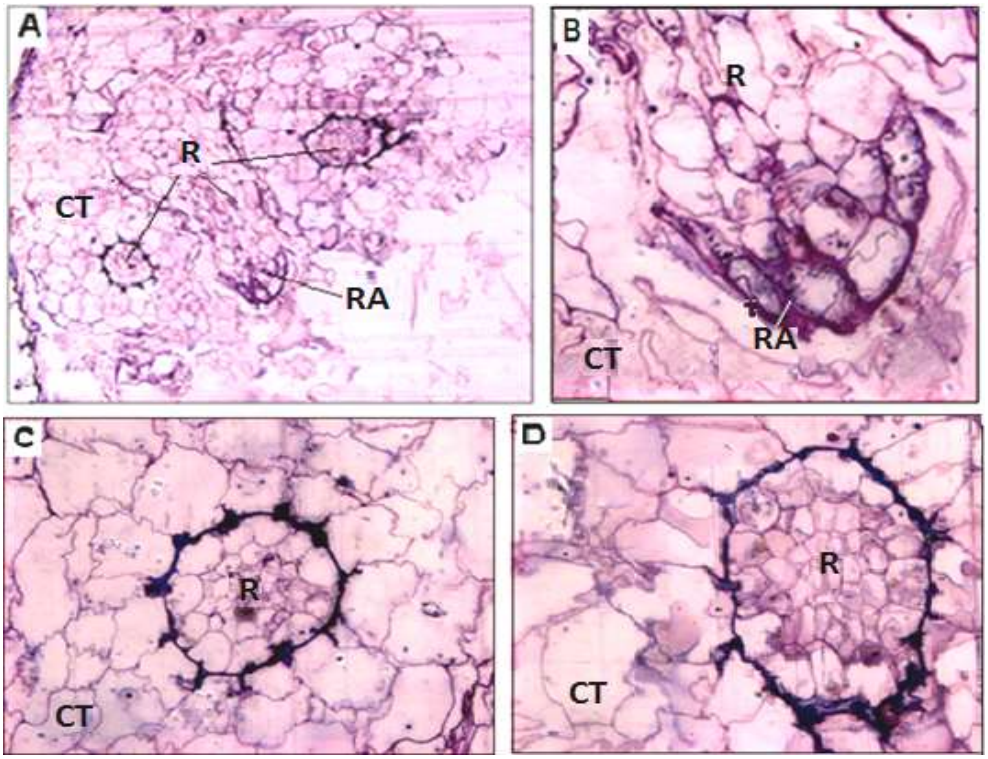

Fig. 4 A - D. Optical microscopy aspects observed in callus mass of Sedum telephium spp. L. maximum seedlings, on the 60th day of subculture's basic medium MS (1962) with the addition of 2.5 mg / l 2,4-D plus 1.5 mg / l BAP, increased by blue fluorescent light 16 hours / day, where: A - image seen by ob. 10; B - visualized image ob. 90 conducted a longitudinal section through the apex of a root mass that penetrates callus on its way to its exterior; C and D - images illustrating the appearance of cross sections made by roots that pierce parenchyma observed ob 40 (abbreviations: R - roots that pierce the mass of callus formation; CT- callus tissue; RA – root apex, longitudinal section through the meristematic).

Another aspect not mentioned in the literature is that of how initiation of callus formation in the mass organogenesis is produced. As can be seen in figures 4 A and 5 A both rootedness and caulogenesis begins in the profound layers of the callus tissue.

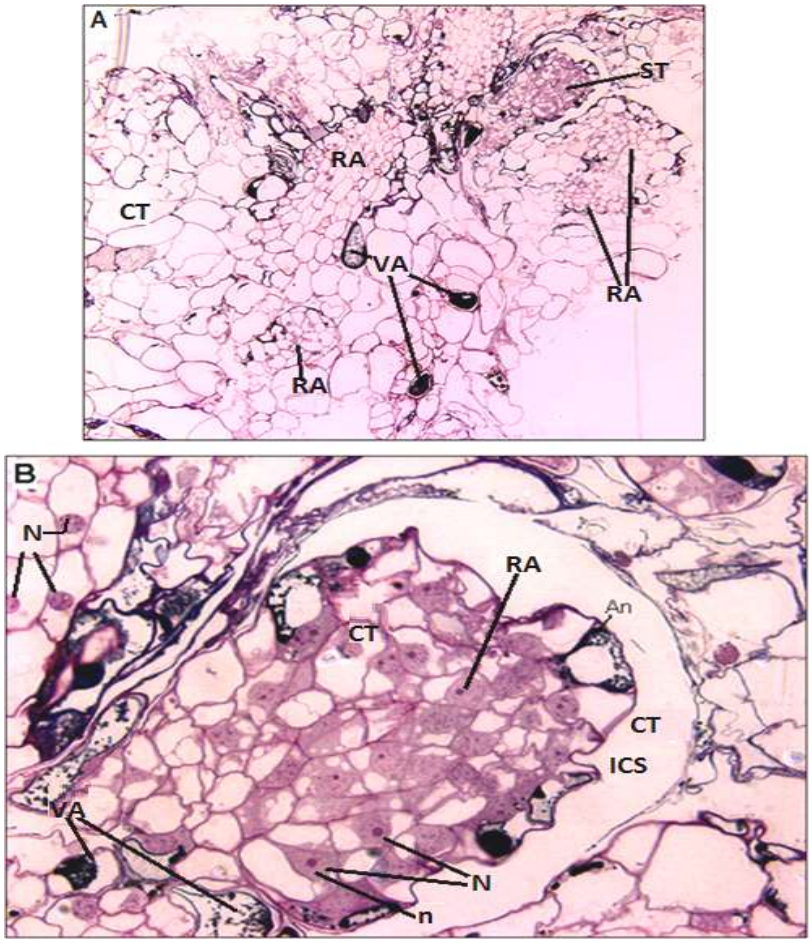

Fig. 5. Optical microscopy aspects observed to *Sedum telephium* spp. L. *maximum* callus , on the 60th day of subculture's basic medium MS (1962) with the addition of 2.5 mg / l 2 4-D plus 1.5 mg / l BAP, increased by blue fluorescent light 16 hours / day, (abbreviations: A - image seen by ob. 20, it can identify a bud neogenesis and caulinar apex seen with ob. 40, in the image of Fig 5 B (abbreviations: R - roots that pierce the mass of callus formation; CT- callus tissue; RA – root apex; ST-stem formation; Va - vacuole with anthocyanins; N – nucleus; n - nucleolus; CT - callus tissue; ICS - intercellular space).

Thus, in figure 4 A, in an area where organogenesis is triggered, roots developing in parenchyma callus can be seen. These show in their apical area (Fig. 4B), a reduced number of cells (probably initial meristematic cells) presenting cell walls stained in shades of purple after cytochemical reactions.

This phenomenon is noticed during young roots advancement throughout callus tissue (Fig. 4 C and D); at interfering points of future rhizodermal cells with parenchyma callus mass, in intercellular spaces - in the contact area - the presence of small conglomerates, black cherry, dark (almost black) can be observed, which we consider mucilaginous type as *Sedum* tissues have - in general - much mucilage; mucilage could facilitate slipping of root apices through the callus mass.

In an adjacent area where roots are produced, structures which will generate stems may arise, structures which present a well defined meristem in their apex (Fig. 5B); some of the future epidermal cells present a red pigment in their vacuolar sap, probably of an anthocyanin type (Fig. 4 A – D).

Conclusions

The experiments were aimed at cultivation "in vitro" explants *Sedum telephium* spp. L. *maximum* explants on *Murashige-Skoog* basal medium (1962) (MS) with the addition of ANA plus KIN in concentrations of 1.5 mg / l, or AIB with BAP, each 1.5 mg / l of each - being exposed to white fluorescent light - it was found that at the level of leaflets or stems randomly some epidermal cells were stained red raspberry, pigment considered by us as being due to accumulation of anthocyanins in their vacuolar juice. Apices of roots regenerated from in vitro cultures were also colored red raspberry about 1-2 mm from the tip (Ardelean et al, 2013), which is quite surprising, with the more so since this pigment is synthesized by the plant cells through a process of secondary metabolism, which was considered absent in meristematic cells.

In this paper, making a green *Sedum* subculturing of callus on a medium MS, but with the addition of 2,4-D 2.5 mg / l BAP plus 1.5 mg / l, exposed to fluorescent light blue (light is known - literature - that the plants stimulates secondary processes of metabolism), we noticed with surprise that not only apices of roots regenerated from the callus were stained red, but the whole callus acquired claret-red coloration. Microscopic examinations revealed that both in some cells from the surface of callus reddened, and the parenchyma thereof, presented their juice vacuolar a conglomeration of

glomerular particles or black plates, interpreted by us as anthocyanins. So, Sedum callus - depending on the nature of the growth regulators present in the culture substratum - under the exposure of light fluorescent blue, has acquired the ability to synthesize the pigment anthocyanin, unreported in the literature, in both apices of roots regenerated from callus and in vitroplantlets.

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GOLGI METHOD: A 140 YEARS OLD YET UNIQUE AND POWERFUL METHOD FOR THE STUDY OF THE CENTRAL NERVOUS SYSTEM

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Abstract

Golgi method is been using for more than 140 years so far for the study of the individual morphological and morphometric characteristics and parameters of the cells of the central nervous system. Although other methods came to light, Golgi method is still unique and one of the most powerful tools in the hands of the neuroscientists. What makes Golgi method unique is the capacity to stain all components of the brain tissue, including neurons, glial cells and the vasculature. The cell soma, the dendritic arborization including the spines and at least a part of the axon are usually visible, providing a panoramic view of the entire neural element. Golgi method can be combined with modern and sophisticated techniques which can reduce the human interference and produce accurate 3D models of the neuronal elements of the central nervous system.

Key words*:* Golgi method, 3D Neuronal Reconstruction, Neuromorphology.

Introduction

The modern scientific investigation of nervous systems started over a century ago with the revolutionary neuron doctrine, posted by Santiago Ramon y Cajal. Cajal showed that, like all the other organs in the body, the brain is constituted by cells and revealed the incredible complexity of the shape and potential connectivity of brain cells. Cajal's findings inspired the principal axiom of modern neuroscience: the key substrate for all the functions performed by nervous systems, from regulation of vital states, reflexes, and motor control, to the storage and retrieval of memories and appreciation of artistic beauty, lies in the structure and assembly of neurons (Mavroudis and Alexiou 2015).

The ultimate, and arguably the hardest, challenge to human knowledge consists of understanding how neurons and their connections give rise to feelings, emotions, and logical thinking.

One of the most powerful methods for the study of the neuronal structure of the central nervous system is the 140 years old yet unique Golgi method.

Golgi method

More than 130 years ago, Camillo Golgi introduced a staining technique for visualizing whole neurons, which are stained black or dark brown, on a yelowish-golden background. Camillo Golgi in his original work used silver nitrate and potassium dichromate; however his method was not reproducible because of the unstable nature of the precipitate of chromate silver on the lipoprotein of cell membrane. Santiago Ramon y Cajal improved Golgi's original method, adding osmium tetraoxide to the potassium dichromate solution, which is stabilizing cell membranes, allowing the visualization of more neurons (Rapid Golgi method). Working with his modification of Golgi method Cajal described for first time the neuronal cells as distinct entities and visualized dendritic spines and growth cones. Several modifications of Golgi method have been developed so far, which all have in common the impregnation of neuronal cytoplasm with metallic salts (Baloyannis, et al. 2011) (Raju, et al. 2004). The most important Golgi modifications are the Golgi-Cox method, which was developed by Cox and is particulalry useful for tracing dendritic arborization, the method which was developed by Hortega del Rio using formalin with the dichromate salt and chloral hydrate, and which is useful for the study of neuroglia, small granule cells and the cerebellum, and finally the Golgi-Fox method, which was first introduced by Fox and is useful for adult formalin fixed brain tissue (Das, Reuhl and Zhou 2013).

After the development of intracellular labeling techniques using horseradish peroxidase and biocytin, Golgi methods have taken second place, yet the new methods never came close to matching the overview of entire brain areas that Golgi methods can provide. Positively even after 130 years Golgi technique is increasingly used for qualitative histology and neuromorphology, neurobiology, experimental neurology and neuropathology (Raju, et al. 2004) (Overdijk, et al. 1978).

What makes Golgi method unique is the capacity to stain all components of the brain tissue, including neurons, glial cells and the vasculature. The

percentage of the neurons that are impregnated varies from 1 to 10%. The cell soma, the dendritic arborization including the spines and at least a part of the axon are usually visible, providing a panoramic view of the entire neural element (Glaser and van der Loos 1965)(Fig. **1**).

The basic steps of Golgi method include:

1. The selection of brain area that will be studied
2. Immersion in formaline solution for more than 25 days
3. Fixation in potassium dichromate solution
4. Immersion in silver nitrate aquous solution
5. Fixation in alcohol
6. Cut in thick sections of 100-120μm
7. Study in a light microscope

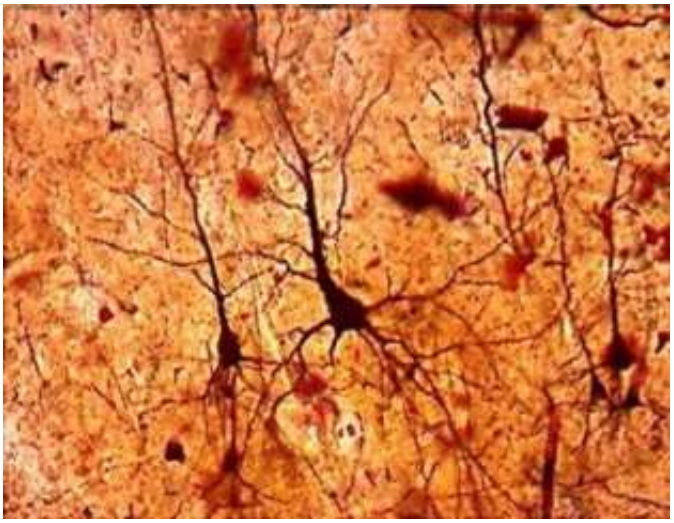


Fig. 1: Golgi stained Pyramidal neurons from the human visual cortex. Magnification 100X

Although Golgi method has been using for more than 140 years now, there are many questions that have to be answered. The studies of Blackstad (Blackstad 1958) and Stell (Stell 1965) showed that the main chemical reaction is that of the creation of a lipoprotein-chrome-nitric compound.

It is not easy for one to provide safe guidance and instructions for the success of the method, however one of the most important factors is the condition of the tissue before the staining process, and in general the intensity of the stain on human dervived material seems to be related to the age of the person, the study area (cortical vs subcortical areas) and the overall fixation process.

Any effort on identifying environmental factors that could contribute to the improvement of the quality of Golgi method had no clear results so far, but after many years of experience and observation in the laboratory of Neuropathology of the 1st Department of Neurology of the Aristotle University of Thessaloniki, we have concluded that a temperature between 18-22ºC showed the best results and a fixation time of 3-7 days in the potassium dichromate solution and 2-7 days in the silver nitrate, depending on the concentration of solutions ranging from 2.66 to 3% and between 0.75-2% respectively. Additional observations led us to the addition of formaldehyde solution 37% 1ml per 100ml of potassium dichromate solution, which probably contributes to the building of bridges between cysteine residues of proteins of the cell membrane, and the lipoproteino-chrome-nitrate cluster, thus staining a larger number of neurons reducing in parallel the chromic artefact deposition.

Neuronal staining starts from the cell body, and then the apical dendrite and the basal dendrites are following with the difference of staining speed to be related to the protein concentration in every different part of the dendritic tree. The next step in the the tracing of Golgi stained neurons which will provide accurate 3D models and will extract the investigated morphological parameters.

Neuronal Tracing

The first and critical task in the study of neuronal morphology is the selection of neurons, which will be traced. The selection of neurons is based on the criteria set forth by Jacobs and requires that all quantified neurons should appear fully impregnated and possessed relatively complete, uninterrupted basilar dendritic systems, consisting of at least three primary dendritic branches, and subsequent higher-order branching (Jacobs, Driscoll and Schall 1997).

Neuronal tracing and reproduction of an accurate 3D model of a neuron can be done either with manual, semi manual, semi-automatic or completely automatic methods with the help of specific software commercial or freeware, Neurolucida and MicroBrightField or Neuromantic, NeuroMorpho and NeuronStudio respectively.

In contrast with early approaches to neuron tracing using specialized computer controlled microscope systems, which stored only the morphological features measured directly from the imaged samples but not the images themselves, the preferred way nowadays is to first acquire the full image data, as it guarantees a permanent record of the original samples and allows the use of

more flexible and more powerful data processing method (Parekh and Ascoli 2013) (Myatt, et al. 2012).

In our method, for each one of the neurons a video of 1 min is recorded, while the microscope table is moving with a stable velocity, so the whole neuron including the dendritic field to be integrated. Then the video is inserted to Image J application, and is analyzed to 120 serial images which are saved as image stack. After uploading the image stack to Neuromantic software the quantification of cell some begins in manual mode, while tracing of dendritic field follows in semi-automatic mode (**Eroare! Fără sursă de referință.**). When the neuronal tracing has been finished full statistical analysis is available and the reconstruction is saved in swc or xml file type. Digital reconstructions enable quantitative analysis of neuronal shapes by means of morphometric parameters describing the metrical and topological properties and the spatial embedding of the three-dimensional structures (Mavroudis and Alexiou 2015). These morphometric parameters make it possible to statistically describe the variability in neuronal morphologies. Three dimensional analysis is completed with Sholl's analysis, which gives the neuronal field density as a function of the distance from cell soma (Sholl 1955).

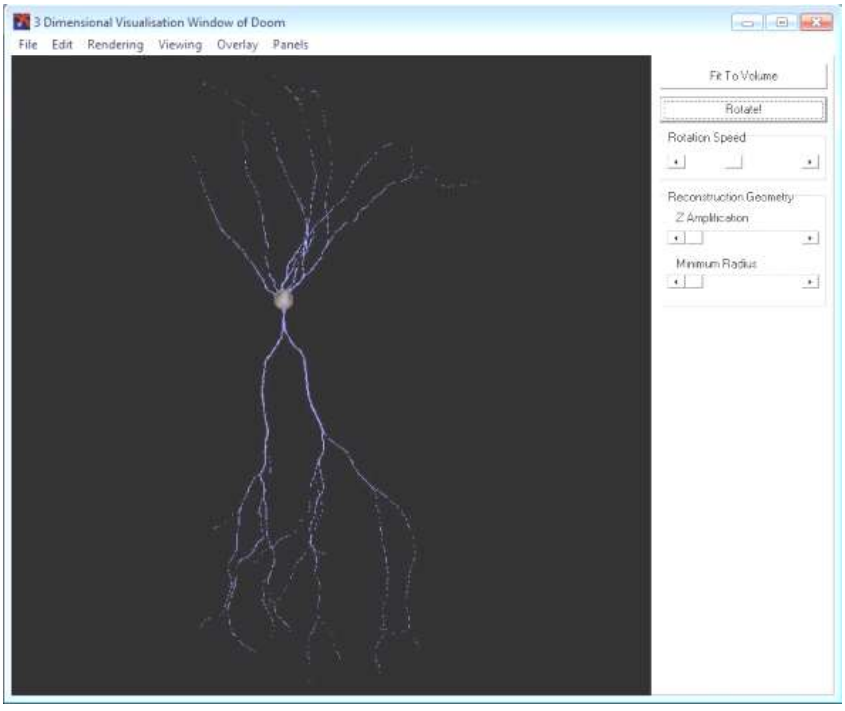


Fig. 2: 3D Visualisation with Neuromantic software of a Pyramidal neuron from the human visual cortex

Sholl dendritic tree analysis

Sholl analysis is a method of quantitative analysis of neuronal dendritic trees, first used to describe the differences in the visual and motor cortices of cats. Initial quantification of a neuron is performed by counting the number of dendrite intersections for concentric circles, usually centered at the centroid of the cell body, of gradually increasing radius (Sholl 1955)(**Fig.**). Curves produced by this initial counting are usually of somewhat irregular shape, and much work has been done to determine appropriate means of analyzing the results.

