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THE FUNCTIONAL ACTIVITY OF CHO CELL LINES EXPRESSING DIFFERENT ATP7B MUTATIONS (THE CYTOTOXICITY OF THE CERTAIN CONCENTRATIONS OF COPPER AND ZINC)

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Rezumat

ACTIVITATEA FUNCȚIONALĂ A LINIILOR DE CELULE *CHO*, CE EXPRESEAZA DIFERITE MUTAȚII ALE GENEI *ATP7B* (STUDIUL CITOTOXICITAȚII DETERMINATE DE ANUMITE CONCENTRAȚII DE CUPRU SI ZINC)

Cuvinte ceie: *Boala Wilson, ATP7B, liniilor de celule CHO, metoda MTT*

Boala Wilson este un bun exemplu modern pentru dezvoltarea ascensiva a ştinţei medicale. Cercetările moderne în domeniul biologiei moleculare şi celulare încearca să descopere multe mistere a bolii Wilson. În timpul actual există un interes crescut în cercetarile legate cu fiziologia metalelor. În timpul de faţa., noi observăm discuţii fierbinţi în cercurile ştiinţifice cu privire la utilizarea preparatelor ce conţin Zn la tratarea bolii Wilson. Lucrarea dată decernează studiile activităţii funcţionale a liniilor de celule CHO, ce se expresează în diferite mutaţii ATP7B prin influenţa anumitor soluţii cu concentraţie determinată de Cu şi Zn şi determinarea citotoxicitaţii legăturilor date.

Material şi metode. *Au fost utilizate culturile celulare din colecţia germană de culturi celulare (DSMZ). Folosind metoda transducţiei retrovirale, sau obţinut 12 tulpini celulare CHO, ce se expreseaza în diferite mutaţii ATP7B. Mutaţiile se localizează în domeniul de legatură cu Cu (n=2), TM (n=4), TGE bucle (n=1) legături de bucle ATP (n=5) gene ATP7B. Pentru aprecierea citotoxicităţii se foloseşte testul MTT.*

Rezultate. *În rezultatul cercetarilor noastre, noi am obţinut următoarele date: concentraţia soluţiei de Zn 500mikroM reprezintă o concentraţie toxică procent de moarte a celulelor se echivalează cu 0,22%. Concentraţia soluţiei de 50microM stimulează diviziunea celulară. Procentul de celule CHO ce au supravieţuit, şi ce expresează gena ATP7B, în soluţie de 1mM de Cu creşte pîna la 20% la adaugarea în soluţia dată 50microM de soluţie cu Zn. Celulele CHO, ce nu expresează gena ATP7B îşi măresc cu 57% capacitatea de supravieţuire în concentraţia de 0,5mM $CuCl_2$ şi 50 Mm $ZnCl_2$ în mediul de cultura.*

Concluzii. *Ionii de Zn preintîmpină toxicitatea ionilor de Cu fără dependenţă de faptul dacă se expresează sau nu gena ATP7B şi independent faţă de ce fel de mutaţie- gravă sau medie după forma clinică*.

Резюме

ФУНКЦИОНАЛЬНАЯ АКТИВНОСТЬ *CHO*- КЛЕТОЧНЫХ ЛИНИЙ, ЭКСПРЕССИРУЮЩИХ РАЗЛИЧНЫЕ МУТАЦИИ *ATP7B* ГЕНА (ИЗУЧЕНИЕ ЦИТОТОКСИЧНОСТИ ОПРЕДЕЛЕННЫХ КОНЦЕНТРАЦИЙ МЕДИ И ЦИНКА).

Ключевые слова: *Болезнь Вильсона, CHO клеточные линии, экстрессирующие различные мутации ATP7B гена, MTT-тест*

Введение. *Болезнь Вильсона является отличным примером современного поступательного развития медицинской науки. Современные исследования в области клеточной и молекулярной биологии пытаются раскрыть многие загадки болезни Вильсона. В настоящее время существует повышенный интерес к исследованиям, связанных с физиологией металлов. В настоящее время, мы наблюдаем горячие дебаты в научных кругах вокруг применения препаратов цинка при лечении болезни Вильсона. Данная работа посвящена исследованию функциональной активности CHO клеточных линий, экстрессирующих различные мутации ATP7B при воздействии определенных концентраций растворов Cu и Zn и определение цитотоксичности данных соединений*.

