THE UNIVERSITY OF MEDICINE AND PHARMACY

"CAROL DAVILA", BUCHAREST

DOCTORAL SCHOOL

MEDICINE

Research on the consequences of chronic ethanol impregnation in the evolution of myelic lesions in patients with spinal cord injury

PHD THESIS SUMMARY

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To my parents, with much love and admiration!

CONTENT

List of abbreviations used in the text

>9w		Mai mult de 9 săptămâni
µL		Microlitru
µM		Micromolar
2w		2 săptămâni
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs	Dezintegrina și metaloproteinază cu motive de trombospondină
ADN		Acid DezoxiriboNucleic
ADP	Adenosine DiPhosphat	Adenozin difosfat
AIF	Apoptosis-inducing factors	Factori inductori de apoptoză
AIM2	Absent In Melanoma 2	Absent în melanom 2
AIS	American Spinal Injury Association Impairment Scale	Scala Asociației Americane de Leziuni ale Coloanei Vertebrale
AK		Adenilat Kinază
ALDOA	ALDOlase, fructose-bisphosphate A	Aldolaza, fructozo-bisfosfat A
AMP	Adenosine Monophosphat	Adenozin monofosfat
AMPA	α Amino-3 hydroxi- 5 Metil- 4 – isoxazol Propionat	Acid α Amino-3 hidroxi- 5 Metil- 4 – isoxazol Propionic
ARE	Antioxidant Response Element	Elemente de răspuns antioxidant
AO	Acridine orange	
Apaf-1	Apoptosis protease activating factor-1	Factorul activator al proteazei apoptozei-1
ARN-m		Acidului RiboNucleic mesager
ASC	Apoptosis-associated Speck-like protein containing a CARD	Proteină cu formă de pată asociată apoptozei ce conține CARD
ATF	Activating Transcription Factor	Factorul activator al transcripției
ATP	Adenosine TriPhosphate	Adenozin trifosfat
Bcl-2	B-cell lymphoma 2	Proteinelor familiei limfomului cu celule B- 2
BDNF	Brain-Derived Neurotrophic Factor	Factorul de creștere neurotrofic derivat din creier
BDNF	Brain-Derived Neurotrophic Factor	Factorul neurotrofic derivat din creier
BH	Bcl-2 Homology	Domeniile de organizare omoloage Bcl-2
Bid	BH3-Interacting-Domain death agonist	Domeniul morții agonist interacțiunii cu BH3
BMK	Big MAPK	

BMP4	Bone morphogenetic proteins 4	Proteinele morfogentice osoase 4
BMPR		Receptor BMP
CA9	Carbonic Anhydrase 9	Anhidraza carbonică 9
CAD	C-terminal transactivation domain	Domeniul de transactivare c-terminal
CARD	Caspase Activation and Recruitment Domain	Domeniul de activare și recrutare al caspazei
Casp	Caspase	Caspază
Caspases	Cysteinyl, aspartate-specific proteases	Proteaze cistenil-aspartat specific
CAT		Catalază
CBP	cAMP response element-binding protein-binding protein	Proteina de legare a proteinei de legare a elementului de răspuns AMPc
CD133		Prominina-1
CDH2		Caderina 2
CDK2	Ciclyn Dependent Kinases	Ciclin dependent kinaza 2
CHI3L1	Chitinase 3–like protein 1	Proteina 1 asemănătoare chitinazei 3
Cited2	Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 2	Transactivatorul de interacțiune Cbp/p300 cu domeniul carboxi-terminal bogat în Glu/Asp 2
CO_2		Dioxide de carbon
$CoCl_2$		Clorură de cobalt
COX-2		CiclOXigenza 2
CREB	cAMP response element-binding protein	Proteina de legare a elementelor de răspuns AMPc (adenozin monofosfat ciclic)
CSTB	CySTatinB	Cistatina B
CTL	C-Type Lectin	Lectinic tip C
CYLD	Cylindromatoză	Acizi grași polinesaturați
DAMP	Damage-Associated Molecular Pattern	Modele moleculare traumatice asociate
DD	Death Domain	Domenii ale morții
DFX		Deferoxamină
DISC	Death-Inducing Signalling Complex	Complexului semnalelor inductoare ale morții
Dkk	Proteinr Dickkopf	
DMEM	Dulbecco's Modified Essential Medium	Mediul de cultură
DMSO	Dimethyl Sulphoxide	Mediu de cultură celulară
EDTA		Acid etilendiaminotetraacetic
EGF-R	Epidermal-Growth Factor Receptor	Receptorul factorului de creștere epidermal
EMEM	Eagle's Minimum Essential Medium	Mediu de cultură celulară

