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INVESTIGATION ON THE RELATIONSHIP OF THE CYTOSKELETON COMPONENTS WITH NORMAL CELLULAR FUNCTIONS AND INJURIES IN NEURO-DEGENERATIVE DISORDERS, USING *IN VITRO*-, *IN VIVO*- AND *EX VIVO*- BIOLOGICAL SYSTEMS WITH MOUSE, RAT AND HUMAN ORIGIN

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ABSTRACT

For a better understanding of the physiological function of cytoskeleton components, several different methods for their quantification and identification were developed and tested. In the concrete study, the main goal was directed to investigation of the importance of these proteins and peptides, as well as of the intra- and extra-cellular interactions between them, for in the normal functions of the tissues and organs, but also of respective abnormalities – for the development of pathological events. Complex biological systems from rat and mouse, but also with human origin for compare with those of both experimental rodent species as appropriate experimental alternatives, were used. In the highly differentiated mature cells in the composition of different tissues and organs, including brain, these abnormalities have been proved as main reason for abnormal functions, leading to development of diseases and disorders on organism level. However, the same injuries in the cytoskeleton components have been suggested as reason for abnormal mitosis in the actively-dividing cells from the same individuals (as different types of undifferentiated leucocytes and their progenitors), subsequently leading to chromosomal aberrations. As one of the key molecules in the intra- and extra-cellular inter-molecular interactions by cascade regulatory pathways, in which these elements participate, has been proved the tri-peptide Glutathione, in particular, its reduced form – GSH. In agreement with the literature data, the current results showed variations in the levels of this molecule in varying external conditions. Future investigations

on direct and/or indirect influence of these components on the function of other biological molecules by cascade regulatory mechanisms with their participation by intra- and extra-cellular interactions are necessary.

KEYWORDS: cytoskeleton components, cascade regulatory pathways, spontaneous chromosomal fragility, chromosomal aberrations, leucocytes, terminally-differentiated mature cells.

1. INTRODUCTION

As a key pathway in the regulation of precursors processing of into active proteins has been characterized the ubiquitin-proteasome mechanism, besides its role in the complete degradation of polypeptides (Palombella et al., 1994). In this way, an essential role of the proteasome complex in two proteolytic processes, required for activation of Nuclear Factor kappaB (NF-κB), as a main transcription factor has been demonstrated. Integration of signals with cell cycle control by mechanisms of Met4 and SCF^{Met30} regulation in response to intra- and environmental conditions have been proposed. In this way, β-catenin has been shown as crucial in cell-cell adhesion in addition to a signaling role as a component of the Wnt/wg pathway (Shtutman et al., 1999). This signal mechanism has been established to result in β-catenin accumulation and transcriptional activation of specific target genes during development. It has also been emerged as a critical during the development of many organ systems. In vertebrates an alternative Ca^{2+}-mediated Wnt-signaling pathway has been established (Kohl et al, 2000). Wnt5A expression has been directly up-regulated through the Smad-complex, as well as through Smad-induced CUX1 and MAP3K7-mediated NF-κB, by TGF-β-signals (Cloutier et al, 2007; Kohl et al, 2000). The conserved NF-κB-binding site within the Wnt5A promoter B region has also been proved to elucidate the up-regulation mechanisms on Wnt via MAP3K7 by the influence of TNF-α and toll-like receptor (TLR) signals.

Similarly, the hormone-like peptide Secretagogin (SCGN) has recently been cloned and characterized as β-cell-expressed EF-"hand" Ca^{2+}-binding protein (Bauer et al, 2011; Gartner et al, 2007; Maj et al., 2010; Rogstam et al, 2007; Wagner et al, 2000; Witcher et al, 1995). Functional analysis of transfected cell lines, expressing SCGN, has revealed an influence on calcium flux and cell proliferation. At the same time, a Ca^{2+}-dependent SCGN-TAU interaction, as well as co-induction of TAU, has been found in the islets of Langerhans and β-cell-derived cell lines with high expression of the neuroendocrine-specific protein SCGN.