Материал и методы. *Культуры клетки были использованы из Немецкой Коллекции Культур Тканей (DSMZ). Используя метод ретровирусной трансдукции, были получены 12 CHO клеточных линий, экспрессирующие различные мутации ATP7B. Мутации находятся в медь-связывающем домене (n = 2), TM (n = 4), TGE петли (n = 1), АТФ-связывающей петли (n = 5) гена ATP7B. Для оценки цитотоксичности использовался MTT-тест.*

Результаты. *В результате наших экспериментов мы получили следующие данные: концентрация раствора цинка 500 mikroM является токсической концентрацией- процент Zn-индуцированной гибели клеток равен 0,22%. Концентрация раствора цинка 50 mikroM стимулирует деление клеток. Процент выживаемости клеток CHO, с экспрессирующим ATP7B геном, в 1 mM растворе меди повышается на 20 процентов при добавлении к данному раствору 50 mikroM раствора цинка. CHO клетки, не экспрессирующие ATP7B ген, на 57% повышается выживаемость клеток при концентрациях 0,5mM CuCl2 и 50uM ZnCl2 в питательной среде.*

Выводы. *Таким образом, ионы цинка предотвращают токсичность ионов меди в CHO клетках независимо от того, экспрессирует или нет ATP7B и независимо от типа мутации – тяжелая или средняя по клиническому проявлению*

«Anyone who has never made a mistake has never tried anything new."
Albert Einstein

Introduction. Wilson disease is an excellent example of contemporary translational medical science. First described in 1912, early clinical observations remain informative. In the mid-20th century the relevance of copper and ceruloplasmin was clear; genetic studies indicated an autosomal recessive pattern of inheritance. Oral chelators provided effective treatment. In 1993, the gene whose mutations result in Wilson disease was identified: *ATP7B*, encoding a metal-transporting P-type ATPase (the Wilson ATPase), mainly expressed in the liver where it participation in both production of holoceruloplasmin and excretion of copper into bile[1]. Subsequent research in cellular and molecular biology has addressed many of the conundrums of Wilson disease.[2]. There is renewed interest in research relating to the physiology of all metals. Metals play an important role in human physiology and in organisms throughout the phyla. Each metal has a functional optimum, and too little or too much of that metal leads to dysfunction.[3].

Copper is essential for life of all organisms, as involved in a wide range of cellular processes from the formation of neurotransmitters to the synthesis of heme. It is part of the prosthetic groups of key enzymes involved in oxidative phosphorylation, the formation of connective tissue, antioxidant defense, bidirectional transport of iron (Karlin, 1993). On the other hand, copper ions can induce the formation of reactive oxygen species, which, acting by a mechanism akin to ionizing radiation, destroying the biopolymers (Linder, 2001). Therefore, in the cells of all types, there are evolutionarily conserved system for the safe import, distribution and excretion of copper (Puig, Thiele, 2002). Any, even minor changes in their work due to genetic or environmental factors that cause the remote development of severe neurodegenerative diseases (Gaggelli et al., 2006).

These include, in addition to diseases associated with inborn errors of metabolism of copper, are atseruloplazminemiya, Alzheimer's, Parkinson's disease, cardiovascular disease, osteoporosis, cancer, prion diseases (Fox et al., 2000; Llanos and Mercer, 2002; Brown, 2003 ; Bush et al, 2003; Gaggelli et al., 2006; Torsdottir et al., 2006). For all of these diseases dysfunction of the mitochondria, the nature of which remains unknown. Recently it was discovered that an excess or deficiency (Pang and Chau, 1999; Gybina and Prohaska, 2003) of copper ions in cells triggers apoptosis mediated by mitochondrial proteins Bcl-2 family (Wei et al., 2001).

Zinc is essential for the function of numerous enzymes and transcription factors and for neurotransmission. When it is severely deficient, acrodermatitis enterohepatica develops. Zinc interacts with copper in enzymes such as superoxide dismutase 1, and it can affect, or ever regulate, copper uptake. It is not redox active and does not generate activated oxygen species in experimental models where copper does[4]. We do not know much about the perils of zinc overload: most report describe the effects of copper insufficiency consequent to zinc excess.

Also, now we have a discussion in scientific society on the fild of zinc toxicity: from "no, never" to "hardly ever" and many publication that showed that zinc was an effective treatment for Wilson disease by Hoogenraad et al[5], Linn et al found that zinc monotherapy was more effective for neurologic than hepatic Wilson disease[11], or not Weisss KH " Zinc monotherapy is not as effective as chelating agents in treatment of Wilson disease[10]

The purpose of our work was to study of biological activity and evaluation the pharmacological potential of zinc ions in Chinese hamster ovary (CHO) cells, normal and mutant cells lines that carry different types of *ATP7B* gene mutations.

Objectives of work:

Assessing the impact of copper and zinc on the viability and proliferation of CHO cells, study the cytotoxicity of these compounds

To correlate between the functional activity of individual mutant lines of CHO cells to the introduction of copper and zinc in the medium for further assess the treatment possibility of Wilson disease patients.