EPO		Eritropoietină
Et-OH		Consum/ expunere etanolic
ERSR	Endoplasmic reticulum stress response	Răspunsul stresului reticulului endoplasmatic
FABPs	Fatty acid binding proteins	Familia proteinelor de legare ale acizilor grași
FADD	Fas-Associated Death Domain	Asociate domeniilor morții Fas, TRAILR1 și TRAILR2
FasL	Fas ligand	Ligand Fas
FBS		Ser fetal bovin inactivat
FDA	Fluorescein diacetate	Diacetat de fluorosceină
Fetu-B		Fetuin B
Fig.		Figură
FIH	Factor inhibiting HIF	Factorul inhibitor al HIF
Flk-1 (KDR)	Kinase insert domain receptor	Receptorul domenului de inserție al kinazei
Flt	Fms related receptor tyrosine kinase	Tirozin kinaza receptorului fms
GABA	γ-AminoButyric Acid	Acid Gama-AminoButiric
GDP	Guanin DiPhosphat	Guanin difosfat
GFAP	Glial fibrillary acidic protein	Proteină acidă glială
GH18)	Glycosyl hydrolases	18 glicozil hidrolazelor
Glut	Glucose transporter	Transportorul de glucoză
GPX4	Glutathione peroxidase 4	Glutation peroxidaza 4
GSDMD	Gasdermin	Gasdermină D
GSDMD-NT	Gasdermin- N Terminal domain	Domeniu N terminal al gasderminei D
GTP	Guanin TriPhosphat	Guanin trifosfat
Hes	Hairy/enhancer of split	Factor transcriptional
Hey	Hairy Ears, Y-linked	Factor transcriptional
HIF	Hypoxia Inducible Factor	Factor inducibil al hipoxiei
HMGB1	High Mobility Group Box 1	Categoria de grup cu mobilitate ridicată 1
HMOX		Hemoxigenază
Hmsc	Human Mesenchymal Stem Cells	Celule stem mezenchimale umane
Housekeeping		Gena de referință Rt-PCR
HRE	Hypoxic Response Element	Element de răspuns hipoxic
HSP	Hat Shock Protein	Proteină de șoc termic
HSR	Heat shock response	Răspunsul proteinelor de șoc termic
HTB-11 (SK-N-SH)		Linie celulară de neuroblastoma
HTB-14	Human Tumoral Brain 14	Linie celulară de gliobastom
IAP	Inhibitor of apoptosis proteins	Proteine inhibitorii ale apoptozei