As one of the key molecules, included in these cascade regulatory pathways, has been determined the tri-peptide Glutathione (L-glutamil-L-cysteinyl-glycine), in particular its reduced form (GSH) (Jahngen-Hodge et al, 1997; Meister, 1983), but also the enzymes, participating in its metabolism as Glutathione-S-transferase (GST), Superoxide dismutase (SOD), Catalase (CAT) and β-galactosidase. GSH has been proved as able to neutralize the oxidative stress, and to detoxify toxins and scavenges the so called reactive oxygen species (ROS) and reactive nitrogen species (RNS), formed during the normal metabolic processes or as a result of trauma, infection or medication (Hayes & McLellan, 1999; Meister, 1983; White et al., 1994). The liver, as a key organ for xenobiotic detoxification and elimination, has been proved as the major site of GSH synthesis (Kaplowitz et al, 1985). The activity of hydrosulfide group in the molecule of GSH determines its biological significance and activity in many antioxidant and detoxifying reactions (Meister, 1983). This substance is responsible for keeping proper thiol-disulfide balance and related redox-potential in the cells. Moreover, the nucleophilic glutathione thiol (–SH) group enters reactions with electrophilic substances, either endogenous or exogenous (xenobiotics, including drugs), yielding glutathione S-conjugates, (i. e GSH thioeters), which are then transformed to mercapturic acids and excreted (Kwiecien et al, 2006). GSH deficit has been found to disrupt the redox-status and upsets the physiological cellular balance between pro-oxidants and antioxidants (Injac & Strukelj, 2008; Jahngen-Hodge et al, 1997; Minotti et al, 2004). Hence, GSH modulation could represent a supportive measure to achieve a therapeutic goal.

As one of the main mechanisms of the action of antioxidant molecules has been characterized the protective influence against the chemically-induced oxidative stress (Attia et al, 2010; Han et al, 2012; Kokotkiewicz et al, 2010). Also, cascade mechanisms, underlining the differentiation of immune cells and immune system, have been proved (Jahngen-Hodge et al, 1997; Kong et al, 2003; Zhu et al, 2012). These results indicated a strong scavenging effect of anthocyanins in *Chokeberry* (*Aronia melanocarpa*) on the intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Alexieva et al, 2010).

In this connection, the main goal of the current study was directed to investigation on the role of the cytoskeleton proteins and peptides in support the normal function of the cells, but also of injuries in the structure of these components in diseases, connected with pathological processes in internal tissues and organs, including in neuro-degenerative disorders. Several

anatomic organs from rat and mouse, but also with human origin for compare with both rodent cell types as appropriate experimental model alternatives, were used.

2. MATERIALS AND METHODS

2. 1. In Vitro-Incubated Cell Cultures

Normal fibroblasts from embryonic mouse Balb/c 3T3 line, malignant mouse myeloma cells, as well as mixed cultures from both cell types, were prepared. All cell cultures (1 x 10^6 cells/ml), were incubated at 37^0C in incubator with 5% CO_2 and 95% air humidification, in RPMI 1640, Dulbecco's Modified Minimal Essential Medium (DMEM) or a mixture of both media (1:1), supplemented with 10% Fetal Calf Serum (FCS), 100 UI/ml Penicillin, 0.25 mg/ml Streptomycin and 0.25 mg/ml Amphotericin-B, in 24-well plaques. In separate sub-populations from each of both cell types used, as well as in mixed cultures of them, were added total Aronia melanocarpa Plant extract; 0.05M solution of Doxorubicin in distilled water, as well as from both tested substances, but respective untreated controls were also prepared. All cell cultures were observed by inverted light microscope, supplied with mega-pixel CCD-camera. Cells from all groups, treated with both substances, with each one of them, as well as the respective untreated controls, treated with 10% trichloroacetic acid (Cl_3CCOOH) and 0.48M solution of K_3PO_4. GSH levels from all of them were determined by a spectrophotometric method and absorbance were measured at 412nm (Ellman, 1959). The levels of GSH were defined from the standard curve with commercially available GSH and the results are expressed as milimole per 1 ml cell suspension (mM/ml cell suspension).