Neuronal morphometric parameters

The morphological parameters for each traced cell are automatically extracted in a .txt format file (**Fig.**). Although for every traced neuron more than 30 parameters are estimated, the most important of them are the number of stems, which refers to the total number of segments leaving from the dendritic root, the number of branch points which refers to the total number of branch points, the branching orders, which refers to the topological distance from the dendritic root, the total dendritic length which is the summed length of all segments in a tree, the segment length which is the path length of the incoming segments toward a node, the stem length which refers to the path length between a branch point with order =1 and the dendritic root, the number of terminal branches which refers to the total number of terminal branches of the dendritic tree, the Euclidean distance which is used to measure the distance between the soma and the termination points, the neuronal contraction which refers to the Euclidean length of a branch divided by the path length, and finally the Asymmetry of the dendritic tree which refers to the topological complexity of a tree, with completely asymmetric tree having an asymmetry index of 1, and completely symmetric an index of 0 .

Neuronal spines

Appart from dendritic arborization and the morphometric parameters of each one of the neurons, Golgi method also provides excellent images of the dendritic spines, allowing the estimation of their density along the dendritic tree, and the study of the morphological characteristics of them (**Fig.**). Spinal density can be assessed either by semiautomatic tracing with Neuromantic or any equivalent software, or in a more manually defined way on the grounds of multiple images of different segments of the dendritic tree.

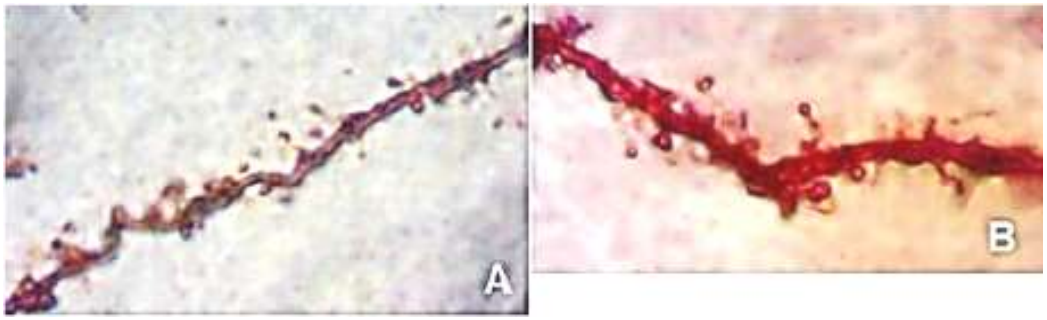


Fig. 3: Dendritic spines as they are demonstrated using Golgi method. Magnification 1000X

Neuronal reconstruction and neuronal function

How neuronal tracing and the study of neuronal morphology can be useful in the understanding of neuronal functionality? From the experimental standpoint, studying the structure–activity relationship in neurons directly is extremely difficult, but computer simulations constitute a powerful alternative. In classical computational models, anatomy is simplified or kept "constant", and the influence of various distributions of active and passive properties on neuronal firing is assessed (Sholl 1955). With a complementary approach, one can keep the biophysical model constant and implement it on different dendritic structures. In this way, investigators characterized the effect of morphological differences among different neuronal classes on their firing patterns and on the dendritic back- and forward-propagation of action potentials (Poirazi and Mel 2001). These findings were recently extended by an analysis of topological influences of firing properties and by studies of the electrophysiological effect of dendritic variability within the same morphological class (van Elburg and van Ooyen 2010).

Model simulation experiments can be carried out in simulation environments like Neuron and Genesis. The NEURON simulation environment can be obtained via the World Wide Web (WWW) at (www.neuron.yale.edu) (**Fig.**). Neuron works on hoc programming language, however a graphic user interface is also available. Neuronal reconstructions can be uploaded and be used for simulation experiments. The user is able to work on passive model or on active models using specific neurophysiological data that are available in the literature (Carnevale and Hines 2008).

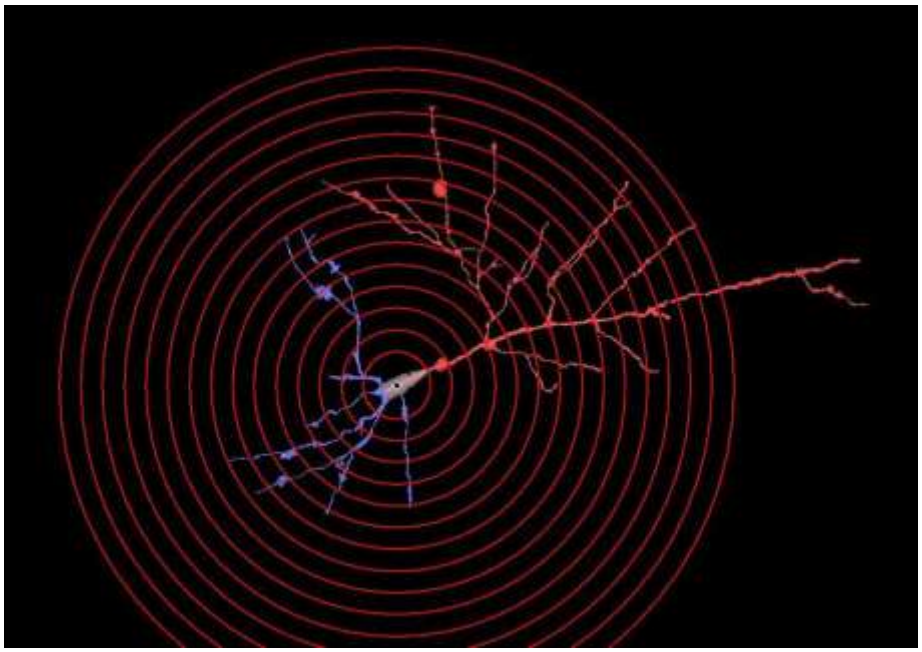
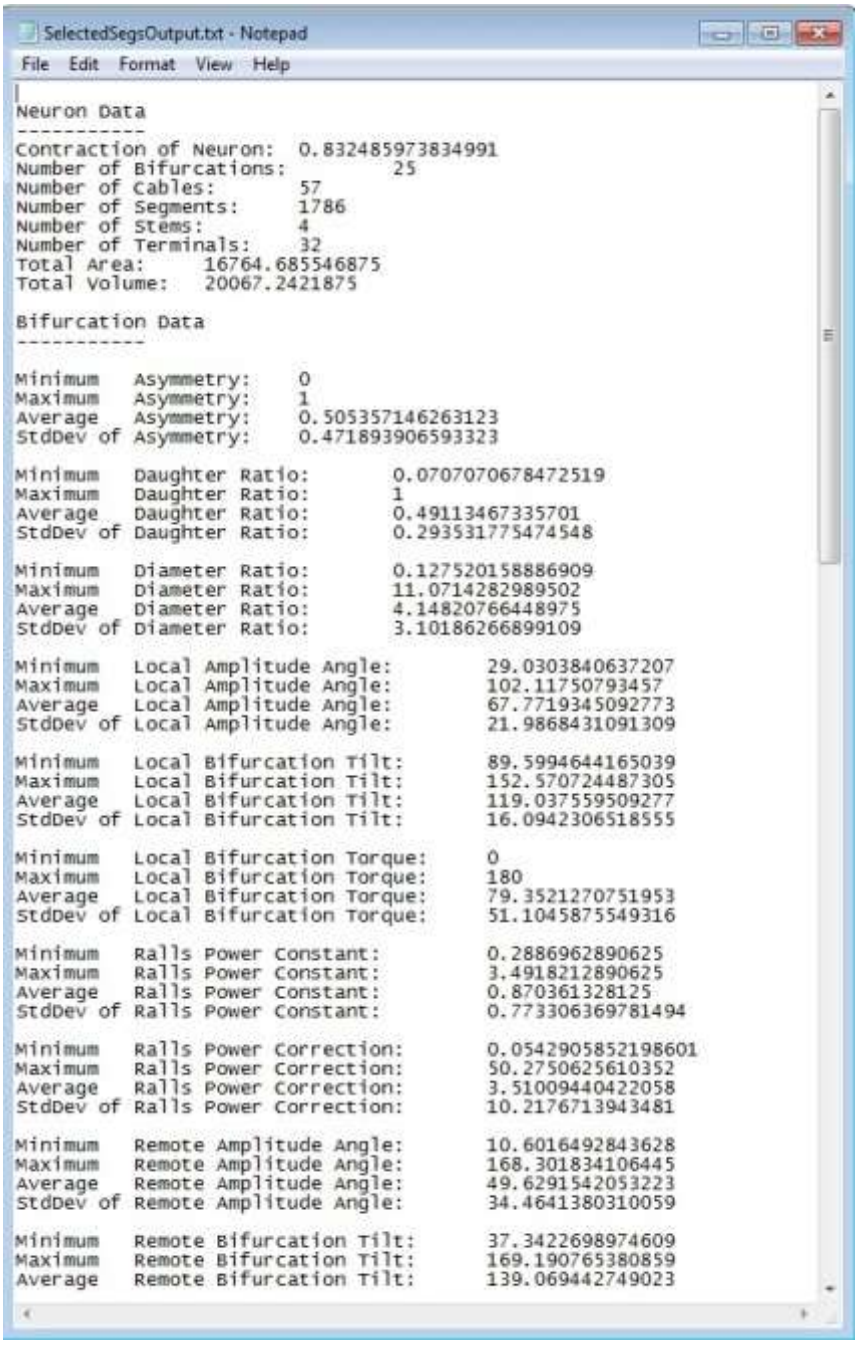


Fig. 4: Sholl analysis is performed by counting the number of dendrite intersections for concentric circles, which are centered at the centroid of the cell body, of gradually increasing radius

Limitations

Neuronal dendrites are not static structures; rather they exhibit dynamic changes that apparently reflect functional changes in the central nervous system. Therefore the data that we obtain from the morphological analysis and 3D reconstruction represent snapshots that may not be entirely representative. Moreover there are numerous practical difficulties in gathering accurate quantitative measurements of neuronal arborisations using conventional light microscopy, namely factors that are related to tissue shrinkage, operator errors, and the limited resolution of the light microscope.

Fixation shrinkage of an entire slice can be assessed by measuring slice size and thickness before and after fixation. Shrinkage factors are estimated from these measurements and applied to the obtained neural reconstructions. These methods assume, however, that shrinkage was uniform throughout the slice and that individual cells shrink at the same rate as the entire slice. This assumption may not hold true in some cases. Shrinkage at the edge of a slice can be different than in the centre, leading to a distortion of cells. Also, individual dendrites may curl up rather than shrink, and curled dendrites may retain their original length (Jaeger 2015).


SelectedSegsOutput.txt - Notepad

File Edit Format View Help

```
Neuron Data
-----------
Contraction of Neuron:  0.832485973834991
Number of Bifurcations:         25
Number of Cables:       57
Number of Segments:     1786
Number of Stems:        4
Number of Terminals:    32
Total Area:     16764.685546875
Total Volume:   20067.2421875

Bifurcation Data
----------

Minimum   Asymmetry:    0
Maximum   Asymmetry:    1
Average   Asymmetry:    0.505357146263123
StdDev of Asymmetry:    0.471893906593323

Minimum   Daughter Ratio:       0.0707070678472519
Maximum   Daughter Ratio:       1
Average   Daughter Ratio:       0.49113467335701
StdDev of Daughter Ratio:       0.293531775474548

Minimum   Diameter Ratio:       0.127520158886909
Maximum   Diameter Ratio:       11.0714282989502
Average   Diameter Ratio:       4.14820766448975
StdDev of Diameter Ratio:       3.10186266899109

Minimum   Local Amplitude Angle:        29.0303840637207
Maximum   Local Amplitude Angle:        102.11750793457
Average   Local Amplitude Angle:        67.7719345092773
StdDev of Local Amplitude Angle:        21.9868431091309

Minimum   Local Bifurcation Tilt:       89.5994644165039
Maximum   Local Bifurcation Tilt:       152.570724487305
Average   Local Bifurcation Tilt:       119.037559509277
StdDev of Local Bifurcation Tilt:       16.0942306518555

Minimum   Local Bifurcation Torque:     0
Maximum   Local Bifurcation Torque:     180
Average   Local Bifurcation Torque:     79.3521270751953
StdDev of Local Bifurcation Torque:     51.1045875549316

Minimum   Ralls Power Constant:         0.2886962890625
Maximum   Ralls Power Constant:         3.4918212890625
Average   Ralls Power Constant:         0.870361328125
StdDev of Ralls Power Constant:         0.773306369781494

Minimum   Ralls Power Correction:       0.0542905852198601
Maximum   Ralls Power Correction:       50.2750625610352
Average   Ralls Power Correction:       3.51009440422058
StdDev of Ralls Power Correction:       10.2176713943481

Minimum   Remote Amplitude Angle:       10.6016492843628
Maximum   Remote Amplitude Angle:       168.301834106445
Average   Remote Amplitude Angle:       49.6291542053223
StdDev of Remote Amplitude Angle:       34.4641380310059

Minimum   Remote Bifurcation Tilt:      37.3422698974609
Maximum   Remote Bifurcation Tilt:      169.190765380859
Average   Remote Bifurcation Tilt:      139.069442749023
```

Fig. 5: A draft presentation of a txt format file containing morphological parameters of a traced neuron, as it is automatically extracted by Neuromantic software

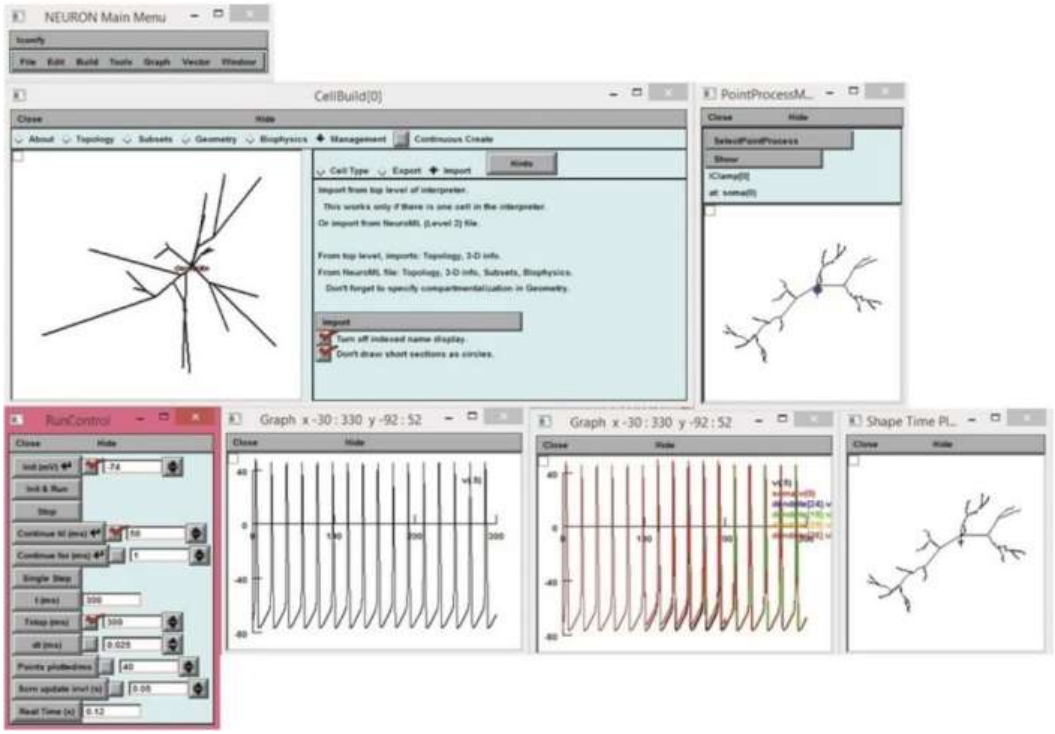


Fig. 6: NEURON Simulation environment is used to study the influence of the morphological parameters to the neuronal functionality

From the time the brain is removed until coverslipping, neurons undergo significant changes in morphological structure. To ensure that autolysis time does not affect dendritic measurements, two-tailed Pearson product correlations can be performed between all dependent measures and autolysis time and in the case that a significant correlation is noticed this has to be taken into account.

Other options such as confocal microscopy of neurons filled with fluorescent tracers could, in principle, be more accurate and might even contribute an element of automation to the reconstruction process.

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FUTURE PERSPECTIVES ON ALZHEIMER'S DISEASE COMPUTATIONAL MODELING – A REVIEW

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Abstract
Alzheimer's disease (AD), which is one of the most common age – associated neurodegenerative disorders, has been proven to be of a multifactorial and polygenic disease. No single gene mutations alone or environmental factors can be associated to AD development, except for the close and complex interactions in human genome and metabolome. This is the reason why AD exhibits many phenotypic features correlated to common and specific neuropsychiatric symptoms. Such complex diseases can only be understood by correlating a large amount of data and knowledge which can be possible only in a high performance algorithmic system available nowadays through computational modeling. There are many computational models to correlate different features of AD and to highlight new features of AD. Based on a rigorous scientific articles database analysis, this review aims to bring together some of the most representative computational AD modeling studies and to propose new ways of using computational algorithms in AD research.

Abbreviations: AD - Alzheimer's disease, ADMET - Absorption, Digestion, Metabolism, Excretion and Toxicity, APOE – apolipoprotein E, CN - Computational Neuroscience, CoMFA - comparative molecular field analysis, CoMSIA - comparative molecular similarity indices analysis, ECSIT - evolutionarily conserved signaling intermediate in Toll pathway, QSAR - quantitative structure-activity relationship, PDCD4 - Programmed cell death protein 4, P2X7 – purinoreceptor 7, P2Y2 – purinoreceptor 2.

Key words*:* memory loss, neuronal loss, brain waves, computational modeling, microarray, MRI, brain neuronal network, drug targets, APOE.

Introduction

Human brain is the most surprising natural computing machine that humanity could ever see and get the occasion to study. Due to its immense capacity of understanding, analyzing, compiling and reordering huge amounts of information brought not only by outside environmental conditions, sensations, language, gestures but also due to its fabulous way of controlling a natural perfect clockwork system, a true living and moving actual algorithmic body, it is believed that the brain is the most complex organ. However, sometimes this perfectly balanced machine gets to be affected by internal or external factors that perturb its normal activity. Many of the brain's pathologies are exhibited as a result of a complex interaction of factors.

AD is one of the most common but highly destructive neuropsychiatric syndromes of whom cause is yet to be discovered. The most visible feature of AD is cognitive defect acquiring, marked by memory loss. In many years of research and etiology desperate search, it has been proven that this may be just an externally manifested effect of the true disease's cause. Along time findings show that memory loss is due to key brain regions active neuron loss and synapse number decrease (Wenk et al., 2003). This gradual loss leads to mass atrophy of the impaired regions to almost entire inactivation (Braak et al., 2007). Neurons disable and synapse discrepancy is further caused by a lower level of organization problem. Amyloid plaques and neurofibrillary tangles are highly present in AD patients' brain tissue (Maurer et al., 1997). These are due to molecular mechanisms disturbance. Protein missfolding, enzymatic activity disturbance, or even genetic expression mistakes or genetic mutations can be keys of the true AD cause (Priller et al., 2006 ; Hernandez et al., 2007). While its biochemical mechanisms are well documented, the exact cause of AD is still not known. Just a small percent (1-5%) of the AD cases are surely confirmed of a molecular genetic cause (AS, 2014). Due to this aspect, AD was categorized in early-onset AD (hereditary gene mutations specific to AD mechanisms) and late-onset AD (idiopathic, multifactorial and occurring commonly after 70 years old) (AS, 2014). The best available interventions in AD patients support remain merely symptomatic. This is the reason why detection and diagnosis in clinical research, and techniques using brain imaging, biochemical and genetic markers must be considered as tools for early detection of AD and further research for a suitable way of preventing AD.