IDO	Indoleamine 2,3-DiOxygenase	Indolamin-2,3-dioxigenază
IGF-1	Insulin-like Growth Factor-1	Factorul de creştere asemămător insulinei-1
IkB		Familia inhibitorilor kB
IKK	kB Inhibitor complex Kinase	Kinazei complexului inhibitor Kb
IKK	kB inhibitor complex kinase	Kinaza complexului inhibitor Kb
IL-		InterLeuchina -
iNOS	Inducible Nitric Oxide Synthase	Sintetazei inductibile a oxidului nitric
IRE-1a	Inositol-requiring protein-1a	Element component ERSR
JNK	c-Jun N-terminal protein Kinase	Protein kinaza c-Jun N-terminală
KCl		Clorură de potasiu
KH_2PO_4		Fosfat monopotasic
LAMA	Laminin	Laminină
LCN2		Lipocalina 2
LDH	Lactate DeHydrogenase	Lactat dehirogenaza
LNGFR	Low-affinity Nerve Growth Factor Receptor	Receptorul cu afinitate scăzută pentru factorul de ceşterea nervilor
LPS	Liposaccharides	Lipozaharide
LRR	Leucine Rich Repeat	repetititve bogate în leucină
LTh		Limfocite Thelper
MAPK	Mitogen-Activated Protein Kinases	Protein kinazele activate de mitogeni
miRNA	MicroRNA	MicroARN
mM		Millimolar
MMP		Matrix metaloproteaze
MOMP	Mitochondria Outer Membrane Permeabilisation	Permeabilizarea membranei mitocondriale externe
MPT	Mitochondrial Permeability Transition pore	Porilor de tranziție ai permeabilității mitocondriale
Na_2HPO_4		Fosfat acid de sodiu
NaCl		Clorură de sodiu
NAD	Nicotinamide-Adenine Dinucleotide	Nicotinamin dinucleotid andenină
NADPH	Nicotinamide adenine dinucleotide phosphate	Nicotinamidă adenin dinucleotidă fosfat,
NALP1	NACHT leucine-rich-repeat protein 1	Proteina repetitivă bogată în leucină NACHT 1
NCAM-L1	Neural cell adhesion molecule L1	Moleculele de adeziune neuronală L1
NCCD	Nomenclature Committee on Cell Death	Comitetul de nomenclatură pentru moartea celulelor
NCS	Neural stem cells	Celulelor stem neuronale
NEK7	NIMA-related kinase 7	Kinaza înrudită cu NIMA-7

Nemo		Modulatorul esențial al NF-kB
NeuroD1	Neuronal differentiation 1	Factor transcpriţional
NFkB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells	Factor nuclear kappa-amplificator de lanțuri ușoare ale celulelor B activate
NGF	Nerve Growth Factor	Factorul de creștere al nervilor
NIK		Kinaza indusă de NF-kB
NLR	The Nucleotide binding domain Leucine-rich Repeat	Domeniul nucleotidic de legare bogat în secvențe leucinice repetitive
NLRP3 (NALP3)	NLR family pyrin domain containing protein 3 (NACHT, LRR and PYD domains containing protein)	NLR și domeniul pirinic conținând Proteina 3
NLS	Nuclear localization signal	Semnalul de localizare nucleară
NMDA		N-metil D-aspartat
NOD-like receptor	Nucleotide Oligomerization Domain-like receptor	Receptori similari domeniului de oligomerizare a nucleotidelor
Non ET-OH		Fără consum/ expunere la etanol
Non TVM		Fără antecedente de TVM
Notch 1	Neurogenic locus notch homolog protein 1	Proteina omoloagă 1 a inciziei locusului neurogen
Npn		Neuropilin
Nrf2/ARE	Nuclear factor erytroid-related factor 2/ antioxidant response elements	Cale de semnalizare celulară
NT-3	NeuroTrophin-3	Neurotrofina 3
Olig2	Oligodendrocyte transcription factor 2	Factor transcriptional
OMI/ HTRA2		Familie de proteaze serinice
p38		Protein kinaza de 38 kDa
PAMP	Pathogen Associated Molecular Pattern	Modele moleculare patogene asociate
PARP	Poly (ADP-Ribose) Polymerase	Polimeraza poli-ADP riboză
PCDH		Protocaderine
PCR	Polymerase Chain Reaction	Reacției de polimerizare în lanț
PCSK1	Proprotein Convertase Subtilisin/Kexin type 1	Proprotein convertază subtilizină/ kexină tip 1
PEG		PoliEtilien Glicol
PERK	ERSR-activated protein kinase ribonucleic acid (RNA)(PKR)-like kinase	Element component ERSR
PFKL1	PhosphoFructoKinase, Liver Type 1	Fosfofructokinaza tip hepatic 1
PGK	PhosphoGlycerate Kinase	Fosfoglicerat kinaza
PHD	Prolin-hidroxilază	
PI	Propidium iodide	