2. 2. Experimental mice

Male and female Balb/c mice, aged 3 months and weighing 20-25 g, came from Slivnica animal breeding house, Sofia. All animal procedures were performed in accordance with Animal Ethics Committee. They were separated into 4 groups of 6 animals: treated with total plant extract (Plant group); with Doxorubicin (DOX-group), with both Plant extract and Doxorubicin (Plant + DOX), as well as untreated controls (Control group), respectively. The supplementation with both Plant extract was made for 28 consecutive days and the fruit total extract was given to mice from the Plant and (Plant + DOX) groups as 20% water solution instead of water. Doxorubicin hydrochloride (Sigma-Aldrich), was freshly prepared in Phosphate Buffered Saline (PBS) and given to animals as intra-peritoneal (i. p.) injection of 20 mg/kg/body weight to DOX- and (DOX + Plant) groups on the 24^{-rd} day from the beginning of the experiment. Mice from both Control- and Plant-groups were injected with

PBS i. p. on the same day. After 28 days of pre-treatment with the Plant extract and 4 days from injection of Doxorubicin, all mice were sacrificed. Heart, small intestine and liver samples were taken and proceeded separately for biochemical measurement of reduced GSH. Tissue samples of the three anatomic organs tested from the experimental and control mice were isolated and after mechanical homogenization were treated with 10% tri-chloroacetic acid (Cl_3CCOOH), 0.48M solution of K_3PO_4 and centrifuged at 3000 x for 10 minutes. The supernatants were used to determine the GSH levels by a spectrophotometric method (Ellman, 1959) and the absorption was measured at 412nm (SPEKOL 1500, Analytik Jena). The level of GSH was defined from the standard curve with commercially available GSH (Sigma-Aldrich) and the results are expressed as micromole per 1 g wet tissue (μM/g wet tissue).

2. 3. Experiment-al rats

Recombinant rat peptide Secretagogin (SCGN), isolated from recombinant DNA-plasmids *E. coli* bacteria strains, transfected with rat *scgn* gene, was incubated in previously prepared rat pancreatic and brain lysates, respectively, as anatomic organs, known as the most actively expression of *scgn* gene in building cells. Because of the revealed very near protein profiles in both organs, different concentrations of the isolated from them proteins were also used in the current study – protein lysate from rat pancreas was diluted 10 folds. Bacteria cells and both organs were treated with lysis buffer and put on 4^0C for 2 hours. After addition of specific anti-rat SCGN antibody to the so obtained bacteria cell protein lysate and subsequent centrifugation, the received pellet/precipitate was resuspended in PBS. The so prepared protein suspension was mixed with the obtained protein lysates from rat brain and rat pancreas, respectively and both mixtures were subjected on CNBr-Sepharose and/or GSH-Agarose columns separation for 2 hours with intensive shaking for connection of proteins with affinity to the recombinant rat peptide SCGN of both organs. After elution of protein materials from both columns with Sodium dodecylsulphate (SDS) and/or Lithium dodecylsulphate (LDS) buffers, the so prepared probes were put at 70^0C for 10 minutes, and subsequent addition of SDS- or LDS-leading buffer and Reducing agent, they were separated by SDS/LDS-Polyacrylamide Gel Electrophorhesis (SDS/LDS-PAGE). The so isolated protein fractions were precipitated in cold 100% EtOH overnight, after which the so formed precipitate were washed with cold 80% EtOH and after centrifugation, the supernatants were turned off and the pellets were diluted in SDS/LDS buffer. After SDS/LDS-PAGE and consequent Comasie-blue staining, the gel was washed and sliced with an in-house tool. Gel

lanes were fractioned into slices, which were subjected on trypsin digestion. The so obtained gel slices were then washed with water and acetonitrile, followed by reduction and alkylation of cysteine residues by DTT and iodoacetamide. Following overnight trypsin digestion, the peptides were extracted by acetonitrile and 5% formic acid, and subsequently concentrated in a speed-vacuum centrifuge. In this way, all probes were prepared for label-free tandem mass spectrometry-liquid chromatography (LC-MS/MS) assay.