Computational approaches in Neurosciences

One of the boldest approaches in Neurosciences is computational modeling. At the same time, because the brain acts like as of an unmeasured power computer, the only most accurate mean to study and understand how human brain works, is by using high performance computer simulations. Nowadays, this forms a distinct branch in Neurosciences called Theoretical Neuroscience or CN. CN studies brain functions as an interdisciplinary complex connecting cognitive science and psychology with engineering, computer science, mathematics, and physics (Churchland et al., 1993). CN almost always refers to essential features of biological systems studied in multiple spatial-temporal contexts (membrane currents, proteins, and chemical coupling to electrochemical oscillations, columnar and topographic architecture, and learning and memory) being used to study hypothesis that can be tested experimentally.

Computational approaches in Neurosciences can be organized in several lines of interest. Often, new models in experimental biology are tested and analyzed by both experimentalists and IT engineers. Topics such as neuron modeling, sensory modeling, synapse and memory formation, cognition, learning, and consciousness are considered main guidelines in CN.

As it has been stated before, the human brain works as a whole in order to guide whole's body movement, responses and actions towards environmental and inside factors. This makes it a complex analysis and coordinating system that computes and calculates each and every literal move of the body. It has been shown that neurons can perform computations through their complex biophysical characteristics (Forrest, 2014). Neurons are themselves complex computational machines. Theories of dendritic, somatic, and axonal functions have matured well beyond the traditional scheme of "input–integration–output". Single neurons and their arbors are considered sophisticated time filters, coincidence detectors, internally distributed devices of local memory storage, and dynamic metabolic assemblies with high internal spatial specificity (Ascoli 2002; Senft and Ascoli 1999). Though a very accurate computed system in predicting timing and qualitative features of neuron's action potential, it has never been engineered a model that can compute and simulate adaptation or shunting. Therefore, nowadays CN terms neuron science as an important topic that includes voltage-sensitive currents and their dynamics, modulations, and sensitivity studies (Wu et al., 1995). Geometric properties of neurons and computational functions of dendrite

are also a subject of intense study (Koch et al., 1999). Many questions about developmental tendencies of neuronal axons and dendrites, neuronal migrations, chemical signaling, growth factor and hormones releasing and modulation have also been answered through theoretical investigations often involving computational modeling and computer simulations (Chkolovskii et al., 2004). Since the early models in sensory percepts represented by Barlow models and minimal wiring hypothesis pursue, many computational models that study sensation perception are guided in one way or another through computational modeling also pursuing minimal wiring hypothesis (Durgin et al., 2005). Furthermore the establishment of neuroanatomical databases and the development of computer graphics have resulted in a plethora of high-level research projects focusing on computational modeling (Ascoli 2002; Ascoli 1999). These studies range from the description of dendritic morphology and the characterization of its relationship with electrophysiology to the analysis of the structural determinants of higher brain functions via the detailed mechanism of neuronal assemblage into functional networks. Another highly questioned brain process is memory formation. Early modeling based on Hebbian learning postulates and Hopfield net model of associative memory. These models addressed to hippocampal memory (medium and long-termed). Prefrontal cortex related memory was modeled through theories such as network oscillations and persistent activity (Durstewitz et al., 2000). The latest models comprise synapse models with multiple timescale function (Fusi et al., 2005) and Monte Carlo method to detail acetylcholine receptor based synapses (Coggan et al., 2005). An even more challenging modeling research is made on the behavior of neuron networks because no artificial model has ever managed to simulate the recurrent, sparse and specific traits of biological networks. Ising model refers to such simulation in which small artificial neuronal networks interactions are closely followed (Schneidman et al., 2006).

Computational modeling of higher cognitive functions has recently showed that even cognition and learning can be modeled. Through single-unit recording in primates, studies proved that frontal and parietal lobes function as integrators of information from the main sensoria.

One of the ultimate goals in Neuroscience is explaining the everyday experience of conscious life. Crick and Koch formulated a consistent framework for neural correlates of consciousness, but the hypothesis remains speculative (Crick et al., 2003). The newest branch of CN, Computational clinical neuroscience brings

neuroscience, neurology, psychiatry, and computational modeling together to define and investigate neurological and psychiatric diseases, and to train scientists and clinicians that wish to apply these models to diagnosis and treatment (Adaszewski et al., 2013; Friston et al., 2014).

Computational models in AD research

In the past decades, many studies have been conducted in computational modeling, from non-invasive imaging of the human brain to uncovering the molecular mechanisms of some complex processes and disease states. Initial studies relied on classical brainwaves modeling and neuronal oscillator markers monitoring (Başar-Eroglu et al., 1992; Schurmann et al., 2000). Years later a part of these were proposed as markers in cognitive disorders diagnosis (Başar et al., 2008). Many of the computational modeling studies were unfortunately conducted mostly on schizophrenia and lesser in other neuro-affective disorders.

In Alzheimer's disease, computational approaches referred to many branches of neuroscience and brain research. The elder studies used classical and non-invasive approaches such as brain waves measurement, computational age/dementia development marker screening, and brain volume calculation algorithms and so on. The new progress in computational science today permits approaches like microarray algorithms, molecular modeling, inhibitor computational simulations, and brain activity matrix pre-evaluations. All of these genuinely aim to find a way to cure human kind's intelligence and personality plague or, at least, to previsualize AD development in individuals.

One of the many studies in AD computational modeling refers to changes in representations in subjects correlated to AD and age (Conley et al., 2001). In this way, the group used a matrix system based on HAL (hyperspace analogue to language) in order to compute and correlate semantic density (using several criteria such as amount of text, topic breadth, and number of unique words) with memory decline rate and age of subjects. This can be considered a rudimentary computational study based on cognitive algorithms (in this case, speech, semantics) that can easily calculate a dependency function between correlative criteria in a high dimensional model of memory. Thus, it has been shown that older adults tend to have denser language representations than youngers. More than that, attributing this model in Alzheimer study, it seems that age correlates with Alzheimer status in the way that adults with Alzheimer's have still denser

representations than normal older adults. These results came to support the hypothesis that greater density, normally associated in the model with good semantic depth, may reach "saturation points" and affect retrieval in older adults and especially adults with Alzheimer's.

Another computational approach was designed by De Haan et al. (2012) in order to study activity dependent degeneration and hub vulnerability. It seems that highly connected 'hub' regions are particularly vulnerable to Alzheimer pathology in the way that they show marked beta-amyloid depositions possibly caused by excessive local neuronal activity. In this way, using a computational model and testing hub regions that possess the highest level of activity or vulnerability in Alzheimer's disease, it has been shown that the modeled neural masses described average activity. The computational modeling of neural masses was based on human DTI-based cortical topology and registration of spike densities and spectral power. In this way, the higher activity of hub regions was confirmed. Also, simulations of activity dependent degeneration were conducted and compared to random degeneration. Conclusively, this model of computational study showed that excessive neuronal activity may lead to degeneration this being a possible existence and explanation for hub vulnerability in Alzheimer's disease.

Later on, computational modeling studies even got further by combining computational neuroscience and body sensor networking (Bergmann et al., 2012). They emphasized the importance of early intervention to the efficacy of treatments by the imperious need for ways of detection and diagnosis in clinical research. Based on the hypothesis the techniques using imaging, biomarkers and genetic information as tools for detection, it has been proved that multifaceted non-invasive screening tools that incorporate computational algorithms, but not relying on imaging, can be in fact a more useful tool than the others. It has been stated that a computation method originally developed to explain mental processes can be adapted to assist in the early detection of Alzheimer's disease by taking advantages of the observable changes in behavior and speech. In this way, a body sensor network system can be used to collect temporal information and computed to an algorithm also simulating natural randomness. Thus, it has been shown that body sensor networking can be used as a computational model for diagnostic purposes or treatment screening in Alzheimer's disease.

On the opposite site of domain methods stand the imaging correlated computational models. A correlation between gray matter volume and depression has been made in the context of putting together common imaging techniques and

highly specialized computational algorithmics (Son et al., 2014). In order to determine whether if depression is an aggravating factor in Alzheimer's disease, depressive symptoms and grey matter volume were compared in a computational system of algorithms based on Consortium to Establish a Registry for Alzheimer's disease (CERAD), Geriatric Depression Scale (GDS), magnetic resonance imaging and voxel-based morphometry (VBM). In this way, it have been shown that decreased gray matter volume in the right hippocampal gyrus correlated to depressive symptoms may be associated with the volume changes of frontal and temporal lobe in patients with Alzheimer's disease.

The correlation between depressive symptoms effects on Alzheimer's disease brain was also demonstrated by the study of regional cerebral brain flow in depressive state of patients (Terada et al., 2014). Thus, regional cerebral blood flow was tracked by single photon emission computed tomography with correction for gamma ray attenuation (Chang method). Depressive status was measures by Neuropsychiatric Inventory, Addenbrooke's Cognitive Examination, Mini Mental State Examination, Frontal Assessment Battery and according to the criteria formulated by the NINCDS-ADRDA. Spatial reprocessing and statistical analysis of images was performed on a voxel-by-voxel basis using Statistical Parametric Mapping 8 on MATLAB. In this way, it has been shown that a group of classical evaluation techniques can be associated with high precision computational modeling in order to correlate visual behavioral effects of Alzheimer's with cerebral fine changes. Thus, the study showed that clusters of voxels in the left middle frontal gyrus were similar to the areas in the simple correlation analysis and the dorsolateral prefrontal area is significantly involved in the pathogenesis of depressive symptoms in AD.

Computational modeling is not limited only in imaging or behavioral areas, but also in more complex and profound levels of organization. In this way, many genetic studies often include computational strategies due to the complex and yet not fully known gene expression mechanisms. A relevant study mentions that microarray analysis may be a useful tool in gene expression evaluation (Panigrahi et al., 2013). In order to determine the regulatory patterns in associated pathways of Alzheimer's disease, it has been used several computational algorithms such as microarray matrix analysis, genetic variation tendencies computing with gene interaction and phenotype determinism. This complex data analysis revealed a common sharing of important biological processes and putative candidate genes

among Alzheimer's and aging. Thus, ten major classes of transcriptional factors were associated with Alzheimer's and other diseases.

Another relevant genetic computational study compares grey matter density in Alzheimer's disease (Zieselman et al., 2014). It have been stated that a bioinformatics approach to the genetic analysis of grey matter density in the context of endophenotyping of late onset Alzheimer's disease. In this way, a machine learning analysis of gene-gene interactions and a large-scale functional genomics data were compiled in order to demonstrate that two single nucleotide polymorphisms interact synergically. Unfortunately, this model could not be replicated in independent datasets, but the genes of which SNPs were connected have high-confidence biological relationships confirmed in sensory processes implication. In this way, it has been proven that missing heritability percents are due to gene-gene or gene-environment interaction models.

Even drug testing and molecular metabolism simulations have been studied using computational modeling. A review study in 2010 (Gupta et al., 2010) shows that there were many computational studies conducted on acetilcholinesterase and beta-secretase 1 interaction hypothesis. More than that, further computational studies were based on finding a strategy to identify dual inhibitors. In this way, multi-targeted directed drugs have been found effective in many central nervous system diseases by drugs and leading inhibitor molecules compiling in ADMET analysis. Three-dimensional QSAR models for 43-hydroxyethylamine derivatives and beta-secretase inhibitors were developed by CoMFA and CoMSIA techniques. Thus, information gathered from the 3D-QSAR contribution maps, and the developed models revealed some of the effects of the substitutional patterns related to the biological activity of anti-Alzheimer compounds.

Computational modeling also revealed important features of Alzheimer's disease mecanistics. In this way, a case study (Charzyńska et al., 2014) describes an interesting computational model for sphingolipid metabolism as a point of origin for further analysis in Alzheimer's disease mechanisms. Based on the fact that sphingolipids are surprisingly involved in apoptosis and cell signaling, the group developed a modeled pattern using computational science in order to describe that sphigolipid metabolism models must not be concentrated only on synthesis form ceramide and biochemical transformations in particular subspecies, but also to other biochemical features such as organelle compartimentization. In this way, the authors claim that a similar computational model can be used for the study of molecular processes underlying Alzheimer's disease.

Apolipoprotein E4 inhibitors were also studied using computational design (Huang et al., 2014). As APOE4 is a confirmed major genetic risk factor for Alzheimer's disease, finding an inhibitor for it can be a step forward in therapy and pharmacology. In this way, using virtual screening of traditional Chinese medicine database and investigating potential compounds for the inhibition of ApoE4, major candidates have been selected: solapalmitine, isodesacetyluvaricin and budmunchiamine L5. Computational dynamics analysis and molecular dynamics in simulated conditions were used to observe protein-ligand complexes interactions and variations. Conclusively, it seems that budmunchiamine L5 can be easily absorbed, can penetrate blood brain barrier, and be lesser toxic according to ADMET prediction.

Ethical considerations

Computational methods and simulations when conducted effectively and ethically could have a substantial impact on medical progression and could lead to a better understanding of Alzheimer's disease underlying pathophysiological mechanisms.

There is no meaning in using sophisticated computer models in medical research if they are not reliable, safe and if they cannot provide a genuine improvement to the humanity. According to the Code of Professional Ethics for Simulations, for every computer model or simulation a full disclosure of system design and assumptions and known limitations should be provided, the researchers should be explicit and unequivocal about the conditions of applicability of specific models and associated simulation results, and furthermore they should assure thorough and unbiased interpretations and evaluations of the results of modelling and simulation studies (Oren, 2016).

Computer models can be easily repeated, usually with low time and money cost. Moreover computer models and simulations can significantly decrease the use of animals in medical research, and they could also prove beneficial because they allow for valuable resources to be better prioritized on successful plans rather than on works in progress, securing that a plan is in proper working order and therefore decreasing to the least the possible detrimental side effects or shortcomings, being safe for use by humans before it is officially set into action.

Tongen and Adam proposed a verification procedure for computational models and simulations that would be used for human clinical tests (Tongen & Adam, 2016). According to this procedure a proper verification of the mathematical model, proper understanding of the model to human pyshiology relationship,

proper verification of margins of error, and proper verification of the rists and benefits of the technology should be ensured in order to eliminate the possible risk on human beings. As medicines and medical tools must be assessed for safety before use on human subjects, computational models used in medical decision-making should be evaluated as methodically, while gone unchecked, could have detrimental effects (Tongen & Adam, 2016).

Future perspectives

Many of the already conducted studies give interestingly perspectives in Alzheimer's disease research. In this way, future perspectives can aim to any of the existent in queue studies' conclusions targeting pathways description or simulation, drug testing simulations, and even revolutionary to bearer diagnostic or monitoring techniques. Considering that computational models reflect human neural mechanism, some therapeutics suggestions can arise: slowing of stopping the degeneration of synaptic connections and thus the development of the disease in its early stages of further understanding of neuronal networking of brain in terms of describing psychiatric symptomatology or psychopharmacology (Duch, 1997; Duch, 2000; Duch, 2007).

As AD reveals itself as an extremely complex disease, it seems that a complex system of algorithms is needed in order to correlate all the pathophysiological features and physiological contexts with the known molecular background and to find new information regarding the complicated brain network function and connection to normal and pathological pathways. For example, only by using a complex computational genetic tool (Panigrahi et al., 2013) it was possible to reveal the association between aging and AD and also the differences between the normal aging process and the neurodegenerative states which lead to dementia. Obviously, all of these findings were possible only by correlating huge amounts of data obtained from complex RNA microarray analysis which is actually a very delicate genetic tool, but which can provide innovative responses to unsolved chapters in brain physiology and pathology.

More than that, tens of AD risk factors were revealed also through computational analysis of information obtained through genetic studies. For example, many of the interactions between AD causative/susceptibility genes were revealed (Soler-Lopez et al., 2011) in a study which provided the most complete interactome of AD by analyzing the global properties, its functional modularity. In this way, it has been shown that a putative role in the case of PDCD4 as a neuronal death

regulator or ECSIT which is the key molecule linking oxidative stress, inflammation, and mitochondrial dysfunction to AD pathology.

In the same way, by complex computational modeling and in vivo observation, it was possible to asses and investigate many receptors thought to be involved in AD pathology. P2X7 and P2Y2 receptors were shown to be linked to APP processing through secretase activity (Miras-Portugal et al., 2015) in a rather far-reaching study reviewing immunochemistry, genetic and computational tools.

Also, new targets in treatment and new active molecules were found or designed through computational modeling. An interesting computational study reveals a new binding site on the acetylcholine esterase molecule on which inhibitors may form stable complexes which can be further exploited in the drug design of new inhibitors of the acetylcholine esterase based on C60 fullerene derivatives (Goncalves et al., 2015).

Therefore, it seems that the most optimistic approach due to the complex features of AD is the computational modeling. In this way, computational modeling together with animal modeling can provide answers to the AD complicated implications and links at a molecular level which are extremely delicate to handle even through the revolutionary molecular biology techniques. In the biotechnological context, these answers can easily be converted to revolutionary lines in drug biotechnology, computational engineering or laboratory animal model engineering. Moreover, it seems that computational modeling is rather the best tool to predict and evaluate in a simulative manner the capacity of a certain potentially therapeutic molecules to interact with the human complex molecular systems such as the active sites of the proteins involved in the APP metabolism, or the beta amyloid receptors (Barman et al., 2014, Devarajan et al., 2014).

Conclusions

1. Alzheimer's disease is a multifactorial disease that affects central nervous system and cause neuronal loss exhibited in memory loss of unknown etiology. Furthermore, many behavioral, physiological, biochemical and even genetic features have been described through revolutionary computational modeling, all of these in the effort of finding causes or interactions that can lead to the so complex pathology of Alzheimer's disease.

2. Many studies describe various computing analysis techniques meant to shed some light on the pathological pathways of Alzheimer's, but until now, it remains unclear although clear progress have been made.
3. Computational science can offer great opportunities in further research in order to understand neuronal networking of brain, information that can correlate behavior to biochemical pathways and genetics.
4. Computational modeling can provide answers to essential matters which molecular biology alone could not solve.
5. Computational modeling could be a real breakthrgouh in the realm of neuroscience and medical research, but only when and if it is done with full respect to the basic principles of Ethics on Medical Research, and under the Code of Professionall Ethics for Simulations

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STUDY ON THE CRYOPRESERVED RAM SPERM CELL ULTRASTRUCTURE AFTER THE VARIATION OF THAWING TIME AND TEMPERATURE

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Abstract
The study aimed to determine the ultrastructural changes of ram spermatozoa after freezing-thawing at different rates (temperature, time) and the correlations between them and the cytological parameters. The thawing variants tested were: thawing at 90° C for 2 seconds, thawing at 75° C for 5 seconds, thawing at 75° C for 10 seconds, thawing at 50° C for 30 seconds, and thawing at 39° C for 120 seconds. The results of this research on the degree of damage to the plasma membrane after freezing-thawing at different rates show that the best thawing rates are obtained when the fine straws were thawed at 39° C and 50° C as compared with other thawing temperatures.
Key words: : ram, cryopreservation, sperm cell ultrastructure, T.E.M.

Introduction

Semen cryopreservation is a widely used method for artificial insemination because it facilitates the dissemination of valuable genetic material, even in small herds, leading to the increase of the improvement of the gene pool.

While the basic cryogenic damage can be morphological and often lead to dysfunction, physical stress suffered by the sperm membrane during the freezing process must be considered as the most limiting factor [8]. Even though sperm motility and morphology assessments can be used as a quick analysis of semen samples, these tests do not reveal morphological changes in nanometer-sized defects [3].

Different regions of the plasma membrane of sperm cells plays different roles in sperm cell function and survival. Structural components of the head, intermediate piece and flagellum respond differently to factors such as heat and osmotic shock during storage in a liquid or frozen state [7].