PI-3K	Phosphoinositide 3-kinase	Fosfoinozitolkinazei 3
PMN		Polimorfonucleare
PON		Paraoxonaza
Pro-		Reziduu de prolină
PRR	Pattern Recognition Receptor	Modele de receptori de recunoaștere
PUFA	Polyunsaturated fatty acid chain	Acizi grași polinesaturați
pVHL	protein Von Hipple-Lindau	Proteina Von Hipple-Lindau
PYD	PYrin Domain	Domeniul pirinic
qRT-PCR	Quantitative RT-PCR	RT-PCR cantitativ
RCD	Regulated Cell Death	Moarte celulară programată/ reglementată
RE		Reticul endoplasmatic
RIP	Receptor interacting protein	Proteinelor de interacțiune cu receptorul
RLR	Retinoic acid– inducible gene (RIG)-I-Like Receptor	Similari receptorilor inductibili ai genelor acidului retinoic
RLU	Relative Light Unit	Unități de lumină relative, ce măsoară chemoluminiscența la citometria în flux
RNM		Recuperare Neuromusculară
ROS	Reactive Oxygen Species	Specii reactive de oxygen
RT-PCR	Real time PCR	PCR în timp real
SCP1	Synaptonemal complex protein 1	Proteinei complexului sinaptonemal 1
SCUBA		Spitalului Clinic de Urgență ”Bagdasar Arseni”
SDF-1	Stromal cell-derived factor 1	Factorul 1 derivat din celulele stromale
Sema	Semaphorin	Semaforină
SHH- wnt	Sonic hedgehog signaling	Semnalizare pe calea sonic heddgehog
SIRT2		Sirtuina 2
SMAC/ DIABLO	Small mitochondria-derived activator of caspases/ direct inhibitor of apoptosis-binding protein with low pI	Mici activatori ai caspazelor derivați din mitocondrii/ inhibitor direct al proteinei de legătură a apoptozei cu punct izoelectric (pI) scăzut
Smad1	Mothers against decapentaplegic homologue 1	Factor transcriptional
SOD1		Superoxid-dismutaza 1
Sox	Sulfur oxidation	Enzimă oxidantă a sulfului
SCI	Spinal Cord Injury	Traaumatism vertebra-medular
TAD	Transactivation domain	Domeniul de transactivare
TAK-1	Transforming growth factor-β-activated kinase 1	Kinaza activată de factorul de creștere transformant-β1
TDO	Tryptophan DiOxygenase	Triptofan dioxigenază

TEHBA	Teaching Emergency Hospital “Bagdasar-Arseni”	Spitalului Clinic de Urgență ”Bagdasar Arseni”
TFS		Tampon fosfat salin
TGF-β	Transforming Growth Factor beta	Factorul de creștere transformant-β
TIE1	Tyrosine kinase with immunoglobulin like and EGF like domains 1	Tirozin kinaza cu domenii asemănătoare imunoglobulinei și EGF 1
TIMP1	Tissue inhibitor of metalloproteinase-1	Inhibitorul tisular al metaloproteinazei 1
TLR	Toll-Like Receptor	Receptor Toll-asemănător
TNF-α	Tumor Necrosis Factor α	Factorului de Necroză Tumorală α
TRADD	TNF Receptor-Associated Death Domain	Proteine adaptoare asociate domeniului TNFR-1
TRAF2	TNFR-Associated Factor 2	Factorul 2 asociat TNFR
TRAIL	TNF-Related Apoptosis Inducing Ligand	Ligandul corelat cu apoptoza indusă de TNF-
TrkB	Tyrosine receptor kinase B	Kinaza receptorului tirozinei B
TVM		Traumatism Vertebro-Medular
U87 MG		Linie celulară de glioblastoma
UPR	Unfolded protein response	Răspunsul proteinelpr neplicaturate
UV		Ultraviolete
VEGF	Vascular Endothelial Growth Factor	Factorul de creștere endotelial vascular
VEGF-R	VEGF- Receptor	Receptor VEGF
Wnt	Wingless/Integrated	Cale de semnalizare celulară
Wnt/PCP	Wnt/Planar Cell Polarity	
XBP1	X-box binding protein 1	Element component ERSR
XIAP	X-linked inhibitor of apoptosis protein	Inhibitorii proteinelor apoptotice X-linkați