Materials and methods. Chinese hamster ovary (CHO) cells are the most widely used mammalian cells for transfection, expression, and large-scale recombinant protein production. Since CHO cells provide stable and accurate glycosylation, they offer a post-translationally modified product and thus a more accurate *in vitro* rendition of the natural protein (Shee-

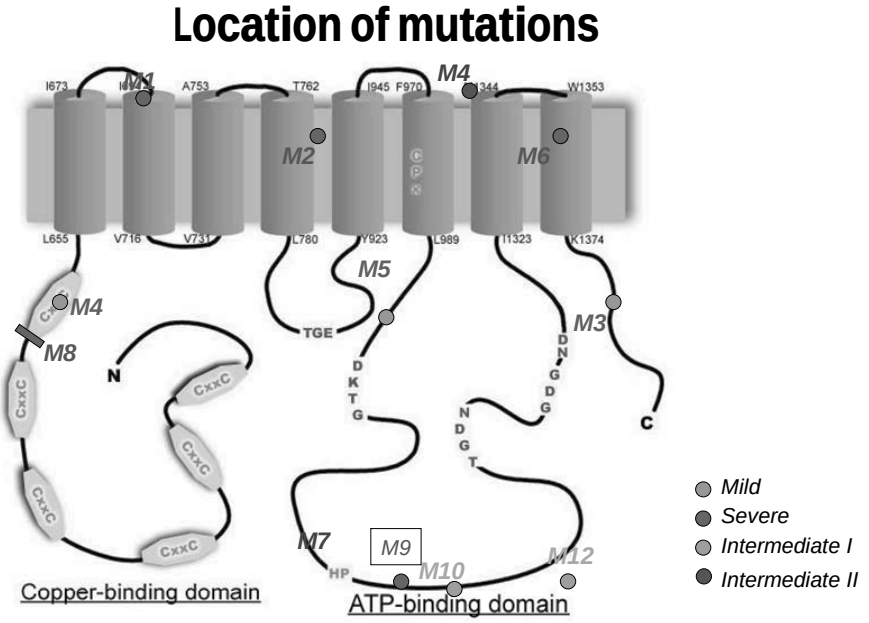

Fig. 1 Location of mutation in ATP7B gene.

ley et al. 1997; Werner et al.1998). CHO cells were used in our study because of their lack of *ATP7B* expression (CHO-vec), which makes possible to receive, cell lines which demonstrate Cu resistance similar to that of the homozygote mutations.

Cells culture were purchased from German tissue culture collection (DSMZ) Using retroviral transduction have been generated 12 CHO cell lines expressing different *ATP7B* mutations. Mutations are located in the copper binding domain (n=2), TM (n=4), TGE loop (n=1), ATP binding loop (n=5) of *ATP7B* gene (Fig. 1).

Chemical reagents were obtained from firms "Sigma". The culture plastics, media and serum - from firms "Greiner", "Costar".

Cells of Chinese hamster ovary (CHO), were grown in 96-well plates and flasks of 75 cm2. CHO were cultured in DMEM high glucose, respectively, containing 10% FCS, in an atmosphere of 5% CO2 at 37°. Test compounds (CuCl2 and ZnCl2) were added to the culture medium in the culture solution according protocols.

Methods . Cytotoxicity of copper and zinc compounds. Evaluate the toxicity of copper and zinc in CHO cells were performed using the color reaction tetrazolium dye 3 - (4,5-dimetiltiazol-2-yl) -2,5-difeniltetrazoly bromide (MTT) with living cells. .(Fig .2) MTT assay for 12 CHO *ATP7B* mutants including positive and negative controls was performed after 48h incubation with 0.06-1mM copper and 50mikroM zinc. All samples were analyzed in triplicate The absorption spectrum were recorded on a spectrophotometer and statistical analysis by Excel

Results and discution. The first task of our study was to evaluate the effect of zinc ion (different concentration 10, 50, 100, 200, 500 mikroM Zn solution) on cell proliferation of CHOwt line and to establish the optimal concentration for subsequent experiments

CHO cells overexpressing *ATP7B* (CHO wt) cell proliferation was increased as time went by in a concentration-dependent manner (Fig.3).Cell proliferation was stimulated even at low zinc (10-50uM) at the first 3 days, compared to no Zn incubated cells. As time went by, this zinc-stimulated cell proliferation was more prominent at high range of zinc concentration at 200uM on 5 and 6 day, while low zinc level showed the adverse effect of cell proliferation during this culture period.