Using TEM (Transmission Electron Microscopy) as a complementary diagnostic for qualitative assessment of semen lead to more accurate determination of its fertilizing ability.

The study aimed to determine the ultrastructural changes of ram spermatozoa after freezing-thawing at different rates (temperature, time) and the correlations between them and the cytological parameters.

Materials and methods

Animals: sperm samples were collected from five adult Merinos de Palas rams with known fertility. Collection was made with an artificial vagina, 2 times per week. Sperm samples from each animal were analyzed separately in order to take into account the variability in the individual. For each male 1-2 ejaculates were collected (every 15-30 minutes), which were subsequently mixed and subjected to experiments. A total of 86 ejaculate were processed. Semen was cryopreserved in 0.25 ml fine straws.

The activity of freezing ram semen was performed according to the freezing technology developed in the Laboratory of Biotechnology of Reproduction, Institute of Research-Development for Sheep and Goats Breeding of Palas, Constanta [12], [13]. Experiments were conducted in the normal breeding season, during October 2012 - December 2012. Thawing and testing semen samples was performed in the Laboratory of Cell Biology, University Ovidius, from March to June 2013.

As dilution medium a diluent of Tris base 20% (v/v) egg yolk was used. The cryoprotectant used for freezing ram semen was glycerol (5% final concentration).

The following thawing variants were tested:

- Thawing at 90 ° C for 2 seconds
- Thawing at 75 ° C for 5 seconds
- Thawing at 75 ° C for 10 seconds
- Thawing at 50 ° C for 30 seconds
- Thawing at 39 ° C for 120 seconds

Sperm samples from the experimental variants were processed and analyzed in terms of ultrastructural view using transmission electron microscopy. The samples were processed in the usual way, namely prefixing the samples in a cacodylate buffer with 2.7% glutaraldehyde, fixing in osmic acid, dehydrating in serial alcohol baths and including in epoxy resins. The fine sections were double

stained with uranyl acetate and lead acetate, after which they were examined under a Philips 320M microscope.

Evaluation of sections

Sperm cells were assessed at all levels of the cell for plasma membrane integrity. Transversal and longitudinal sections at the level of the main part and the intermediate part of the flagella and sagittal sections through the sperm head were examined. Sections were categorized as having undamaged or damaged membranes. Also, the acrosome integrity was analyzed, which has been classified as undamaged or vesicular. The method consisted of counting at least 200 spermatozoa, serially sectioned in various planes of the sperm cells for each experimental variant [6], [13]. Characterization of whole cell was made after the appearance of the membrane on photographs taken on a small scale x 3500-6000.

Results

The results regarding the integrity of the plasma membrane at the flagella and sperm cell head level and the acrosome integrity are shown in Table 1.

Table 1. The integrity of the plasma membrane and the acrosome (%, mean ± SE, n = 10) after freeze-thaw

Variant	n	Plasma membrane		Acrosome
		Head	Flagella	
Thawing at 39 ° C for 120 seconds	10	41.59 ± 2.56^{a}	44.25 ± 3.12^{a}	46.99 ± 2.39^{a}
Thawing at 50 ° C for 30 seconds	10	46.21 ± 1.79^{a}	53.12 ± 2.72^{a}	60.02 ± 4.52^{a}
Thawing at 75 ° C for 10 seconds	10	24.66 ± 1.23^{b}	28.03 ± 1.85^{b}	27.86 ± 1.42^{b}
Thawing at 75 ° C for 5 seconds	10	29.04 ± 2.19^{c}	34.16 ± 1.62^{c}	37.92 ± 3.23^{c}
Thawing at 90 ° C for 2 seconds	10	12.66 ± 0.78^{d}	15.25 ± 1.65^{d}	17.23 ± 0.83^{d}

Sperm cell ultrastructure analysis results are in agreement with those obtained by optical microscopy analysis and flow cytometry. The best results regarding the

integrity of the plasma membrane, both at the head and the flagellum level were obtained for thawing at 50 °C for 30" and the worst results for thawing at 90° C for 2". There were no statistically significant differences between thawing at 39 º C and thawing at 50 º C, but there were statistically significant differences ($p < 0.05$) compared to the rest of the thawing variants.

1. Ultrastructure of frozen-thawed spermatozoa at 39 ° C for 120"

In general, all the head level sections shows normal membranes and a rate of 41.59% of the cell plasma shows non lysed membranes at the head level (figure 1). The acrosome has a intact structure in 42% of cells and the acrosome external membrane is generally unaffected. At the flagella level, 44.25% of the cells have integral plasma membrane.

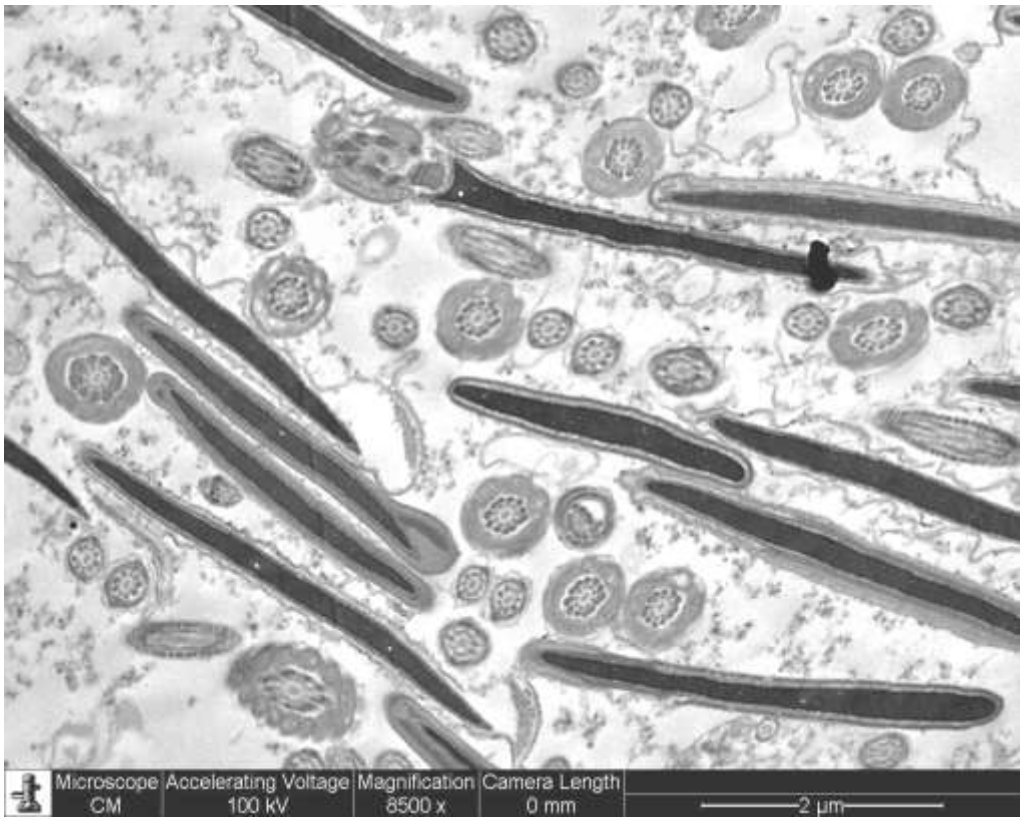

Fig. 1 (original). Sagittal section at the head level (x8500), acrosome contains an acrosome matrix with an electron-dense layout, in which the apical ridge is observed

2. Ultrastructure of frozen-thawed spermatozoa at 50 ° C for 30"

It is found that most of the membrane damage occurs at the head level, similar to the other experimental variants, although differences between the damage of the flagella are very small. Interrupted membranes occur in 46% of ram spermatozoa thawed at 50 ° C. At the intermediate piece and the main piece level damage occurs in 49% of cells (figure 2). The acrosome has a intact structure in 60% of the cells.

The increased motility observed implies the existence of a cytoskeleton and a motor system of the microtubule and intact mitochondria.

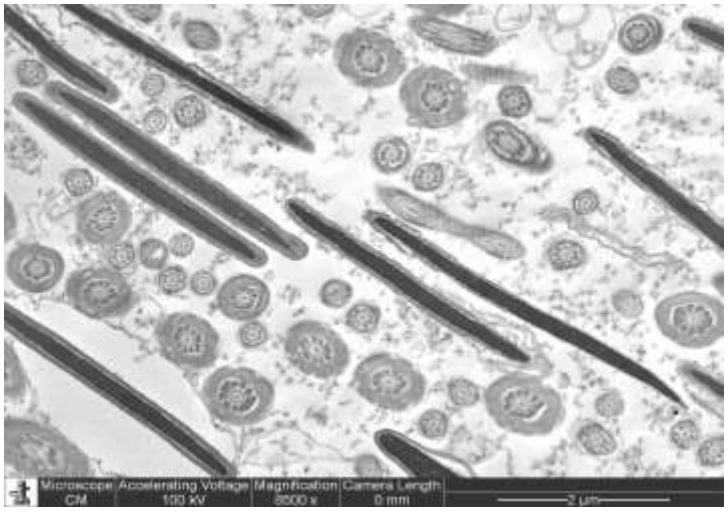

Fig. 1 (original). Ram sperm cells thawed at 50 O C (x8500)

3. Ultrastructure of frozen-thawed spermatozoa at 75 ° C for 5" and 10"

Thawing at 75 ° C, both for the period of 5 seconds and 10 seconds lead to significantly lower results compared to thawing at 39º C and 50º C. Better results were obtained thawing for 5 seconds.

At the flagella level the plasma membrane has a serrated look, is partially detached or completely broken (figure 3).

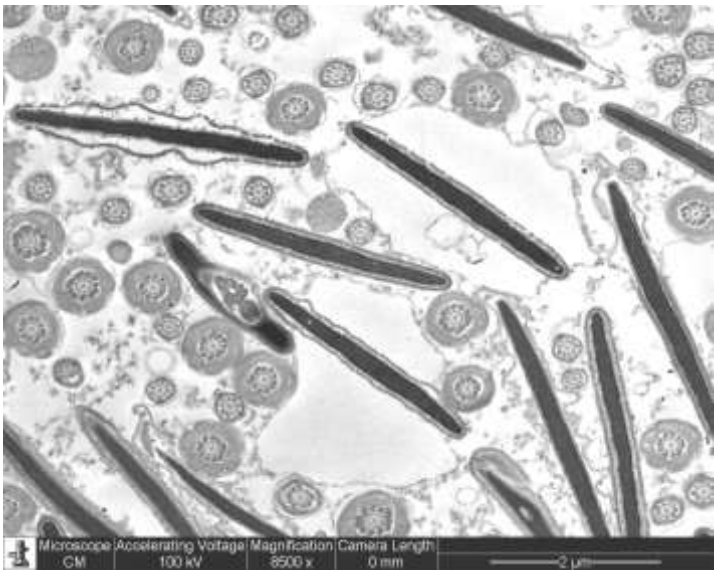

Fig. 3 (original). Ram sperm cells thawed at 75 O C for 5" (x8500)

4. Ultrastructure of frozen-thawed spermatozoa at 90° C for 2"

In the case of thawing at 90º C statistically significantly lower results were obtained compared to the rest of variants. Only 12.66% of the cells shows integral plasma membranes at the head level and 15.25% at the flagella level (figure 4). The acrosome has intact structure only in 17.23% of cells.

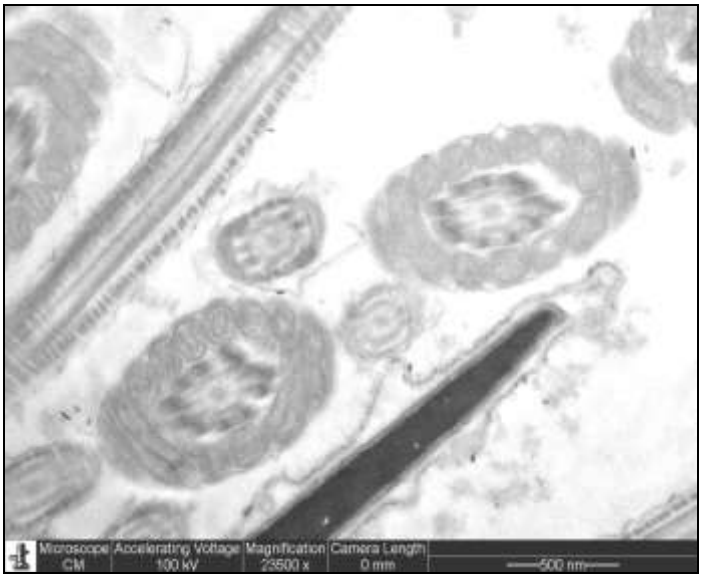

Fig. 4 (original). Ram sperm cells thawed at 90 O C for 2" (x23500)

DISCUSIONS

During the process of cryopreservation sperm cells suffers ultrastructural changes (plasma membrane, mitochondrial, acrosome), biochemical and functional [10]. Damage can occur at any stage of the process, but to a greater extent during cooling at 0° C and thawing and less during storage at -180 ° C. Structures and spermatozoa organelles respond differently in different phases to the osmotic or environment temperature changes. Ultrastructural damage are accompanied by biochemical changes or cell loss of vital content.

Plasma membrane integrity and mitochondrial function are the main attributes of a sperm cell to fertilize an egg. Damage to the plasma membrane and mitochondrial function may lead to membrane destabilization and impaired mitochondrial energy metabolism and cell viability [9], [11]. After freeze-thawing the alteration of mitochondrial filament was observed, also in a smaller proportion at the axonema, the filament and the fibril flagellation level.

The main target of the damage caused by cryopreservation is the spermatozoa plasma membrane. Due to variations in temperature and osmolarity, such as freeze-thaw induced alternations of the cell volume of water generating considerable mechanical stress on the cell membrane [5]. Since only sperm with intact membranes may be subject to capacitation and acrosome reaction, it is important to know the type and location of changes in order to optimize the technology of freezing [2].

In mammals, sperm cell membrane has a specific lipid content, distinct from that of other cells. It contains high levels of phospholipid, sterols, saturated and unsaturated fatty acids, plasminogen and sphingomyelins . This structure is responsible for the specific flow, flexibility and the ability of sperm cell operation. Polyunsaturated fatty acids have an important role in ensuring fluidity and in regulation of spermatogenesis [1]. Sperm plasma membrane has a heterogeneous structure in five specific areas: acrosome, equatorial segment, basal segment, intermediate and terminal piece. The differences between these regions are related to different physiological functions. Before and after ejaculation the plasma membrane suffers from some changes in the integration of lipid, modification of the degree of saturation of fatty acids and the loss of cholesterol from its composition resulting in a marked decrease in the cholesterol / phospholipid ratio. The various regions of the membranes differ in respect to this report.

Microscopic examination of ram sperm labeled with an membrane integrity indicator demonstrated that exposure to low temperature followed by

heating has a different effect on plasma membrane, especially at the head and intermediate piece level [6]. Ultrastructural analysis results show that the head plasma membranes are more affected than the flagella. While several cytoskeletal proteins were identified, their role in maintaining the integrity of the plasma membrane remains unclear [4]. Also, research shows that the acrosome is less affected, although the sperm plasma membrane that surrounds the sperm cell head presents major detachment, vacuolation and even interruptions. Similar results were obtained in studies [8] showing that the sperm plasma membrane surrounding the head is considerably more labile than the one at flagellum level and the outer acrosome membrane is more vulnerable than the internal one .

CONCLUSIONS

Freezing-thawing leads to alterations in the plasma membrane that include rupture, especially at the head level, and membrane detachment and vacuolation at the head and flagella level. Analysis of photomicrographs shows that the acrosome were only partially affected by the freezing-thawing process. Although many cells shows a bloated acrosome, the internal acrosome membrane is intact. Plasma membrane has several degenerative changes at the head level compared to the flagella level.

The results regarding the degree of damage to the plasma membrane after freezing-thawing at different rates show that the best rates are thawing at 39 ° C for 120" and thawing at 50° C for 30".

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THE EVALUATION OF SERUM ELECTROLYTES CALCIUM, IONIC CALCIUM, MAGNESIUM AND HEMOGLOBIN IN SULINA'S PATIENTS, DANUBE DELTA, ROMANIA

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Abstract

According to literature data, the normal values of biochemical parameters in blood vary by sex, age, geographical region and type of diet. The aim of this study was to analyze the benefits of a fish-based diet among the population of Sulina Town, in the Danube Delta. The batch of patients underwent a set of biochemical tests in the RoutineMed Laboratory of Sulina. The novelty of the research is represented by the geographic area covered, as the Danube Delta had no medical analysis laboratory until 2010, when RoutineMed Laboratory was opened in Sulina. Blood samples were collected from 260 patients (of 3663 residents) for the evaluation of the serum electrolytes: total calcium, ionic calcium, magnesium and hemoglobin. Both women and men were involved in the research and patients were grouped into age ranges: 20-40 years, 40-60 years, > 60 years. The study included 260 patients, of which 90 men (34.6%) and 170 women (65.4%), who declared they eat fish or fish-based products at least once a week. The values obtained were statistically analyzed using the SPSS v. 20 software and then compared to the ranges considered normal for these parameters. The results obtained showed that patients with a fish-based diet seem to be healthier than those with a diet in which fish meat is scarce, as their blood biochemical parameters values are closer to normal, which leads to the conclusion that including fish and fish products in people's regular diet is beneficial.

Keywords: calcium, ionic calcium, magnesium, hemoglobin, Sulina

Introduction

The aim of this study was to analyse the benefits of fish diet in the Danube Delta, Sulina's population. Based on literature data, normal values of biochemical parameters vary by gender, age, diet and geographical region (Wallach, 2001).

Calcium is the major mineral component of bone. Calcium ions play an important role in transmitting nerve impulses, muscle contraction, heart function and coagulation processes (Fischbach, 2009). Hormonal regulation of calcium metabolism as the phosphorus is complex (Rosoiu, 2005). Interrelation between the small intestine, skeleton, kidney and the endocrine system, particularly parathyroid, maintain homeostasis of calcium and phosphorus. Also, calcitonin, vitamin D, estrogen, androgens are factors that influence calcium levels (Thomas,1998). 55% of plasma calcium is in the ionic form or free fraction and physiologically active processes regulating hemostasis and neuromuscular excitability and its concentration in plasma is directly regulated by PTH and 1,25 (OH) 2D3 (Brudasca, Cucuianu, 2003).

Magnesium is an element which, although is found in small proportion in the body (0.05% of total body weight), shows great importance of structurally and functionally (Rosoiu,2008). Actions of calcium and magnesium are closely linked. The one deficit of these elements significantly affect the metabolism of other (magnesium is required for both intestinal absorption and metabolism of calcium) (Fischbach, 2009). Magnesium ions with Na^+, K^+ and Ca^{2+} regulates neuromuscular excitability and coagulation mechanism (Rosoiu, 2010).

Most of the iron in the body is found in compounds heme, special in hemoglobin and myoglobin. Most of the non-hemic iron is stored as ferritin or hemosiderin in macrophages and hepatocytes. Only a very small fraction (~ 0.1%) circulates in plasma in the form of Fe^{3+} linked to a carrier protein - transferrin. Hemoglobin is the main component of red blood cell (95% of erythrocyte cytoplasmic proteins) and serves as a vehicle for the transport of O_2 and CO_2. Iron excretion occurs by cell loss in the gastrointestinal, skin, urinary and menstrual losses in women. Most of the functional iron in the body comes from reusing existing iron derived from senescent red blood cells destroyed in the reticuloendothelial system, mainly the spleen (Andrews, 2004).

Material and Methods

For serum biochemistry were used following measuring instruments: automatic biochemistry unit SAPPHIRE 350, centrifuges Rotofix 32 A and reagents Audit Diagnostics (Procedura specifică –determinări de biochimie, 2013). Blood samples were collected from 260 patients (of 3663 residents) for the evaluation of the serum electrolytes: total calcium, ionic calcium, magnesium and hemoglobin. Both women and men were involved in the research and patients were grouped into age ranges: 20-40 years, 40-60 years, > 60 years. The experimental data were processed using IBM SPSS Statistics 20. The procedures used were: Descriptive statistics (characterization variables discrete and continuous defined in the database), Graphs, Statistical tests parametric (t-test to compare the average of two independent samples, t-test to compare the average of a sample value specified test One-Way ANOVA), correlation analysis.