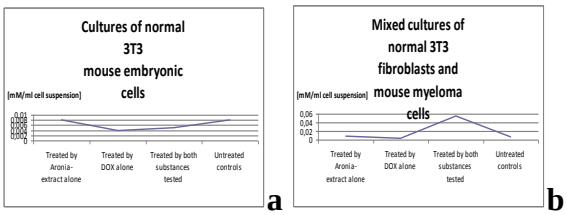
2. 4. Ex vivo-preparations of smears from human blood, containing cells from different types and in various maturation stages

Human peripheral blood probes (3-5 ml) were *ex vivo*-incubated, in 4 ml medium RPMI 1640 (Sigma-Aldrich) with added 0.8-1 ml Fetal Bovine Serum (FBS) and 0.2 ml phyto-hemagglutinin (PHG), at 37^0C in incubator with 5% CO_2 and 95% air humidification for 68-70 hours, and the further cell growth and proliferation was then blocked by addition of 0.2 ml Colhicin and the probes were put again at 37^0C for 50 minutes. After careful shaking, they were centrifuged for 10 minutes at 1000 rpm. The supernatants were taken off, and 0.3 ml from the pellets were resuspended, after which 10 ml warm hypotonic 0.555% KCl solution was added to each suspension, and the probes were put at 37^0C for 10 minutes. After centrifugation at 1000 rpm for 10 minutes, the supernatants were taken off, and after resuspendation of 0.3 ml of the pellets, 10 ml fixative mixture of methanol-glacial acetic acid (3:1) were added to each one probe/pellet. After centrifugation at 1000 rpm for 10 minutes, this step was repeated 3-4 times by changing of the fixative mixture. After the last centrifugation procedure, the supernatants were turned off, and the resuspended 0.3 ml of the pellets were put at 4^0C used for preparation of metaphase plates on light microscopy slides, put on 30-40 sm, which were dried, stained by G-banding technique and observed as fixed chromosomal preparations by immersion light microscope Amplival.

3. RESULTS AND DISCUSSION

3. 1. Cell Cultures

Decreased levels of GSH in normal embryonic fibroblasts, malignant cells and mixed cultures were noted in all cases of cultivation in the presence of the chemotherapeutic drug alone (Figure 1).



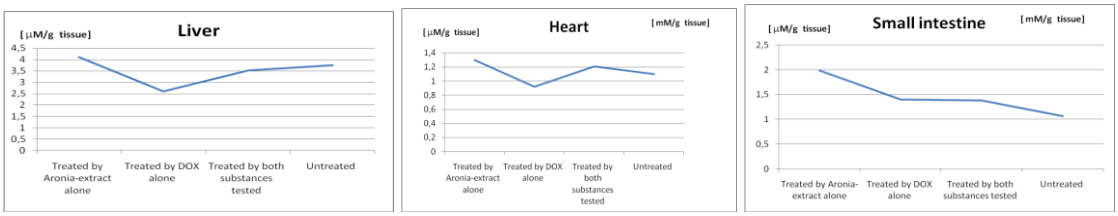
a **b**

Figure 1. Influence of Doxorubicin and total Plant extract on the intracellular GSH levels [mM/ml cell suspension], in normal 3T3 mouse embryonic fibroblasts (a), and mixed cultures of normal 3T3 and mouse malignant myeloma cells (b)

These levels were partially restored in the presence of Plant extract in the three groups of cell cultures, both in the presence and absence of Doxorubicin. Similar effects have been observed in investigation of other plant extracts or their components (Attia et al, 2010; Kähkönen et al, 1999; Syng-ai et al, 2004; Zdunczyk et al, 2002). Restoration in GSH-levels in the presence of Plant extract was observed in all cases, both in the presence and absence of the chemotherapeutic drug.

3. 2. Experimental mice

Statistically significant differences in GSH levels were assessed in probes from the three different organs of experimental rodents from the four groups tested (Figure 2).



A **b** **c**

Figure 2. GSH-levels [µM/g wet tissue] of DOX-treated mice with and without Plant extract total extract pre-treatment, in liver (a); heart (b) and small intestine (c): Significantly different from control group ($p<0.001$): Significantly different from DOX-treated group ($p<0.001$); significantly different from DOX-treated group ($p<0.05$). Values are expressed as mean±S.D., n=6.