Similar results were obtained by several researchers in studies with other cells line.: osteoblastic MC3T3-E1[6]. They established that the low Zn

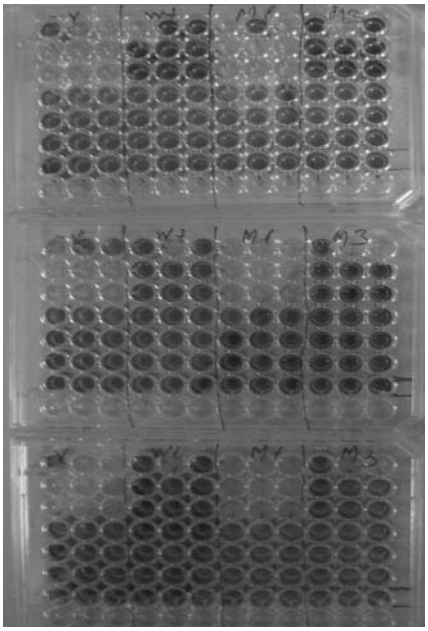

Fig 2. Copper induced cytotoxicity - MTT assay.

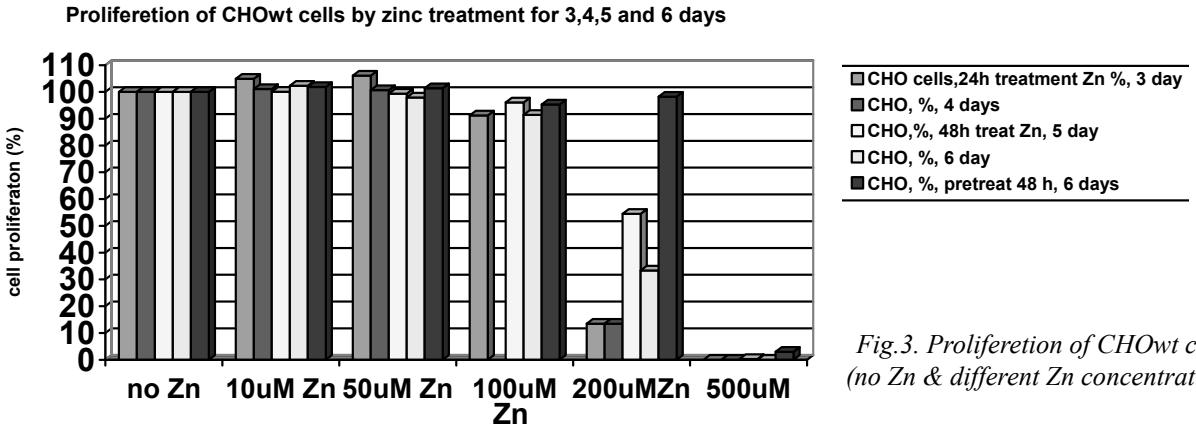

Fig.3. Proliferetion of CHOwt cells (no Zn & different Zn concentration)

concentration (1-3uM) increase cellular proliferation in osteoblastic MC3T3-E1 cells as time by duration 1-10 days, which is considered as stimulating osteoblast differentiation by bone marker protein synthesis by osteoblasts.

Perez MJ, Cederbaum AI from Mount Sinai School of Medcine, New York in there study have been investigate if zinc or metallothionein (MT) induction by zinc could afford protection against *CYP2E1*-dependent toxicity on the HepG2 cells overexpressing *CYP2E1* (E47 cells). Preincubation overnight with 150 microM zinc sulfate induced a 20- to 30-fold increase of MT2A mRNA [9].

Next objective of our study was to study the effect of different zinc concentrations on the CHO- wt cells and to investigate if zinc could afford protection against Cu dependent toxicity

*Description of the methodology***:**

CHO cells overexpressing *ATP7B* (CHOwt) were treated with different concentrations of Zn and Cu - plate 1 during 48 hours. (see fig.4)

Other 2 plates (2 and 3) were pre-treated with different concentration of Zn during 48 hours respectively.

In the 1 plate we add MTT (incubated 3 hour, 37C) and ELISA measure have been performed after 24 h

In the 2 plate we add only deferens concentration of CuCl2. In the 3 plate we add ZnCl2 and CuCl2

Then we incubate these 2 plates during 48 h. After that we add MTT (incubated 3 hour, 37C) and ELISA measure have been performed after 24 h

Plate design

column 1--2: only cells +media lacking phenol red no ZnCl2 addition

column 3-4 : 10 mikroM ZnCl2,

column 5-6: 50 mikroM ZnCl2

column 7-8: 100 mikroM ZnCl2

column 9-10: 200 mikroM ZnCl2

column 11-12: 500 mikroM ZnCl2

Row A: 1mM CuCl2

Row B: 0,75mM CuCl2

Row C: 0,5mM CuCl2

Row D: 0,25mM CuCl2

Row E: 0,125mM CuCl2

Row F: 0,06mM CuCl2

Row G: Zn additions different concentration (according to the column), no CuCl2 addition, only cells (media lacking phenol red)

Row H: no CuCl2 addition, no cells, only medium (media lacking phenol red)