Results and Discussion

The study included 260 patients, 90 were males (34.62%) and 160 were females (65.38%) (Figure 1). The distribution by age groups on male was: 25 were in the age range (20-40) years, 38 were in the age range (40-60) years and 27 were in the age range (60 -...) years. The distribution by age groups on female was: 46 were in the age range (20-40) years, 74 were in the age range (40-60) years and 50 were in the age range (60 -...) years (Figure 2).

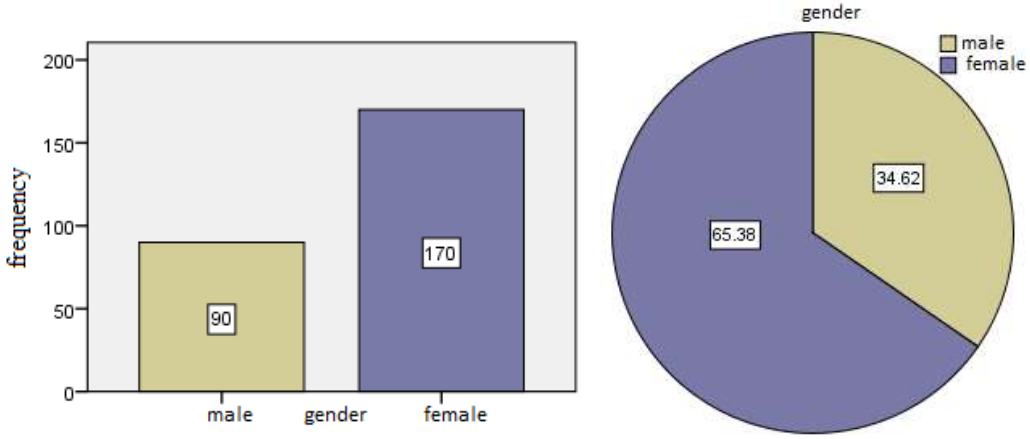


Fig. 1: The distribution by gender and percent

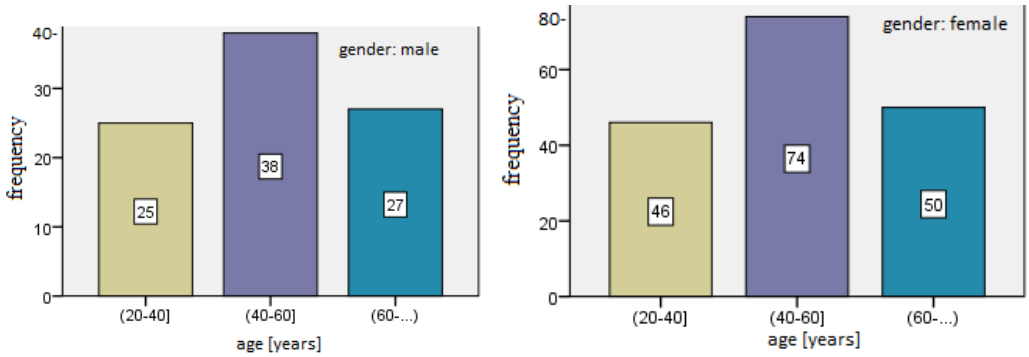


Fig. 2: The distribution by age groups on male and female

The average value of total calcium : For male patients (N = 90) the mean of total calcium is M_M = 9.104 mg / dL For female patients (N = 170) the mean of total calcium is M_F = 9.179 mg / dL.

When the mean of total calcium are compared in the two groups of patients with a reference value L_{sup} = 10.2 mg / dL: for the group of male patients average amount of total calcium - M_M = 9.104 mg / dL and for the group of female patients average amount of total calcium - M_F = 9.179 mg / dL indicate that both women and men results are within normal limits (Figure 3). The amount of protein in the blood affects the level of calcium, since 45% of the calcium in serum is protein-bound (Fischbach, 2004). Hypercalcemia and hypokalemia often accompanies it always leads to dehydration because excess calcium cause nephrogenic diabetes insipidus (Laboratory Corporation of America, 2015). Women have a diurnal variation ionic calcium and intact PTH hormone higher than men.

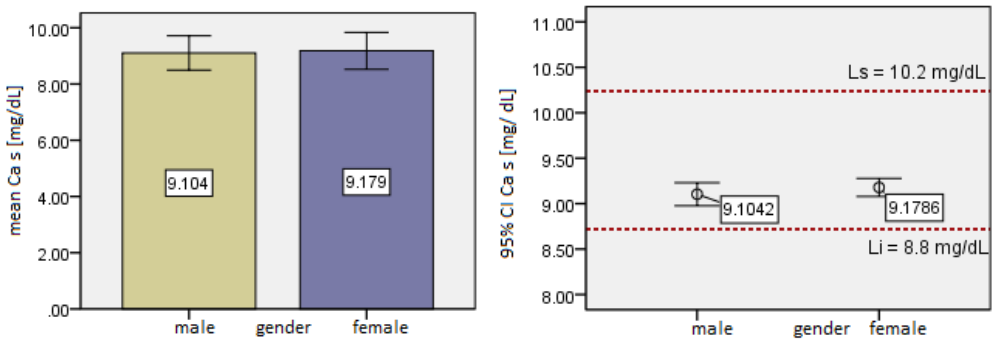


Fig. 3: Mean total calcium on male and female

The average value of ionic calcium : For male patients (N = 90) the mean of ionic calcium is M_M = 4.1197 mg / dL For female patients (N = 170) the mean of ionic calcium is M_F = 4.125 mg / dL.

When the mean of ionic calcium are compared in the two groups of patients with a reference value L_{sup} = 5.2 mg / dL: for the group of male patients average amount of ionic calcium - M_M = 4.11 mg / dL and for the group of female patients average amount of ionic calcium - M_F = 4.12 mg / dL indicate that both women and men results are within normal limits (Figure 4). Ionized serum calcium fraction tends to decline in case alkalizing blood, they increase the ability of proteins to fix calcium (Brudasca, Cucuianu, 2003). Circadian variations in ionic calcium shows significant increases after exercise and decreases postprandial.

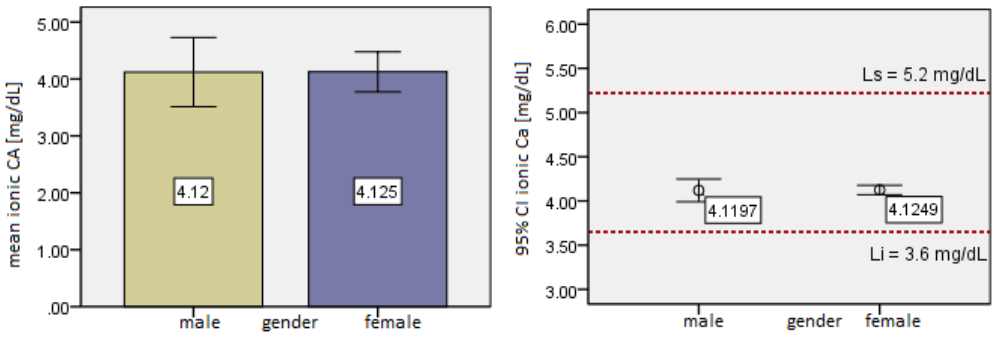


Fig. 4: Mean ionic calcium on male and female

The average value of magnesium : For male patients (N = 90) the mean of magnesium is M_M = 2.457 mg / dL. For female patients (N = 170) the mean of magnesium is M_F = 2.349 mg / dL.

When the mean of magnesium are compared in the two groups of patients with a reference value L_{sup} = 2.6 mg / dL: for the group of male patients average amount of magnesium - M_M = 2.457 mg / dL and for the group of female patients average amount of magnesium - M_F = 2.349 mg / dL indicate that both women and men results are within normal limits (Figure 5).

Magnesium deficiency will generate bone calcium mobilization, the possible occurrence of abnormal calcification in the aorta and kidney. It is therefore important to consider both levels when evaluating calcium and magnesium. Also, hypokalemia and hypomagnesemia was associated with 60%

of cases. (Thomas,1998). From clinical point of view, magnesium deficiency cause neuromuscular disorders (muscle weakness, tremor, tetany and convulsions) and on the heart may cause arrhythmias (Laboratory Corporation of America, 2010). Serum levels of magnesium may remain normal even in the presence of a depletion up to 20% of the total reserves of the body.

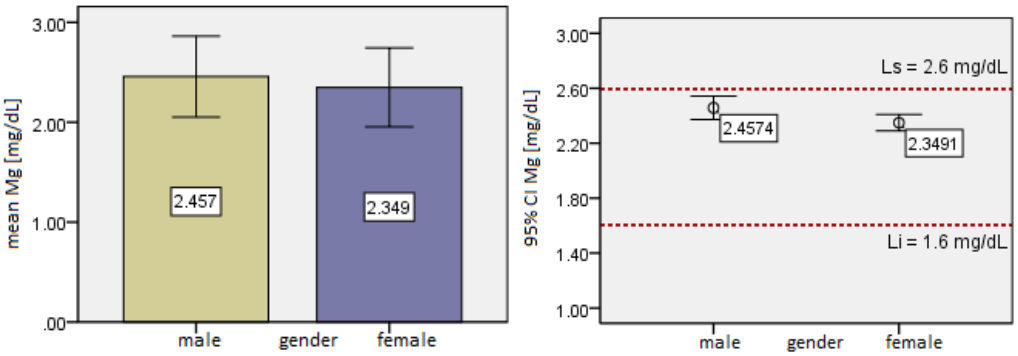


Fig. 5: Mean magnesium on male and female

The average value of hemoglobin : For male patients (N = 90) the mean of hemoglobin is M_M = 15.04 mg / dL. For female patients (N = 170) the mean of hemoglobin is M_F = 13.91 mg / dL.

When the mean of hemoglobin are compared in the two groups of patients with a reference value L_{sup} = 18 mg / dL: for the group of male patients average amount of hemoglobin - M_M = 15.04 mg / dL and for the group of female patients average amount of hemoglobin - M_F = 13.91 mg / dL indicate that both women and men results are within normal limits (Figure 6).

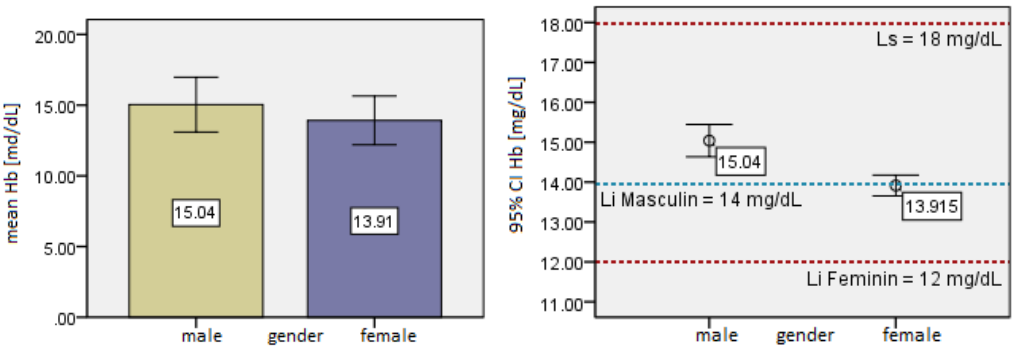
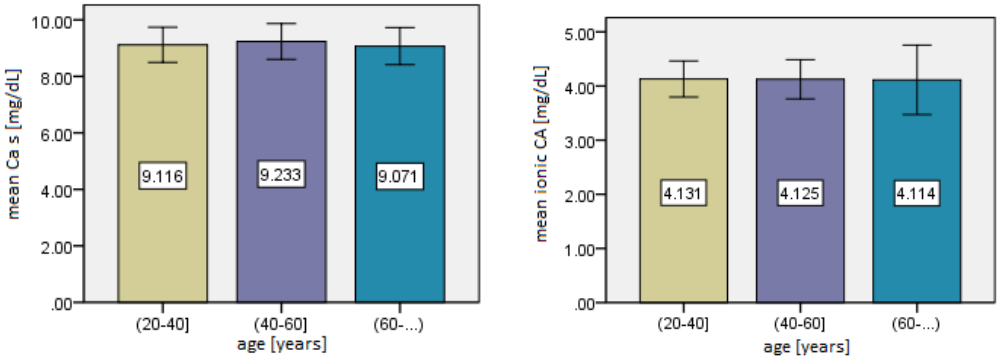


Fig. 6: Mean hemoglobin on male and female

Serum iron level without transferrin / ferritin and transferrin saturation has limited clinical value. Decreased hemoglobin below baseline levels cause anemia. Hemoglobin should be evaluated together with hematocrit, erythrocytes, erythrocyte indices and cell morphology on smear for classification of anemia. A normal value of the concentration of hemoblobina does not exclude the anemia due to acute hemorrhage.

The average value of total calcium by age groups – Male and Female: For the group (20-40] years (N = 71) the average of total calcium is $M_{(20-40]}$ = 9.11 mg / dL; in group (40-60] years (N = 112) the average value of the total calcium is $M_{(40-60]}$ =9.23 mg / dL; in the group (60- ...] years (N = 77) the average value of the total calcium is $M_{(60-...]}$ = 9.07 mg / dL (Figure 7a).



a) b)

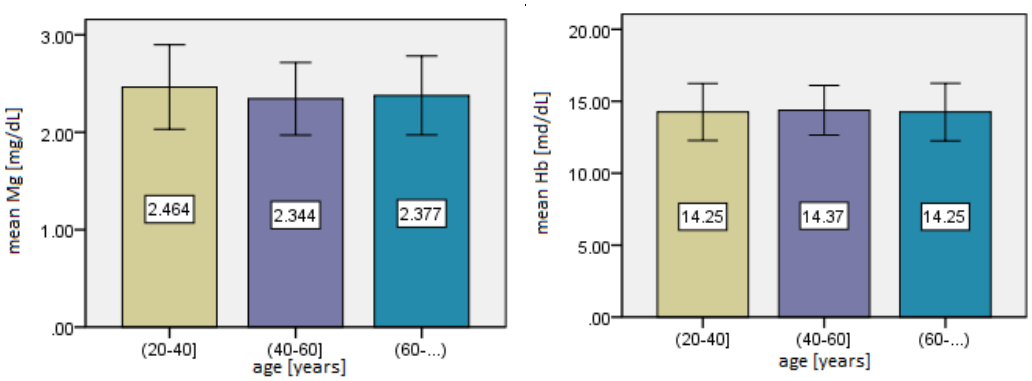
Fig. 7a (left) Mean total calcium on male and female by age groups
Fig. 7b (right) Mean ionic calcium on male and female by age groups

The average value of ionic calcium by age group – Male and Female: For the group (20-40] years (N = 71) the average of ionic calcium is $M_{(20-40]}$ = 4.13 mg / dL; in group (40-60] years (N = 112) the average value of the ionic calcium is $M_{(40-60]}$ =4.12 mg / dL; in the group (60- ...] years (N = 77) the average value of the ionic calcium is $M_{(60-...]}$ = 4.11 mg / dL (Figure 7b).

The average value of magnesium by age groups – Male and Female: For the group (20-40] years (N = 71) the average of magnesium is $M_{(20-40]}$ = 2.46 mg / dL; in group (40-60] years (N = 112) the average value of the

magnesium is M $_{(40\text{-}60]}$ =2.34 mg / dL; in the group (60- ...] years (N = 77) the average value of the magnesium is M $_{(60\text{ -...}]}$ = 2.37 mg / dL (Figure 8a).

The average value of hemoglobin by age groups – Male and Female: For the group (20-40] years (N = 71) the average of hemoglobin is M $_{(20\text{-}40]}$ = 14.25 mg / dL; in group (40-60] years (N = 112) the average value of the hemoglobin is M $_{(40\text{-}60]}$ =14.37 mg / dL; in the group (60- ...] years (N = 77) the average value of the hemoglobin is M $_{(60\text{ -...}]}$ = 14.25 mg / dL (Figure 8b).



a) b)

Fig. 8a(left) Mean magnesium on male and female by age groups
Fig. 8b(right) Mean hemoglobin on male and female by age groups

Conclusions

The values obtained were statistically analyzed using the SPSS v. 20 software and then compared to the ranges considered normal for these parameters. The results obtained showed that patients with a fish-based diet seem to be healthier than those with a diet in which fish meat is scarce, as their blood biochemical parameters values are closer to normal, which leads to the conclusion that including fish and fish products in people's regular diet is beneficial.

Including fish and fish products in people's regular diet is beneficial in preventing and screening osteoporosis in patients over 50 years, evaluation of

biologically active fraction of calcium in pregnant women, detection of certain metabolic disorders of kidneys and hemoglobin is useful for detection and monitoring of anemia and polycythemia

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AGE DETERMINATION ASPECTS IN ANCHOVY (*Engraulis encrasicolus*, LINNAEUS, 1758) AT THE ROMANIAN BLACK SEA COAST

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Abstract

Age is a factor providing significant information on species evolution at population level. Anchovy is a pelagic, gregarious, small-sized species and, until 20 years ago, it represented an important share of Black Sea fish catches. The Black Sea anchovy stock has suffered in the past decades from various causes, among which overexploitation, penetration of alien species, pollution etc.

Age determination of Romanian Black Sea anchovies was made by otolith reading using an inverted microscope. A decrease of the 2+ years share and an increase of the 1+ years age classes share were reported.

Key words*:* anchovy, Black Sea, otoliths, age

Introduction

In recent years, marine fisheries along the Romanian Black Sea coast have been limited to performing stationary fishing, in the coastal and shallow area (forage and spawning grounds for many fish species), using fixed fishing gears.

As a follow-up of analyzing the samples collected during 2010-2014, it was noticed that the biomass of commercial fish species recorded a continuous drop (Table 1). This decrease may be the result of several factors: eutrophication (sources from agriculture, municipal waste, industry etc.), harmful substances (sources from agriculture, industry, municipal waste etc.), commercial fisheries, alien species. The anchovy biomass and catches were largest during the 1980s. The first signs of overfishing appeared after 1984, when anchovy shoals were difficult to be found and the fishery enterprises incurred losses; it appears that the

dramatic reduction of the Black Sea anchovy stock in the late 1980s was due to the combined action of two factors: the excessive fishing and *Mnemiopsis leidyi* outburst [1].

Table 1. Biomass (tons) of the main commercial fish species of the Romanian Black Sea coasts (original, according to internal NIMRD Reports).

Species	**2010**	**2011**	**2012**	**2013**	**2014**
Engraulis encrasicolus (anchovy)	**50**	**41**	**57**	**44**	**30**
Sprattus sprattus (sprat)	59634	60000	68887	56429	60000
Gobiidae sp. (gobies)	500	500	450	300	300
Psetta maeotica (turbot)	1149	1147	628	554	298
Squalus achantias (dogfish)	13051	10000	1550	4483	1520
Merlangius merlangus euxinus (whiting)	20948	21000	5650	19797	15550

As it arises from the table above, anchovy, species with high commercial value and targeted by active fisheries before, has recorded massive biomass drops in the past years. Thorough population studies of the species are needed, in order to better understand the development of events such as biomass decrease. Consequently, the accurate determination of fish age is one of the most important aspects in population dynamics studies. Age is the background for calculation of growth, mortality, recruitment and other fundamental parameters for population studies [2].

Age determination in fish can me made by three methods: skeletochronology (the concentric growth rings identified in a bone cross-section are counted), scalimetry (estimation of the age of fish by examination of concentric peaks on their scales) and otolithometry (identifying the annual growth rings of otoliths in the internal ear**).** Otoliths, sometimes referred to as ear bones, are of unique value for age determination of teleost fishes because across taxa they are the only hard structures that continue growing even after somatic growth has ceased [3], but also because they are easily collected and stored.