Doxorubicin treatment caused significant reduction in GSH content, compared to controls in all investigated organs. Pre-treatment with the Plant extract, however, restored in part GSH level, but it did not reach those of the control group. There was not assessed a statistically significant difference in GSH content between Plant-group and control group. The results obtained were in agreement with literature data about the protective action of the Plant extract

against Doxorubicin-induced side effects (Denev et al, 2012; Kujawska et al, 2011; Sonneveld et al, 1981).

3. 3. Experimental rats

According the results obtained, 82 were appeared as common proteins, possessing affinity to connect with SCGN peptide, from both anatomic organs, 24 – as unique for the brain, and 16 – for the pancreas (Figure 3).

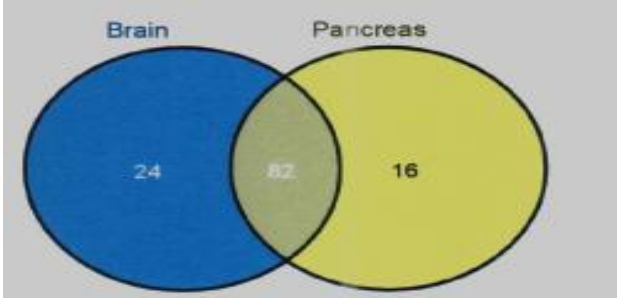


Figure 3. Identifications of common proteins from between rat brain and pancreas, with highest affinity to recombinant rat peptide SCGN, separated on CNBr-Sepharose and/or GSH-Agarose columns

According to the results obtained, the molecules with highest affinity to GSH both rat anatomic organs, were mainly cytoskeleton components as microtubule-associated proteins (MAPs) and cyclins (Table 1).

Table 1. Distribution of cytoskeleton proteins from rat brain and pancreas, possessing high affinity to the recombinant rat peptide SCGN (according values of molecular weight and intensity of fluorescence signal), separated on CNBr-Sepharose and/or GSH-Agarose columns

Cytoskeleton Proteins from Rat Brain, Possessing High Affinity to Recombinant Rat Peptide SCGN		**Cytoskeleton Proteins from Rat Pancreas, Possessing High Affinity to Recombinant Rat Peptide SCGN**	
Molecular Weight [kDa]	**Fluorescence Signal Intensity**	**Molecular Weight [kDa]**	**Fluorescence Signal Intensity**
46-68 (intermediate filament proteins and tubulins) 75-80 (cytokeratin fraction)	tubulin fraction Tau peptide filament proteins microtubule-associated proteins (MAPs) actin fraction cytokeratin fraction	24-29 (cytoskeleton-related proteins) 42-44 (actin fraction) 46-68 (intermediate filament proteins and tubulins) 75-80 (cytokeratin fraction)	tubulin fraction cytokeratin fraction

These data were in agreement with many literature findings in this direction (Aamodt & Culotti, 1986; Oakata et al, 1995; Savage & Chalfie, 1991). These cytoskeleton elements have been proved to play mainly enzyme and secretory functions in the pancreas and as neuro-filaments in the neuro-transmission processes in the brain, respectively, by respective cascade regulatory pathways (Butner & Kirschner, 1991; Putaala et al, 2001; Takeda et al, 1989; Zhang et al., 2010). Furthermore, deformations in the cell cytoskeleton components could lead to high frequency of spontaneous chromosomal fragility and/or of cell spindle structure, which could lead to number chromosomal aberrations because of abnormal cell division (Zheng et al, 1993). Also, the current investigation confirms the power and usability of the applied method (LC-MS/MS assay) identification of proteins/peptides and protein-protein interactions from different biological samples, which was in agreement with many literature data (Cuattrecasas, 1970; Dicker et al., 2010; Haqqani et al., 2008; Old et al., 2005; Pham et al., 2010; Zhu et al., 2010).