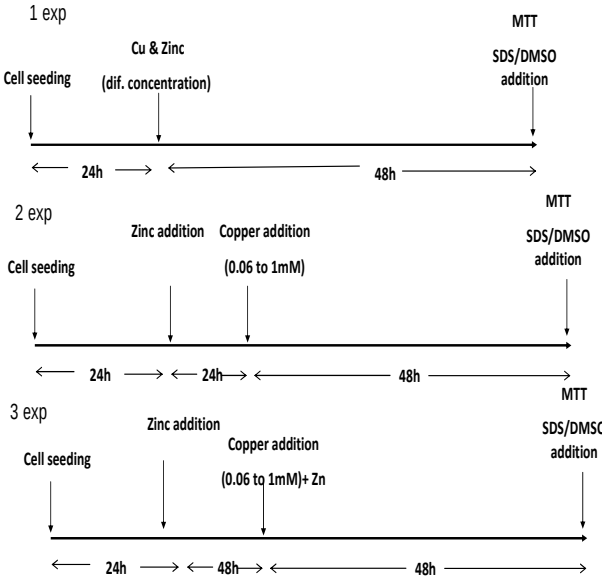

Fig 4. Design of experiments.

Study of cell survival, depending on the concentration of copper showed that the concentration of 0.06 and 0.25mM copper is not effective, whereas with increasing concentration from 0,50 to 1,0 mM observed a dose-dependent increase in cell death from 57% to 28%, respectively. We observed the ability of equimolar concentrations of copper to inhibit the cell survival necessitates understand the nature of cell death. It is known that cell death can occur in two alternative ways, necrosis and apoptosis, which differ in their

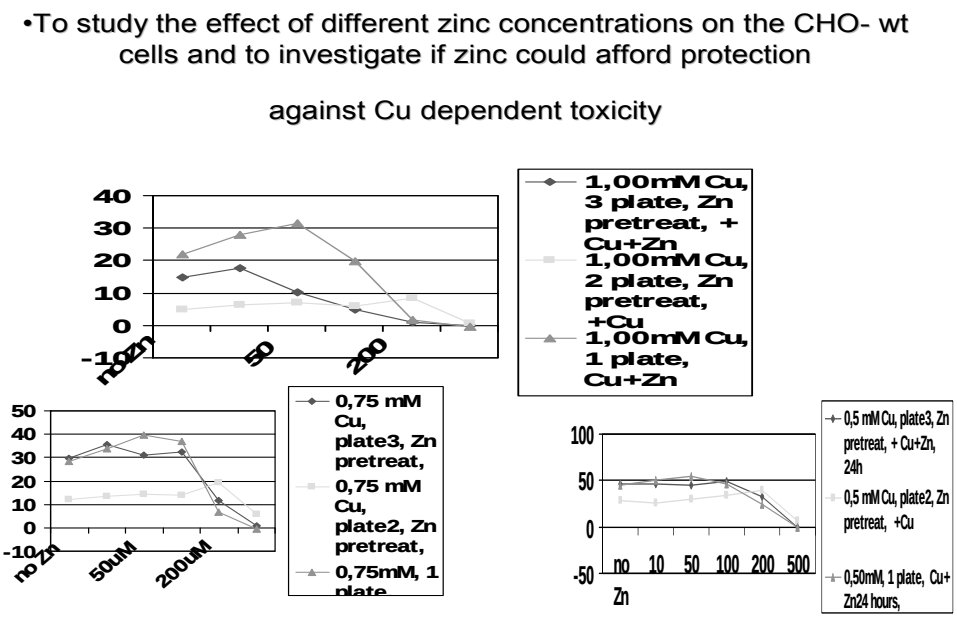

Fig.5. Percent of living cells at various concentrations of copper and zinc.

morphological and biochemical characteristics. So one of the main features of apoptosis is internucleosomal DNA fragmentation of chromatin. In the case of cell death by way of necrosis or fragmentation is not observed at all or generated DNA fragments are of uncertain dimensions, which give a continuous band at the electrophoretic separation.[9]

It is interesting to trace the changes in cell viability at various concentrations of copper and zinc. If we compare the survival of cells, measurements of MTT after 24 hour of DMSO/SDS added, at the 0,75 mM concentration of copper, it may be noted that under the joint incubation of cells in a solution of copper and zinc (50mikroM) cell survival by 20 per cent increase than in the corresponding cells of cells incubated with copper without zinc. (fig.5)

Of course, it is very difficult to find an adequate concentration of zinc and copper. Good protective effect is the concentration of 10 mM solution of zinc. Phenomenon that describes Perez MJ [9] in his article also confirmed in our experiment. Preincubation of cells in a solution of high (200 mikroM) zinc concentration increases the survival of cells approximately 2-fold (4% in the wells without zinc versus 8% in the hole 200 mikroM) at toxic dose of copper - 1,0 mm (plate 2).