The analysis of otoliths for age determination can be carried out in two different ways: at macrostructural level, considering annual bands, and at microstructural level, based on daily increments.

Materials and methods

Aiming at determining the age of anchovy, samples were collected from fixed fishery points located along the Romanian Black Sea coast, during May-September of the past years. Anchovy specimens were brought to the laboratory and analyzed on length classes or individually, with the purpose of assessing the general state of anchovy population at our coast (Fig. 1).

Fig. 1. Anchovy specimens analyzed in the laboratory (original).

A batch of approximately 200 individuals is analyzed for each sampling station; specimens are weighed, measured and, after separation on length or weight classes, sex, maturation degree and parasitization extent (where the case) are determined, and subsequently otoliths are extracted for age determination; growth rings are identified with an inverted microscope (Fig. 2).

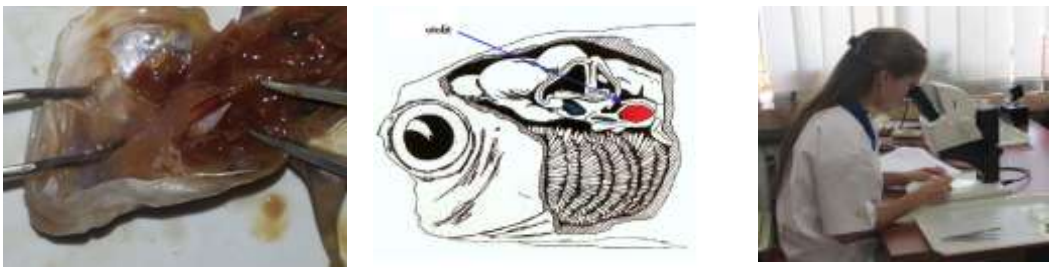

Fig. 2. Identification, extraction and analysis of otoliths (original)

Otoliths were collected by performing a section on the fish head, extracted and subsequently cleaned of the protection membranes with an alcohol-based solution. They were then stored in dry spaces until inverted microscope analysis was performed.

Age determination was made by identifying the growth rings on the surface of otoliths (Fig. 3).

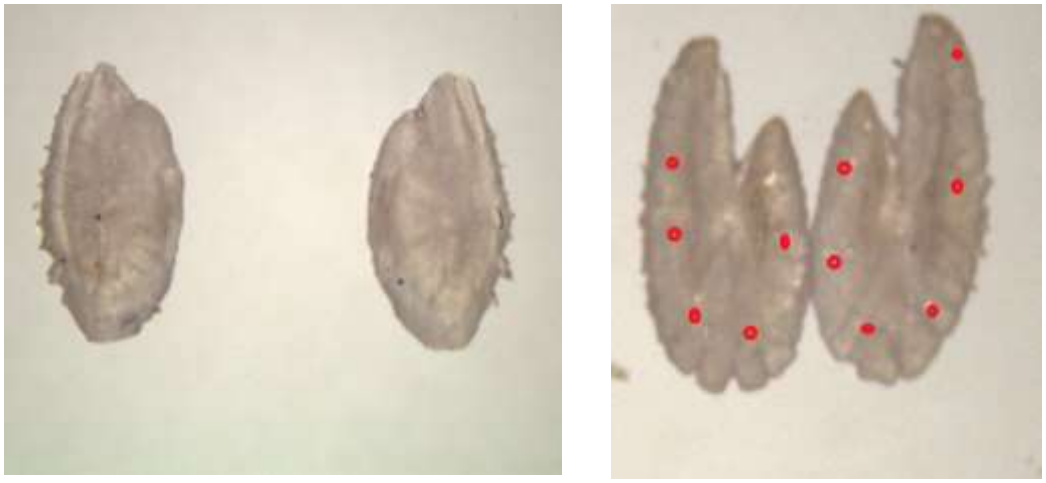

Fig. 3. Anchovy otoliths, age 1 : 1+ (original).

For an accurate age estimation, after identifying and counting annual growth rings, other aspects are also considered, such as likely hatching date, time of fishing etc. [4].

Results and discussions

During the analyzed period, the dominant age classes were between 1 : 1+ and 2 : 2+ years, while the 3 : 3+ age class had a smaller share; individuals belonging to the 4: 4+ years age class were identified only in an isolated manner (Fig. 4).

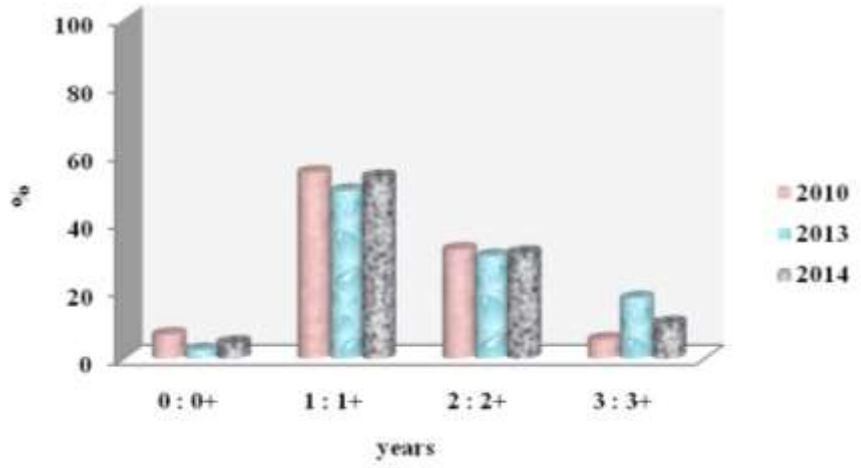

Fig. 4. Percentage of age classes in anchovy 2010-2014 (original)

Compared to previous years, it was noted that the share of individuals aged 1 : 1+ and 2 : 2+ years is dominant, with a slight increase of 3 : 3 + years individuals [5]. As such, it can be concluded that a slight rejuvenation of anchovy population of the Romanian Black Sea coast is documented.

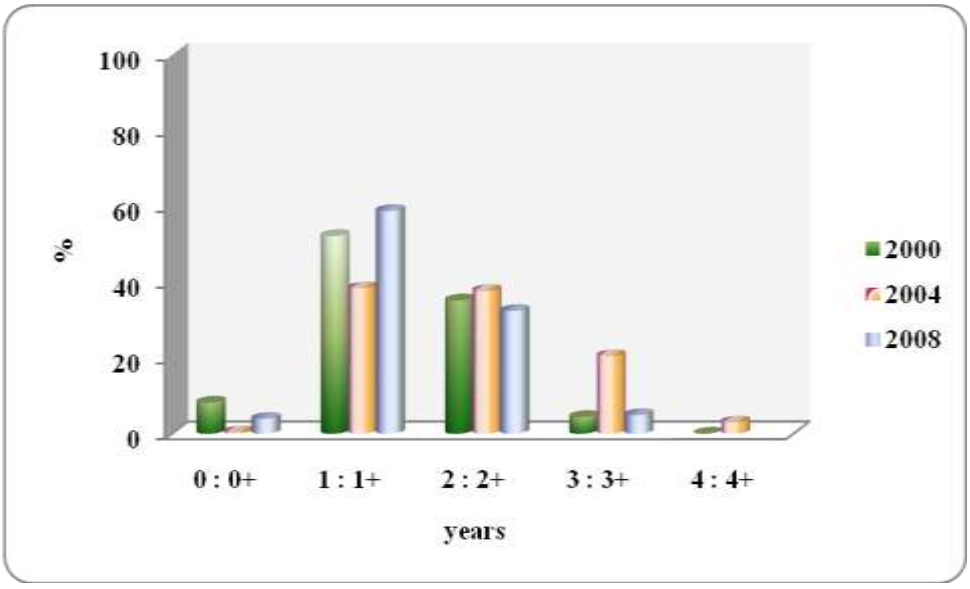

Fig. 5. Percentage of age classes in anchovy 2000-2008

(modified after Maximov et al., 2010).

Representation of several age classes in the population of one species in an ecosystem is an indicator of a good general state.

Conclusions

Age determination in fish can me made by three methods: skeletochronology (the concentric growth rings identified in a bone cross-section are counted), scalimetry (estimation of the age of fish by examination of concentric peaks on their scales) and otolithometry (identifying the annual growth rings of otoliths in the internal ear). In this case, age determination of anchovy of the Romanian Black Sea coast was made by otolith analysis.

The results pointed out that, during the analyzed period, the dominant age classes were between 1 : 1+ and 2 : 2+ years, while the 3 : 3+ age class had a smaller share, while anchovy individuals belonging to the 4: 4+ years age class were identified only as isolated specimens.

Compared to previous years, it was noted that the share of individuals aged 1 : 1+ and 2 : 2+ years is dominant, with a slight increase of 3 : 3 + years individuals [5], thus indicating a rejuvenation of anchovy population of the Romanian Black Sea coast.

Identification of several age classes in the anchovy sampled from the Romanian coastal waters show a reproductive success of the species, as well as a good state of the environment it lives in.

Acknowledgements

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ALFLUTOP® MODULATES "IN VITRO" RELEVANT MECHANISMS OF OSTEOARTHRITIC PATHOLOGY

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Abstract

The osteoarticular injuries have a large complexity, and could be counteract on two main direction: inducing the reconstitutive and regenerative effects at cellular level and proteic core and /or through the antioxidant and antiinflammatory effect. Alflutop® is a standardised injectable solution based on small fish concentrate, with therapeutical indication in degenerative rheumatic diseases. Its pharmacological action is founded on the synergy of the active principles that are present in the formulation of the product. The objectives of this study comprise the identification of more cellular and molecular targets of Alflutop®'s action, relevant for articular degenerative pathologies. Our results reveals an important therapeutic potential in the rising of the intrinsic recovery capacity of the organism, restoring biomechanical stability of viscoelastic matrix of cartilage tissue, leading to chondro-protective action, restoration of inter- and intracellular signaling pathways in the cartilage matrix and thus the improvement of the joint compressive strength and the decrease of inflammation.

Key words: Alflutop®, chondrocytes cell culture, osteoarthritis, cartilage inflammation, chondrogenic therapies

Introduction

The degenerative articular pathologies are chronic disabilities that degrade the quality of life, having as main causes a cumulus of local or systemic risk factors, with high impact on the disease evolution: joint injuries, alignment, bone metabolism, obesity, etc. Advancements in molecular biology reveals osteoarthritis as a very complex, multifactorial disease, characterized by "low-grade inflammation" in cartilage and synovium, resulting in the loss of joint structure and progressive deterioration of cartilage. (1, 14)

Healthy tissue is represented by normal cartilage without any fissures, with no signs of synovial inflammation. Osteoarthritic pathology is characterised by early local degenerate lesion and 'fibrillated' cartilage, as well as remodelling of bone, leading to bony outgrowth and subchondral sclerosis episodically inflammation of synovium, fryed tendons. (2)

The cartilage degradations could have several pathological correspondences, as the main ones: arthrosis, osteoarthritis, rheumatoid arthritis. All of them are progressive degenerative pathology, with an inflammatory component at the synovial membrane level, and is characterized by the degradation of cartilage, osteophyte formation, but are accompanied by a different intensity of the inflammatory components. In degenerative articular pathologies appear cellular and molecular modifications, as degraded extracellular matrix, fragmented proteoglycan network, pre-senescent chondrocyte. Articular chondrocytes exhibit a dose- and time-dependent response to shear stress that results in cytokine release, matrix metalloproteinase expression and activation of intracellular signaling pathways. The release of soluble mediators and extracellular matrix macromolecules as response to mechanical stimulation contributes to the maintenance of articular cartilage homeostasis in vivo. (3).

Osteoarthritic chondrocytes are constantly abnormally extracellularly stimulated by autocrine and paracrine factors, synovial stimuli and protein fragments from the degraded matrix inducing multiple cellular responses at the anabolism, catabolism and phenotype levels. Chondrocyte number decreases by proliferation and apoptosis perturbations. Cells become pre-senescent losing a lot of their functions. OA presents multiple pro-inflammatory extracellular signaling factors like pro-inflammatory cytokines (IL6, IL8, IL1b), matrix metalloproteinases (collagenases), aggrecanases, hyaluronidases, growth factors (TGFb), neuronal mediators. Besides these, free radicals (ROS) hasten progression of this disease together with autoimmune factors.

The strategies of therapeutical approaches should have in mind the biochemical complexity of intra /extra cellular interactions directed by key factors, haveing as final target the treatment of the diseases’ cause, not of the effect. For a complete “in vitro” screening we have to cover all the possible targets of this main pathologies (Fig.1)

The experimental studies performed since the first development of this drug, allow molecular targets identification for Alflutop® and also the optimum active configuration. This paper will present an up to date review of the main “in vitro” effects explored.

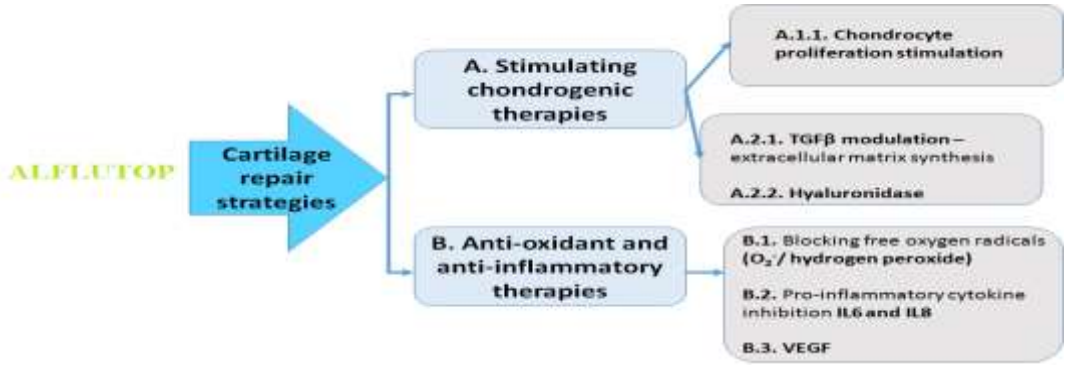


Fig.1: Therapeutic targets for an osteoarthritis treatment, focused in Alflutop® drug development ("in vitro" screening)

Materials and Methods

Cells cultures:

CHON-001 (ATCC® CRL-2846™), human normal chondrocytes from long bone cartilage bring the *advantages of a standardised and reproducible cell model.*

Cultivation: high glucose DMEM media, 10% fetal bovine serum, 0.1mg/ml G-418 antibiotic solution, at 37°C, in 95% humidified air and 5% CO_2 incubator.

Chondrocytes isolated from rabbit cartilage (primary culture) - *advantages of better maintaining the physiological features of the primary source, the cartilage.* Chondrocytes were isolated from cartilage fragments dissected from long bones of 2 years old female rabbits through enzymatic digestion with collagenase II.

Cultivation: high glucose DMEM, 10% fetal bovine serum, supplemented with antibiotic-antimycotic solution, at 37°C, in 95% humidified air and 5% CO_2. They were used within the first two passages.

***In vitro* methods to test the biologic action of Alflutop®:**

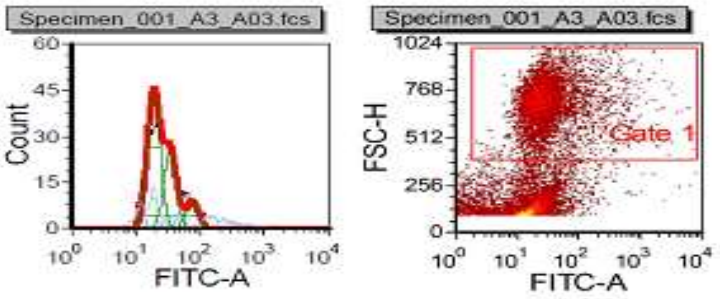
CELL CYCLE AND CELL DIVISION by flow cytometry detection:

Cell cycle: - specific labeling of the DNA with propidium iodide (PI) fluorochrome. (Cycle Test Plus DNA Reagent – BD Pharmingen)

Successive generation proliferation (Cell Trace CFSE Cell Proliferation Kit-Invitrogen): - CFSE (carboxyfluorescein diacetate succinimidyl ester) staining, a cell permeant fluorescein-based dye which covalently attaches to cytoplasmic components of cells, resulting in uniform bright fluorescence. Upon cell division,

the dye is equally distributed between daughter cells, allowing the resolution of up to eight cycles of cell division by flow cytometry. (Fig. 2)

Soluble, key-proteins from extracellular environment (VEGF, TGF-β, pro-inflammatory cytokines: IL6, IL8) quantified through flow cytometry - BD™ Cytometric Bead Array (CBA) - BD Pharmingen - The detection reagent provided in the kit is a mixture of phycoerythrin (PE)–conjugated antibodies, which provides a fluorescent signal proportional to the amount of bound protein. When the capture beads and detector reagent are incubated with an unknown sample containing recognized analytes, sandwich complexes (capture bead + analyte + detection reagent) are formed. These complexes can be measured using flow cytometry to identify particles with fluorescence characteristics of both the bead and the detector. (Detection shown by APC-A / PE-A coordinates) The analysis of the results (standard curve for each cytokine and concentration calculation) is done with FCAP Beads Array software.



Generation	Generation #	Log Std Dev	# of Cells	% of Orig Cells
Undivided Cells	0.00	35.73	502.00	10.32
Generation 1	1.00	35.73	1561.00	32.09
Generation 2	2.00	0.00	0.00	0.00
Generation 3	3.00	35.73	2756.00	56.66

Division Index	Proliferation Index	% Divided	# of Original Cells	RMS Error	R-Chi Square
3.84	2.96	69.15	1627.00	2.95	10.95

Fig.2: A flow cytometry model of cell proliferation experimental data acquired (Data analysis with FACS Express software)

HYALURONIDASE INHIBITION to estimate the extracellular matrix maintenance

Measurement of hyaluronidase reaction rate was based on the Reissing method, which

determines the concentration of reducing β-N-acetyl- D-glucosamine ends generated from hyaluronan acid hydrolysis. N-acetyl-D-glucosamine was used as a standard. For the evaluation of N-acetyl-D-glucosamine quantity formed as consequence of hyaluronidase activity it is necessary to do calibration curves which correlete the N-acetyl-D-glucosamine quantity with the probes absorbance at 585 nm (the wavelenght value where the compound formed by N-acetyl-D-glucosamine and 4-dimethylaminobenzaldehyde has its maximum of absorbance). The experimental procedure using DMAB is detailed in our previous paper(4)

OXIDATIVE BALANCE MODULATION - Intracellular CATALASE (CAT) and SUPEROXIDE DISMUTASE (SOD) assessment

To assess the enzyme activity of CAT and SOD, after detach adherent cells with trypsine / EDTA, cells were washed once with cold PBS and suspended in 300 µl of Cell Lysis Solution per 1 - 5 x 10^6 cells. The suspension was transferred to a 1.5 mL tube and centrifuge for 5 minutes at 12,000 - 14,000 x g at 2 - 8° C. The supernatant contain extracted cellular catalase and superoxide dismutase. Superoxide dismutase (SOD) is metallo-enzyme that catalyzes the dismutation of superoxide anion into oxygen and hydrogen peroxide. We have been used the spectrometric procedures described by Sigma Aldrich to determine the SOD activity in samples(5) method is based on the spectrophotometric evaluation (550nm absorbtion spectra) of the inhibition rate of cytochrome C reduction by competing for the superoxide radical with superoxide dismutase. Catalase (CAT) was assayed according to the method of Aebi (6) The estimation was done spectrophotometrically measuring the decrease in absorbance at 240nm. The reaction mixture contained 0.01M phosphate buffer (pH 7.0), 2mM H_2O_2 and cell lysates. The specific activity of catalase is expressed in terms of units/mg protein. A unit is defined as the velocity constant per second.