3. 4. Ex vivo-preparations from human cells

Injuries in the structure of MAPs, including as a result of their abnormal expression, has been characterized as one of the main reasons, underlining the predisposition for development of neuro-psychiatric disorders in people, in particular MAP2 and MAP5 (Arnold et al., 1991). On the other hand, here again these abnormalities have been suggested as one of the reasons for abnormal mitosis in the actively-dividing cells from the same patients, subsequently leading to chromosomal aberrations (Daniel, 1986; Zheng et al., 1993). The established frequency of spontaneous fragility of chromosomes in leucocytes from peripheral blood of patients with neuro-psychiatric disorders was significantly higher than in the control healthy individuals (Figure 4).

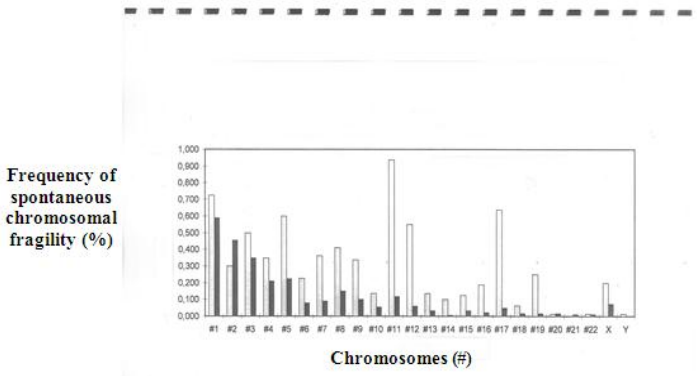


Figure 4. Frequency of spontaneous chromosomal fragility (%) in patients with neuro-psychiatric disorders (light columns) and healthy controls (dark columns)

As is seen, the most often affected is chromosome 11, followed by chromosomes 1 and 17. In all cases, the highest spontaneous fragility was assessed in the centromere chromosomal regions, and the most significant difference between the patients and controls was noted in chromosomes 11, 12 and 17 (Figure 4). In support of the literature data, a possibility the high spontaneous chromosomal fragility frequency in the centromeres predispose abnormal cell division due to injured connection to the mitotic spindle, in this way – to chromosomal aberrations (Daniel, 1986; Zheng et al., 1993).

4. CONCLUSION

The main goal of the current study was connected with a better understanding of the importance of the cytoskeleton components, as well as the intra- and extra-cellular interactions, in which they participate, in the physiological functions of complex biological systems, as well as in the respective abnormalities in injuries of these molecules and interactions. Several different techniques their quantification and identification of cytoskeleton proteins and peptides were developed and tested. The analogy between cells, tissues and organs from human, mouse and rat confirmed the usability of rodent biological systems as convenient experimental model alternatives of these with human origin. In the highly differentiated mature cells in the composition of different tissues and organs, these abnormalities have been proved as main reason for abnormal functions, leading to development of diseases and disorders on organism level. However, in the actively-dividing cells from the same individuals, these injuries were suggested to lead to abnormal mitosis, and subsequently - to chromosomal aberrations. As one of the key molecules in the intra- and extra-cellular inter-molecular interactions by cascade regulatory pathways, in which these elements participate, has been proved the tri-peptide GSH, and variations in its levels in varying external conditions were established. In many studies, this molecule has also been suggested to play a basic role in the prevention of side effects of chemotherapeutic drugs, both *in vitro* and *in vivo*. By cascade mechanisms, including with the participation of GSH, many plant extracts and natural foods have been proved as useful in this aspect, because of the high content of anti-oxidant substances. As one of the main mechanisms of this action is proved their action against the chemically-induces oxidative stress, by cascade pathways, with participation of the internal anti-oxidant molecule GSH, but also enzymes, participating in its metabolism, as GST, SOD, CAT and beta-galactosidase. Over-lapping between different mechanisms of cardio-toxic, anti-malignancy and other protective effects in the three types of biological systems tested could very often be noted, by several different

mechanisms or with resulting and interaction between each other or between different components from them. Future studies on direct and/or indirect influence of these components, in the presence and absence natural extracts, on the levels of GSH and other biological molecules, by cascade regulatory mechanisms with their participation by intra- and extra-cellular interactions, are necessary.

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