According Perez MJ data, zinc pretreatment protect about 50% against the DNA fragmentation, cell necrosis, and the loss of mitochondrial membrane potential induced by arachidonic acid (AA) treatment in HepG2cells, overeexpressing CYP2E1 [9].

VanLandingham JW, 2002 in his work exploredthe ability of Zn to protect human neuron in culture (NT2-N) from Cu-mediated death and tested the hypotheses that the tumor-supressor protein p53 plays a role in Cu-induced neural death and is part of the mechanism of Zn protection. Copper toxicity (100microM) resulted in significant apoptotic neuronal death. Addition of 100 microM Zn to Cu-treated cells increased neuronal death. However, the addition of 700 microM Zn to Cu-treated cells resulted in neuronal viability that was not different from untreated controls through 24h.[8]

The results that we received in the ***first*** experement:

- Statistical significant between data of survival cells in high Cu concentration (0,50mM, 0,75mM and 1,0mM)
- Concentration 500 mikroM Zn is toxic concentration - percent of survival cells is **0,22%**
- The survival rate of CHO-wt cells at 1 mM copper concentration have been increased by 20% by adding 50 microM zinc (24 h measure).
- Concentration 50 mikroM zinc stimulates cell division

The ***second*** experiment consist of per-treatment with Zn during 2 days and then added Cu and incubate again 48 hours.What we received:

- No statistical significant between data of survival cells in high Cu concentration (0,50mM, 0,75mM and 1,0mM) .
- The cells survival on concentration 200 mikroM (1,88% –Zn &Cu (1 exp.) comparative 20,65% pre- treatment Zn in concentration 1,00 mM Cu,
- Pre-treatment needed (necessary) for using high concentration of Zn (more then 100 mikroM)
- Pre-treatment increase cells survival after added 0,75mM & 0,50 mMCu in 2 times (46.01 % comp 28,27% and 73,75% comp. 44,77%)
- Cells with pre-treatment (200mikroM) gives

twice as many living cells at a concentration of copper 0,1 and 0,75 мМ, as the cells with out Zn

The ***third*** experiment consist of per-treatment with Zn during 2 days.After 48 hours we add Zn and Cu and incubate again 48 hours. Thus, these cells grew in medium with zinc within 96 hours. What we received:

- No statistical significant between data of survival cells in high Cu concentration (0,50mM, 0,75mM and 1,0mM) The cells survival on concentration 200 mikroM
- The survival rate of cells at a concentration of 1 mM copper fell by 1.5% by adding 50 nM zinc
- In high concentrations, 200 mikroM and 500 microM more cells are death than in the second experiment.

Accordingly, Zn protect cells against Cu toxic effects. But how does it work, remains an open question. Is Zinc a simple antagonist of copper or includes the work of other proteins and enzymes, particularly metalloproteins, or induction metallothionein?

It can be assumed that zinc accumulates in the cells in the same compartments in which copper accumulates and causes a decrease in oxidase activity of ceruloplasmin (CP) and zinc ions induces the suppression of gene expression and protein metalotransport gene CP. It is required to establish the relative level of gene expression of intracellular copperenzymes changed or unchanged, or increase the proportion of mitochondrial SOD1.

Given these results and the fact that the best indicator of an experiment made with simultaneous incubation of copper and zinc 50mikroM we decided to conduct further experiments, and this mode and with two concentrations of 50 and 80mikroM Zinc solution. Although the doses of zinc 5-10uM is also very interesting for the study, which was confirmed by the literature.

Next step of our research is to study the copper sensitivity of different CHO cell mutant lines under the combined addition of zinc (estimated survival rate of cells)

This experimentation was carried out seeding cells in three plates (96 wells)

- 1 pl. added copper,
- the second – Cu and 50 uM zinc and copper simultaneously,
- third-80 uM zinc and copper

Incubate 48 h. Fourth day: we add MTT and performed ELISA measure after 24 h.

Rezults of the experiment revealed a different sensitivity of cells to the joint addition of zinc and copper. We received 3 categories of cells:*The first* one showed an increase in the number of living cells by adding zinc (M1, M2, M6, M8, M7) (fig.6). *The second* - did not respond (it was resistant) to zinc supplementation (M5 and M12). *the third* category, showing an increase in cell death in zinc supplementation (M9) (fig 7).