OXIDATIVE BALANCE MODULATION - Flow cytometry for reactive oxygen species –ROS – quantification – DCFH-DA (for HYDROGEN

Cellular oxidative stress through intracellular activation of superoxide anionand and hydrogen peroxide is quantified by simultaneous measurement of intracellular levels of H_2O_2 and O_2^- - DCFH-DA (dichlorofluoresceine diacetat), and HE (hydroxiethidium) staining and flow cytometry analysis. DCFH-DA is

embedded in lipid hydrophobic region of membrane where hydrolytic enzymes clive the diacetat residues, releasing the membrane permeant configuration which is oxidized in the cytoplasm by the intercellular hydrogen peroxide, producing FITC-A fluorescence (530nm emission). HE permeates the cell membrane and is oxidized by superoxide anion ethidium bromide which tight bond DNA and emits at 620nm (PE-A). Hydrogen peroxide and superoxide anion quantities are proportional with the variation of mean fluorescence channel: FITC–A mean – for Hydrogen peroxide and PE-A mean – for superoxide anion. Flow cytometry diagrams shows cellular subpopulation (green and blue) producing hydrogen peroxide (FITC-A positive) and superoxide anion (PE-A positive)- fig.3

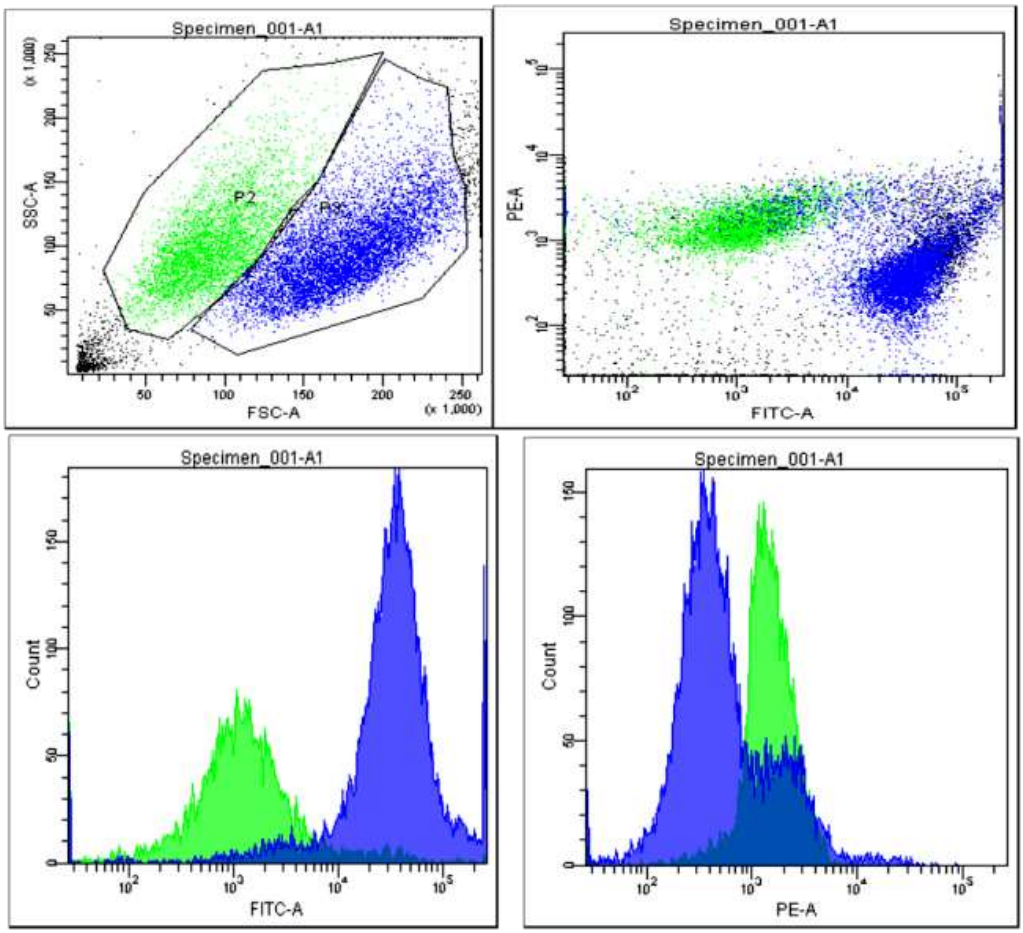


Fig.3: Flow cytometry diagrams showing H_2O_2 and O^{2-} intracellular level

Models of cell stimulation: IL1β and TNFα , dominants cytokines in the inflammatory cascade, but acting with a few differences:

- TNFα - primary inflammatory agent with systemic impact, activates NF-kβ and induce apoptosis
- IL1β triggers production of pro-inflammatory cytokines, stimulates production of stromelysin and collagenase and osteoclast differentiation.

Positive controls:

- Ascobic acid was used as a potent anti-oxidant control, as well as a positive control for condrocytes proliferation
- glycchyrizic acid (Gly) was used as a potent control for hyaluronidase inhibition
- N-acetylcysteine were used as a potent anti-oxidant
- Eicosapentaenoic acid (EPA) is used as a anti-oxidant control
- Dexametasone is a well-established anti-inflammatory agent

Results and Discussion

In vitro models to test the biological action of Alflutop® were choose considering a suitable complete screening of the main pathways of osteo-arthicular pathologies, as described in fig.1.

A.Stimulating chondrogenic therapies (as we presented in fig.1)

A.1.1. Cellular component – proliferative status amplification

The reaction patterns of chondrocytes in osteoarthritis can be summarized in five categories: proliferation and cell death (apoptosis); changes in synthetic activity and degradation; phenotypic modulation of the articular chondrocytes; and formation of osteophytes. (7) Chondrocytes act as the main actor for the maintenance, organization and composition of the cartilage matrix. Additionally, there is a low percentage of chondrocytes (1-5% of the total volume of the cartilage) and they have a slow rate of multiplication, thus stimulating them is an extremely important target for a drug. (8)

Our previous studies shows that Alflutop® significantly increases the % of cells in replicative (S) and mitosis (G2/M) phases of cell cycle. As well as, Alflutop® significantly increases the proliferation index of chondroblasts CHON 001 and primary rabbit chondrocytes (9)

A.2.1. Extracellular matrix maintenance through TGFβ modulation

Transforming growth factor beta (TGF-beta) is an ubiquitous regulator of cellular growth and differentiation. TGF-beta markedly stimulated DNA synthesis in a dose-dependent manner, showing increasing mitogenicity with increasing cellular maturation, with maximal stimulation in the proliferating and early hypertrophic cells, suggesting a potentially important autocrine function for TGF-beta in modulating chondrocyte proliferation and matrix synthesis in endochondral calcification. (10) When TGF- beta bind to chondrocytes cell surface receptor signaling cascades are triggered, among which the TGF- beta - Smad pathway is the most important. TGF- beta also activates protein kinases, including MAPK, PKA and PKC, and modulates gene expression via its delicate interaction with other signaling pathways. Improving the research of mechanisms underlying TGF- beta -mediated signaling pathways and their effects may greatly impact the treatment of many common orthopaedic diseases. (11)

Previous published data (9) showed a significant 7% activation of TGF-β induced by Alflutop® compared with the cellular control. This activation of TGF-β should be considered in respect to the general homeostasis of the cell, haveing in mind that TGF-β regulates the fine balance of protein synthesis/degradation and a high pool of this signal protein could lead to abnormal ossification.

A.2. 2. Extracellular matrix maintenance through Hyaluronidase inhibition

The intracellular matrix is a complex gel containing water, electrolytes, metabolites, vitamins, enzymes, carbohydrates, lipids and proteins. The viscousity of these matrix solution is due to the plenty of macromolecules: acid mucopolysaccharides with long chain strengthen at microscopic level by a three-dimension network of collagen fibres. An important feature of the intracellular substances is its very high viscosity and cohesion. This feature depends on the chemical integrity of the large molecules. High molecular size hyaluronan, hyaluronic acid (HA) occurs in normal tissues and has anti-angiogenic, anti-inflammatory, and anti-immunogenic properties. Fragmented HA, in contrast, is highly angiogenic, very inflammatory and immuno-stimulatory, a reflection of tissues under stress (12). A major function of high molecular mass HA in the synovial joint is to maintain proper viscoelasticity of synovial fluid. This enables the HA to serve as a shock absor-ber and also to ensure smooth joint movements The viscosity can be reduced and structural integrity destroyed by the depolymerizing action of the enzyme called hyaluronidase. In the degenerative

processes, the hyaluronic acid are intensely depolymerized by hyaluronidase, the matrix-ground substance of the connective and cartilaginous tissues being destroyed. Due to the reduction of molecules dimensions, the viscosity of hyaluronic acid is reduced. (13, 16). Since the development of many diseases can be linked to activity of this enzyme, currently many pharmaceutical research focuses on the study of specific inhibitors and potential hyaluronidase.(15) The enzymatic activity of hyaluronidase was determined in the presence of Alflutop and glycyrrhizic acid and results are presented in Fig 4.

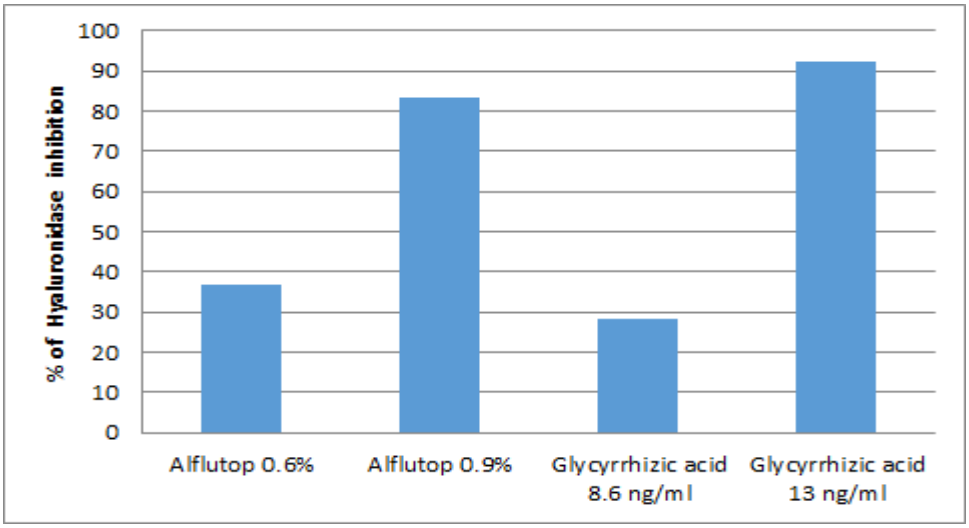


Fig. 4. Hyaluronidase enzymatic activity in the presence of Alflutop and glycyrrhizic acid

Alflutop® shows an inhibition of the hyaluronidase activity in a dose-dependent manner, similar with the positive control, glycyrrhizic acid.

B. Anti-oxidant and anti-inflammatory effects (as we presented in fig.1)

B.1. Oxidative balance modulation

Reactive oxygen species (ROS) can contribute to the onset and progression of degenerative arthritic pathologies by inducing indispensable chondrocyte death and matrix degradation. However, ROS are also key components of many normal physiological processes, and at moderate levels, they act as indispensable second messengers. (16) As a first step in antioxidant/antiradical screening the cellular catalase (CAT) and superoxide – dismutase (SOD) activity was measure,

correlated with further intracellular hydrogen-peroxide and superoxide anion monitoring through flow cytometry.

B.1.1. Catalase and Superoxid dismutase assay from rabbit primary chondrocytes culture

After 48h treatment of rabbit primary chondrocytes in the presence or absence of IL1β stimulation, cells were lysed and enzymatic activity was assessed from the lysate by 1) indirectly measuring quercetin auto-oxidation linearly correlated with SOD activity and with 2) hydrogen peroxide degradation as a direct measure for catalase activity. Results are presented in the graphics below (Fig.5)

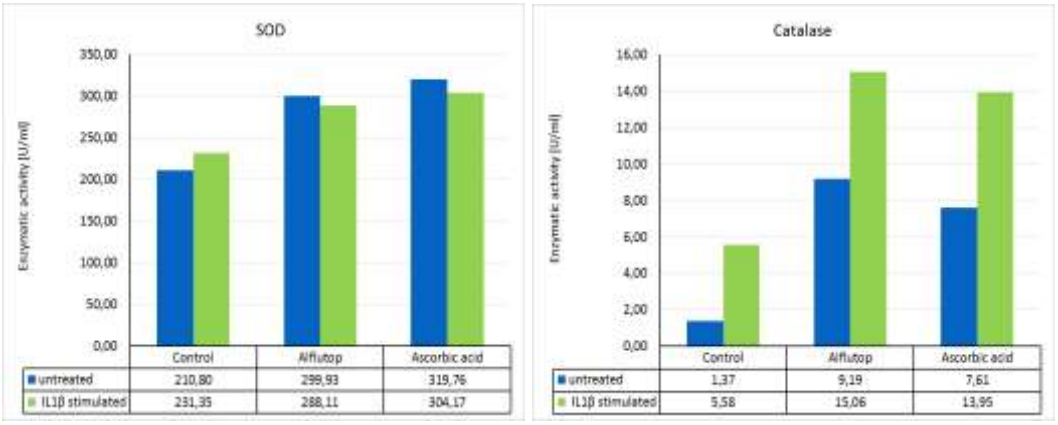


Fig. 5: Intracellular Superoxide dismutase (SOD) and catalase activity modulation by Alflutop® and ascorbic acid - positive control

Alflutop® treatment of primary rabbit chondrocytes stimulates SOD and catalase activity, in both unstimulated conditions and pro-inflammatory degradative injuries mimicked by IL1β stimulation. The rise of the catalytic activity of these antioxidant enzymes induced by Alflutop® leads to a protective effect against oxidative stress, transforming superoxide anion and oxygen peroxide, aggresive reactive oxygen species.

B.1.2. Antioxidant activity evaluation through intracellular reactive oxygen species monitoring from rabbit primary chondrocytes culture

IL1β stimulation generates a cellular oxidative stress expressed at ROS level through the mean rise of the hydrogen peroxide fluorescence (143% compared with the unstimulated control) and of superoxide anion with only 13%. The

massive release of hydrogen peroxide is therefore a characteristic of particular type of stimulation haveing as final result generalised injuries of the articular tissue. In these special conditions, Alflutop® product reduces the hydrogen peroxide with 33% compared with the stimulated control, similar with the positive control, 30% for ω3 fatty acid (eicosapentaenoic acid – EPA 10μM). The intracellular superoxide anion is reduced with 21% by Alflutop® and with 20% by EPA 10 μM. (Figure 6) IL1β stimulation generates a cellular oxidative stress expressed at ROS level through the mean rise of the hydrogen peroxide fluorescence (143% compared with the unstimulated control) and of superoxide anion with only 13%. The massive release of hydrogen peroxide is therefore a characteristic of particular type of stimulation haveing as final result generalised injuries of the articular tissue. In these special conditions, Alflutop® product reduces the hydrogen peroxide with 33% compared with the stimulated control, similar with the positive control, 30% for ω3 fatty acid (eicosapentaenoic acid – EPA 10μM). The intracellular superoxide anion is reduced with 21% by Alflutop® and with 20% by EPA 10 μM. (Fig. 6)

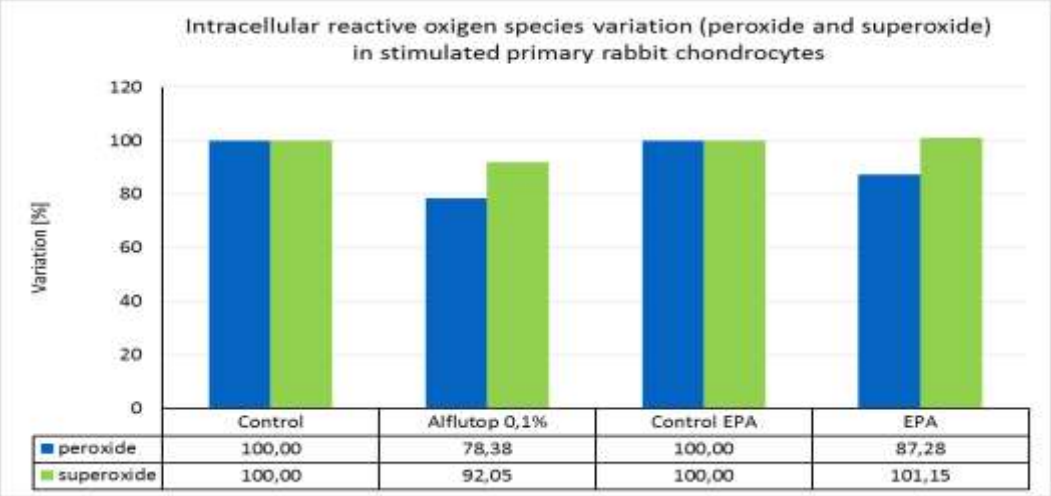


Figure 6: ROS evolution (hydrogen peroxide and superoxide anion) in rabbit primary chondrocytes stimulated with IL1β and treated with Alflutop® and EPA

B.1.3. Antioxidant activity evaluation through intracellular reactive oxygen species detection from human normal chondrocytes –CHON-OO1 standardised cell line

The human chondrocytes from standardised cell line CHON-001 were stimulated 24h. with TNFα 15ng/ml and PMA 0.1μM, the induced oxidative stress being appreciated through the rise of fluorescence with 38% for the hydrogen peroxide and 61% for the superoxid anion. The action of Alflutop® was compared with N- Acetil-Cysteine 5mM and ascorbic acid 45μM, two well known antioxidants. The results are presented in the figure 6, as % of variation of the specific fluorescence channel (H_2O_2 - FITC-A; (O_2^-) – PE-A) compared with the stimulated control.

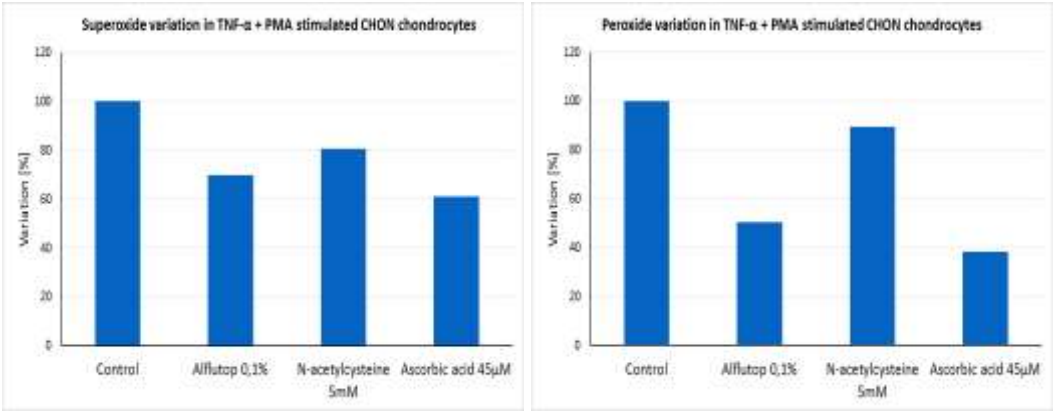


Figure 7: ROS evolution (hydrogen peroxide and superoxide anion) in human chondrocytes –CHON-001, stimulated with TNFα+PMA and treated with Alflutop®, Ascorbic acid and N-Acetil-Cysteine

Alflutop® induces the inhibition of both oxygen reactive species, an important step in ROS mediated perturbancies. The intracellular hydrogen peroxide decrease in the presence of Alflutop® with 50% compared with the stimulated control, the other positive controls effects being of 11% for N-Acetil-Cysteine, and 62% respectively for Ascorbic acid. The action on intracellular superoxide anion is lower, Alflutop® reduce it with 31%, N-Acetil-Cysteine with 20%, and Ascorbic acid with 39% (Fig. 7)

Considerations regarding the antioxidant activity of Alflutop®:

In osteoarthrithic pathology the anion superoxide and hydrogen peroxide production is dramatically stimulated, inducing a marked expression of reactive oxygen species (ROS), in synergy with the rise of pro-inflammatory cytokines and

proteasic activity. SOD improves the pathological events of osteoarthritis. The catalase association amplify the protective effect, both through the quicker elimination of hydrogen peroxide and blocking the SOD degradation by it. The rise of SOD in the presence of Alflutop® induces the oxidative stress inhibition, with superoxide anion implication, considering its role in the chain of oxidative stress aggressive compounds triggering. The rise of SOD prove the inhibition of superoxide anion accumulation, and the experimental data sustain through complementary methods the product's implication in this antioxidant mechanisms. It could be noted the implication of Alflutop® especially in the enzimatic change system of intracellular hydrogen peroxide, neutralizing the production of H_2O_2. This is one of the most aggressive species of oxygen, that inhibits SOD and produces superoxide anion accumulation, initiates pathways that converge to lipids peroxidation and leads to cells injuries and death, block gluthation-peroxidase stopping this way the synthesis of gluthation as intrinsic antioxidant. Decreasing the intracellular H_2O_2, Alflutop® contributes to prevent the escalation of all these processes. Being an activator of SOD and Catalase, Alflutop® decrease the inflammation mediators release in their propagation flow.