Based on these data, we did 9 repeats of mutant lines that are in the category increased cell proliferation under the influence of zinc. In experiment have been involve cells CHO-vec, CHOwt and CHO cells lines with different type of mutation.(Fig 8)

The cell lines have been broken into at least 3 different categories at the functional level (copper sensitivity):

- First type of cells, which give a high percentage of deaths at a concentration of 1 mM, 0.75

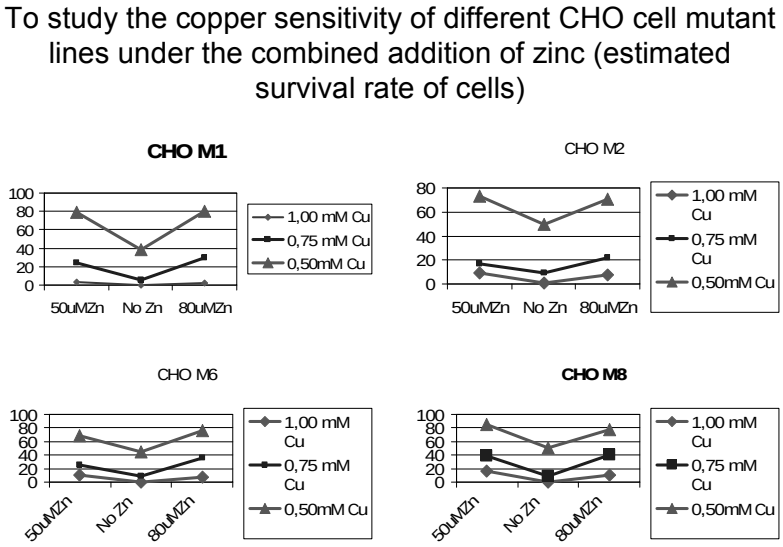

Fig.6. Mutan lines showed an increase in the number of living cells by adding zinc

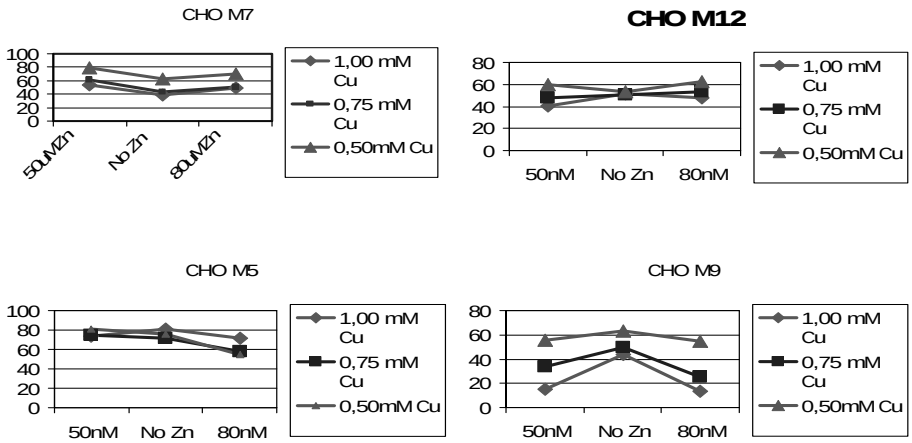

Fig7. The second category of cells line did not respond (it was resistant) to zinc supplementation and the third category, showing an increase in cell death in zinc supplementation

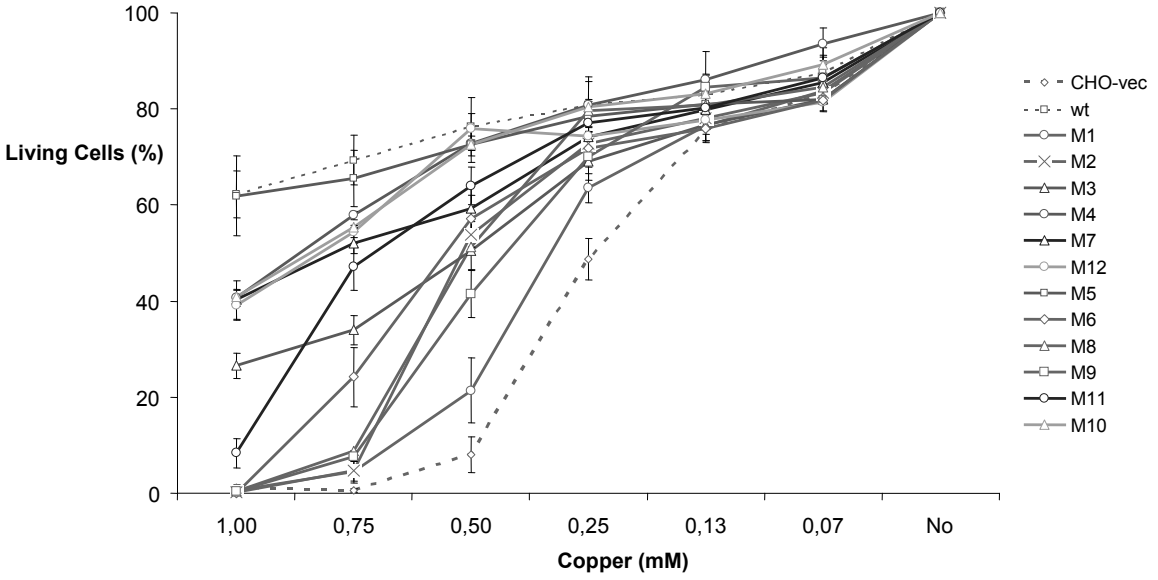

Fig. 8 Functional analysis. Copper induced cytotoxicity assay

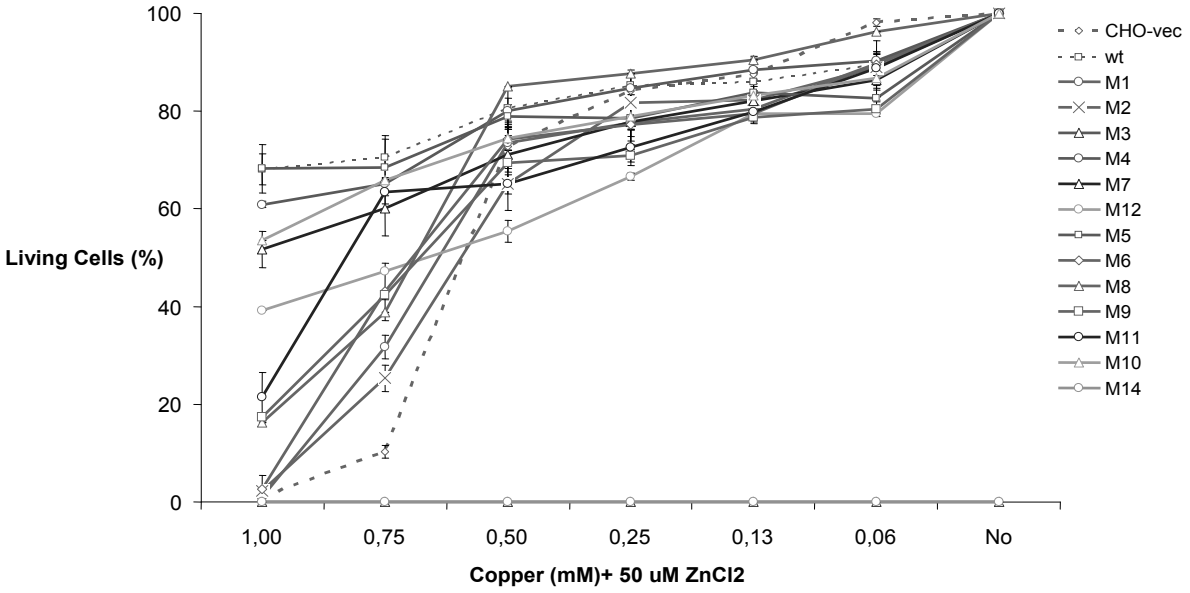

Fig.9. Functional analysis. Copper and Zinc induced cytotoxicity assay.

mM and 50 mM copper – "Sevier" forms for examples M1, M2, M6, M8, M9.

- Second type of cells, which survive in the proposed 38-50% at the above-mentioned copper concentration-"mild" forms for examples M4, M3, M7,.
- Intermediate forms for examples M10, M12

Analyzing the data, increasing of living cells have been established on 23% in CHOwt line; on 20,29% in CHO M4; on 18% -CHO M7 and CHO M10; and on 15,37%- CHO M11 during incubation in 1.0mM copper and 50.0uM zinc solution. We want to note the absence effect (0,6%) of increasing the number of viable cells in CHO-vec lines in the same solution, but show 57% of increasing living cells in 0,5mM CuCl2 plus 50mikroM ZnCl2 solution. "Sevier" forms of mutant lines show increasing % of living cells in 0,5mM CuCl2 plus 50mikroM ZnCl2 solution (Fig.9). May be this combination of Cu-Zn concentration is very important for some metabolic process. At 2000 year Yakugaku Z in his toxicological studies, two biological aspects of metallothionein (MT) i.e., antioxidant and prooxidant depending on the Cu/Zn ration in Cu-containing MT have been proposed [6]

Conclusion:

1. Zn prevents toxicity of Cu in CHO cells regardless whether if *ATP7B* is expressed or not

2. Zn prevents copper toxicity in CHO cells not dependent mutant is severe or moderate.

3. CHO-vec lines show 57% of increasing living cells in 0,5mM CuCl2 plus 50uM ZnCl2 solution

4. Zinc is not a simple antagonist of copper and includes the work of other proteins and enzymes, particularly metalloproteins, metallothionein.

5. It is required to establish the relative level of gene expression of intracellular copper enzymes and mitochondrial SOD1

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