B.2. Modulation of pro-inflammatory cytokines

Osteoarthritic disease in cartilage is driven by both mechanical and inflammatory signals. Specific signaling pathways linked to altered physiological states that drive stressed articular chondrocytes to proliferate or differentiate, are related to inappropriate activation of NF-κB signaling. Inflammation and stress-induced responses orchestrated by canonical NF-κB signaling may impact both directly and indirectly on OA disease onset and/or progression. (16). Different molecular aspects involved in the pathogenesis of osteoarthritis are controlled by inflammatory mediators, their cascade signaling further contributing to the highly catabolic state, chondrocyte apoptosis, and the resultant progressive degeneration of articular cartilage (17). Cytokines produced by inflammatory cells induces synovial inflammation and joint destruction in rheumatoid arthritis.(18,19) Pro-inflammatory cytokines are able to induce apoptosis, whereas IL-4 as an anti-inflammatory cytokine can inhibit the effect of IL-1α and TNF-α on NO production and proliferation of chondrocytes. (20). The catabolism of osteoarthritic cartilage involves the action of proinflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α). Interleukin-1 down-regulates extracellular matrix (ECM) synthesis and up-regulates metalloprotease synthesis through production of nitric oxide (NO) in chondrocytes. Human

synoviocytes spontaneously release IL-6 in a manner that is increased by IL-1 and TNF-α. Interleukin 6 levels have been correlated with pain in the temporomandibular joint, IL-6 synergizes with IL-1 to promote collagen degradation in cartilage. (21)

Alflutop reduces especially the IL6 release in human chondrocytes stimulated with IL1β, that will prevent or slow the inflammatory cascade progression. When human chondrocytes are stimulated with TNFα, Alflutop® inhibits both IL6 and IL8 interleukins release, the main modulators of inflammatory acute phase progression, proving significant anti-inflammatory effect on these classical pathways. "In vitro" modulation of these important mediators of inflammation sustains Alflutop® contribution in the rehabilitation of the cartilage physiology through anti-cytokines action. (22)

B.3. Regulation of VEGF

Angiogenesis is another event of synovial tissue inflammation. It starts early in the firsts stages of disease and could be asymptomatic, IL8, VEGF or FGFβ acting as pro-angiogenic markers. An increasing number of observations suggest that VEGF, for a long time considered to be endothelium specific on the basis of its receptor localization, might instead have effects also on non endothelial cell types, holding active signal transduction.

There is consistent evidence for VEGF being involved in cartilage pathological neovascularization, with factor increase in synovial fluids deriving from rheumatoid arthritis. The presence of VEGF receptor and functional signal transduction in hypertrophic chondrocytes was considered in the light of a possible additional differentiating or morphogen effect of VEGF in endochondral bone formation. Expression of VEGF activates the chondrocytes autocrinally for producing MMP-1, -3, and -13. TIMP-1 and -2, the inhibitors of MMPs, are reduced by mechanical overload. The increase of MMP and the decrease of TIMP contribute to the destruction of the articular cartilage. (24)

ALFLUTOP® inhibits VEGF, an important angiogenesis factor with a recent discovered impact as a biochemical mediator in destructive processes of osteoarthritis (results previously published). (23)

In adidtion with all these effects of Alflutop®, we could also mention: the inhibition of the expression of proteases responsible for the degradation cascade of the core-protein of aggrecan (mRNA expression of ADAMTS-4); the increasing aggrecan and hyaluronan synthesis (activation of hyaluronan synthase mRNA expression - HAS-1); and the activation of a pivotal transcriptional

regulator essential for articular cartilage formation and hypertrophic maturation, SOX 9 (24), in order to prevent the hypertrophy and extracellular matrix decline(24).

Conclusions

Overview of molecular and cellular effects from the *in vitro* studies to determine mechanisms of action for Alflutop®:

This product, through its complex of complementary biological components, shows an integrated algorithm of *in vitro* action specific for osteoarticular diseases. The product influences the mechanisms that restore and configure the architectural matrix at the cartilage level, allowing the recovery of the functional pathways.

- Alflutop® reduces/stops cartilage destruction, inhibiting the expression of proteases responsible for the degradation cascade of the core-protein of aggrecan, improving cellular response in catabolic processes by increasing aggrecan and hyaluronan synthesis, and in the same time inhibiting the action of hyaluronidase.
- Alflutop® helps recover the structural integrity of cartilage, stimulating chondrocyte proliferation.
- Alflutop® restores cellular turn-over and maintains the protein-core, through stimulation of DNA synthesis in chondrocytes and the moderate stimulation of TGFβ, a molecule responsible for the depletion / restoring dynamics of the balance between the synthesis and degradation of the matrix.
- Alflutop® works in numerous ways to reduce inflammation processes and also diminishes oxidative stress having an antioxidant effect demonstrated by scavenging free radicals and inducing the activation of the enzymes implicated in oxidative cascades.

All these effects allow Alflutop® inhibit the physiologic degeneration modulated by intrinsic and external factors, individual variability. The in vitro preclinical action of ALFLUTOP® is a synergism confirmed through convergent cellular mechanisms.

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A MINI-REVIEW ON THE EFFECTS OF EXERCISING AND OBSESITY IN PARKINSON'S DISEASE

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Abstract

Recent knowledge is supporting physical activity as an important preventive factor against the onset of neurological disorders such as Parkinson's disease, even stating that physical exercising is crucial for the maintenance or for slowing down the decline of optimal functional ability levels in these patients, as we will go to detail in the present mini-review. Moreover, even if lately there is a relatively large amount of epidemiological data suggesting that physical activity can have protective functions in the context of Parkinson's disease development, currently there is not sufficient information to enable a precise description for the best exercise regimen for patients with Parkinson's disease. In this way, it seems that it is advisable to combine aerobic exercises with other activities that are beneficial for the neuromuscular system (e.g., strength/power training and stretching), balance function and the performance of motor coordination. Moreover, the development of coordination associated with the stimulation of psychomotor capacity seems particularly relevant for PD subjects, considering the specificity of their pathology involving movement command and control processes.

Key words*:* Parkinson's disease, exercising, neuromuscular system.

Exercising and Parkinson's disease pathology

Neurodegenerative disorders such as Parkinson disease (PD) represent a major medical concern for health professionals and national healthcare societies (Hritcu et al., 2008). This disorder results from progressive neuronal dysfunction and neuronal cell death leading to progressive disability and eventual death

(Ciobica et al., 2012). Classical signs and symptoms specific to this disorder include motor problems, cognitive impairment, behavioural disturbances, and systemic or genetic abnormalities (Foyet et al., 2011, Balmus et al., 2016).

Unfortunately, there is no cure and few cost-effective drug agents for treating people with Parkinson disease (Olanov et al., 2009; Evans et al., 2011). In this way, recent advances in understanding the pathogenic mechanisms responsible for each disorder may aid in the identification and development of cost-effective disease-modifying agents in the future. However, cost-effective treatments, with disease-modifying properties and symptomatic benefits are required in the short term.

Moreover, modern knowledge are supporting physical activity as an important preventive factor against the onset of neurological disorders such Alzheimer's disease (AD) and Parkinson's disease, even stating that physical exercising is crucial for the maintenance or for slowing down the decline of optimal functional ability levels in patients with neuropsychiatric modifications (Honceriu et al., 2016; Sandu et al., 2014, Ciobica et al., 2016), as we will going to detail in the present mini-review, by insisting on PD pathology.

However, currently there is insufficient information to enable a precise definition of the best exercise regimen for patients with AD or PD.

In addition, aerobic exercise is increasingly recommended by therapists because of its role in relation to angiogenesis, as well as the liberation of neurotrophic factors (increases in cerebral blood flow and cerebral plasticity). In fact, as we will insist later on, aerobic exercise is a necessary part of the treatment for AD or PD, but aerobic exercise alone is probably not the best activity. In this way, strength/power training, Tai Chi, balance/coordination, and other types of physical exercises also contribute to reconditioning, maintaining, and improving the cognitive and motor functions of AD and PD subjects.

In fact, there is a relatively large amount of epidemiological data suggesting that physical activity can somehow prevent the development of Parkinson's disease (PD) (Alonso et al., 2011).

In this way, some studies found that the risk of developing this disease appeared to be inversely associated with the amount of physical activity practiced throughout life (Chen et al., 2005, Xu et al., 2010).

Still, it has to be mentioned that a possible important limitation of these observational studies is that subjects predisposed to develop PD may naturally tend to avoid physical activity prior to the onset of the clinical symptoms of the

disease. Furthermore, the protective effect of physical exercises against this pathology appears to be particularly large when practiced at young-to-middle adulthood (i.e., around 35-39 years old) and at the end of life. Thus, Xu et al. suggested that people who practice cardiovascular exercises during these two periods of their life have a 40% lower risk of PD, as compared to people who remained inactive during the same periods (Xu et al., 2010).

Moreover, in a study involving 48,574 men and 77,254 women, Chen et al. found that the direct relationship between the amount of physical activity and the risk of developing PD was only significant in men (Chen et al., 2005).

In addition, strength training has been shown in recent years to be beneficial for people with Parkinson disease.

Still, consensus regarding its utility for these disorders nevertheless remains contentious among healthcare professionals. In this way, increased clarity is required, especially in regards to the type and magnitude of effects, as well as the response differences to strength training between individuals with Parkinson disease.

In fact, in many studies strength training has been shown to improve the muscle strength and walking speed of PD patients which are not severely affected by the disease (Hass et al., 2012 , Lima et al., 2013).

Also, accumulating evidence are suggesting that strength training is a useful therapy for addressing many of the clinical features which are present in individuals with neurodegenerative disorders (Falvo et al., 2008, Hindle et al., 2013).

By definition, strength training refers to an intervention in which participants train a muscle or group of muscles against an external resistance (Esco et al., 2013). Whereas evidence suggests that lower limb strength training (i.e., leg press, knee extension, and knee flexion) is beneficial for individuals with Parkinson disease and multiple sclerosis (Shulman et al., 2013, Schilling et al., 2010), consensus regarding the effects, magnitude of those effects and disease-dependent responses remain contentious.

By contrast, the therapeutic utility of strength training is well recognized in the elderly (Nelson et al., 2007), individuals with mild cognitive impairment and in those that have suffered a stroke. In this way, health benefits associated with strength training in elderly individuals include improvements in strength (Fiatarone et al., 1990), cardiorespiratory capacity (Pereira et al., 2012), mood (Pereira et al., 2013), cognition (Cassilhas et al., 2007) and health-related quality

of life (Levinger et al., 2007).

Moreover, in individuals who have suffered a stroke, strength training has been found to improve muscular strength, upper and lower limb function and performance on functional tasks (Ada et al., 2006). Improvements in selective attention, conflict resolution, associative memory and regional patterns of functional brain activity have also been observed after strength training in seniors with mild cognitive impairment (Nagamatsu et al., 2012).

Regarding the specific effect that resistance training has on patients suffering from PD, three randomized (Shulman et al., 2013; Corcos et al., 2013, Sage et al., 2011) and one nonrandomized controlled trial (Dibble et al., 2009) evaluated the effect of strength training on clinical disease progression using the Unified Parkinson Disease Rating Scale Version 3.

In this way, Corcos et al. in 2013 reported a significant improvement on the Unified Parkinson Disease Rating Scale Version 3 in the intervention group (7.4 point decrease), but not in the control group after 24 months of strength training. In addition, Shulman et al. in another study in 2013 similarly reported a significant improvement on the motor subscale of the Unified Parkinson Disease Rating Scale Version 3 in the strength training group. Furthermore, Sage et al (Sage et al., 2011) found a significant improvement on the Unified Parkinson Disease Rating Scale Version 3 in the strength training group. In contrast, Dibble et al. in 2009 found no improvement on the Unified Parkinson Disease Rating Scale Version 3 in the intervention group after strength training.

In addition, other studies concentrated on the effect that strength training has on specific areas that are affected by PD. Three randomized (Shulman et al., 2013, Prodoehl et al., 2015, Paul et al., 2014) and 3 nonrandomized controlled trials (Schilling et al., 2010, Hass et al., 2012, Dibble et al., 2006) evaluated the effect of strength training on mobility in individuals with Parkinson disease. Mobility in these studies was assessed using the 10 meter timed walk test, 6 minute walk test, 50 feet walk test and timed up and go.

Moreover, one study by Paul et al. in 2014 did not report significant changes in mobility after strength training. In contrast, Prodoehl et al. and Shulman et al. found significant improvements in mobility as a result of strength training. In addition, the 3 nonrandomized controlled trials (Esco et al., 2013, Sabapathy et al., 2011, Fimland et al., 2010) that reported on mobility as an outcome also documented improvements in this matter.

Regarding the effect on balance, two randomized (Prodoehl et al., 2015, Paul

et al., 2014) and 2 nonrandomized controlled trials (Schilling et al., 2010, Sabapathy et al., 2011) examined the effect of strength training on balance outcomes in Parkinson disease. In this way, balance was evaluated by using the single leg stance, choice stepping task, berg balance scale, functional reach test, 5 times sit to stand test, and the activities-specific balance confidence scale.

Moreover, Paul et al did not find a significant improvement in balance as a result of strength training, while Prodoehl et al. reported in contrast a significant improvement in balance after strength training. Still, both nonrandomized controlled trials (Schilling et al., 2010, Sabapathy et al., 2011) were unable to find a significant improvement in balance after strength training.

Another randomized trial (Corcos et al., 2013) examined the effect of strength training on functional capacity, assessing functional capacity by using the modified Physical Performance Test and reporting no significant changes after strength training in the intervention compared to the control group.

Another area of interest in any cognitive impairment is the quality of life of the patients. In this way, two randomized (Shulman et al., 2013; Corcos et al., 2013) and 1 nonrandomized controlled trial (Dibble et al., 2006) evaluated the effect of strength training on quality of life. The quality of life was assessed in all 3 trials using the 39-Item Parkinson Disease Questionnaire. Both randomized controlled trials (Falvo et al., 2008, Hindle et al., 2013) did not report a significant improvement in quality of life after strength training. By contrast only Dibble et al. reported a significant improvement in quality of life in the intervention group after strength training.

Also, another randomized controlled trial (Shulman et al., 2013) evaluated the effect of strength training on mood in Parkinson disease, but found no significant changes in mood after strength training using the Beck Depression Inventory.

In addition, no significant change in fatigue after strength training in a strength training intervention group or high- and low-intensity treadmill intervention groups was found in one randomized controlled trial (Ada et al., 2006), as they used the 16-item Parkinson Fatigue Scale for measuring the fatigue in PD patients.

Moreover, we have to mention that another two randomized controlled trials (Hindle et al., 2013, Medina-Perez et al., 2014) evaluated the effect of strength training on falls in people with Parkinson disease, with falls being assessed by using two scales, the New Freezing of Gait Questionnaire and Falls Efficacy Scale. Still, no trial reported a significant effect on falls outcomes after strength

training.

Also, in regards to the muscle volume, one nonrandomized controlled trial (Dibble et al., 2006) evaluated the effect of strength training on quadriceps muscle volume in Parkinson disease. As expected, their results showed a clear and significant increase in quadriceps muscle volume, by using magnetic resonance imaging after strength training in the intervention group only.

Same aspects were also demonstrated in animal models of Parkinson's disease, including by our group of research, which reported in a preliminary study that physical exercising seems to reduce anxiety, depression and memory deficits (Ciobica et al., 2015) associated with a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced rat model of PD (Ababei et al., 2015). In fact, we mostly want it to see if induced physical exercising in an MPTP-induced rat model of PD (20 mg/kg i.p.), will result in any changes in memory (as tested in Y maze), anxiety (as tested in elevated-plus-maze) and depression-like behaviour (forced-swim-test), as compared to a non-exercised control group of rats which also received MPTP, as the exercising was performed on an adapted treadmill, for 2 weeks (3 series of 5 minutes/day).

Thus, our mostly unpublished results showed an increased time spent by the rats in the open arms of the elevated-plus-maze at in the group of exercised MPTP group, together with a significant decrease of stretching behaviour and increased head dipping, as compared to non-exercised MPTP group, factors which suggested an anxiolytic-like manifestation. In addition, spontaneous alternation in Y maze (index for immediate memory), and swim time (anti-depressive index) in forced swim test were increased in the exercised rats with an MPTP-induced model of PD (Ciobica et al., 2015).

In the same context, we could mention here the fact that oxidative stress status could be another key player in the positive roles exerted sometimes by the various exercising performing, considering for example that our group previously demonstrated the importance of vitamin C and oxidative stress in general in both exercising rats or human patients (Trofin et al., 2014, 2017), by using original or general oxidative stress markers determinations (Cojocaru et al., 2005, 2007, 2010). Of course, this could be also correlated with increased oxidative stress status in most of the neuropsychiatric disorders (Ciobica et al., 2011, Padurariu et al., 2013, Stefanescu et al., 2012, Bild et al., 2013), including in Parkinson's disease (Hritcu et al., 2011; 2013), as we previously mentioned in this mini-report.

Thus, it seems that strength training is having a positive effect on disease

progression in people with Parkinson disease (Unified Parkinson Disease Rating Scale-Version 3). Interestingly, improvements in disease progression were observed also in a cohort with mild-to-advanced disability that were not on medication, suggesting that strength training alone may be capable of positively impacting on disease progression in individuals at all stages of Parkinson disease. In this way, it seems that this positive effect of strength training on disease progression may have been mediated by favourable central changes. For instance, recent evidence showed that repetitive force generation increases neuronal activation in the basal ganglia, thalamus, parietal cortex, cerebellum and motor cortex (Ehrsson et al., 2000).

Furthermore, emerging evidences have shown that exercise interventions can increase regional brain volume and structural connectivity in patients with Parkinson disease and other neurodegenerative disorders (Sehm et al., 2014), suggesting fundamental implications of these aspects in the neuropathology of Parkinson's disease.

Conclusions

Recent knowledge is supporting physical activity as an important preventive factor against the onset of neurological disorders such as Parkinson's disease, even stating that physical exercising is crucial for the maintenance or for slowing down the decline of optimal functional ability levels in these patients. Moreover, even if lately there is a relatively large amount of epidemiological data suggesting that physical activity can have protective functions in the context of Parkinson's disease development, currently there is not sufficient information to enable a precise description for the best exercise regimen for patients with Parkinson's disease. Still, it seems that it is advisable to combine aerobic exercises with other activities that are beneficial for the neuromuscular system (e.g., strength/power training and stretching), balance function, and the performance of motor coordination. Even more, the development of coordination associated with the stimulation of psychomotor capacity seems particularly relevant for PD subjects, considering the specificity of their pathology involving movement command and control processes.

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