List of personal published works

1**. Simona Isabelle Stoica**, Ioana Tănase, Vlad Ciobanu, Gelu Onose

”*Initial researches on neuro-functional status and evolution in chronic ethanol consumers with recent traumatic spinal cord injury.*”

J Med Life. 2019 Apr-Jun;12(2):97-112. doi: 10.25122/jml-2019-0026. PMID: 31406510; PMCID: PMC6685305. https://pubmed.ncbi.nlm.nih.gov/31406510

2. **Simona Isabelle Stoica**, Ioana Tănase, Gelu Onose

”*Influences and consequences resulting in addictions in general and to chronic alcoholism, especially for patients with spinal cord injury* ”

Balneo and PRM Research Journal. Vol.12, No.2 June 2021 p: 129–132 http://dx.doi.org/10.12680/balneo.2021.432

3. **Simona Isabelle Stoica**, Coralia Bleotu, Vlad Ciobanu, Anca Mirela Ionescu, Irina Albadi, Gelu Onose, Constantin Munteanu

”*Considerations about Hypoxic Changes in Neuraxis Tissue Injuries and Recovery*” Biomedicines. 2022 Feb 18;10(2):481. doi: 10.3390/biomedicines10020481. https://www.mdpi.com/2227-9059/10/2/481

4. **Simona Isabelle Stoica**, Gelu Onose, Mihail Hoteteu, Constantin Munteanu

”*Effects of ethanol and deferoxamine on rat primary glial cell cultures, in regard with ischemia induced by traumatic spinal cord injury*”

Balneo and PRM Research Journal. 2022, 13(2): 502 **http://bioclima.ro/Balneo502.pd**

5. **Simona Isabelle Stoica**, Ioana Madalina Pitica, Ana Iulia Neagu, Laura Denisa Dragu, Lacramioara-Elena Radu,Lilia Matei, Mihaela Chivu-Economescu, Gabriela Ion, Laura Georgiana Necula, Constantin Munteanu, Aurelian Anghelescu, Gelu Onose, Carmen Cristina Diaconu, Coralia Bleotu

” The hypoxic stress effect in chronic ethanol exposure of neuronal cells”

Int. J. Mol. Sci. 2022, 23, x FOR PEER REVIEW

INTRODUCTION

This PhD thesis comes to the meeting between problems of the modern world (where the active lifestyle predisposes to severe accidents in the conditions of the technological leap) and the consequences of addictive food choices (which have influenced human society since ancient times).[1]. In these conditions, a pathology with increasing frequency is that of vertebral-medullary traumatology, which affects all population categories, with the risk of dysfunction on multiple levels: individual, family, social [2] [3].

We were contradicted by the observations regarding the case report from RNM of the THEBA regarding frequent differences between the objective neuro-dysfunctional state after spinal cord injury (SCI) between patients known to abuse chronic ethanolic and those who did not have such behavioral disorders[4]. Thus, with the approval of the Ethics Committee of the Hospital (no. 35517/25.11.2015), we initiated the clinical study related to this doctoral thesis with the aim of researching the relationship between alcoholism and neuro-dysfunctional and evolutionary recuperative, neuromuscular status after SCI.

The working hypothesis refers to the objective-statistical verification and, as far as possible, through paraclinical investigations including at the intimate tissue and molecular level of the positive (beneficial) influence of chronic alcoholism on the evolution (acute and subacute) of the neuro-dysfunctional clinical status in patients with SCI, where chronic ethanol abuse is known to cause structural and functional damage in nervous tissue.

The general objective of this doctoral study is the analysis and attempt to explain the above-mentioned, somewhat surprising, phenomenon in the context of the verification of the Working Hypothesis.

The specific objectives are:

- the objectification of the statistical significance of the clinical finding from the working hypothesis (through the retrospective analysis of the cases of patients with SCI, hospitalized in the RNM Department of THEBA between 01.01.2005 and 01.06.2022);
- the prospective attempt to explain and identify, through fundamental research approaches, the intimate mechanisms possibly involved in the generation of the (paradoxically) more favorable evolution of myelic tissues under conditions of aggression, in immortal tumor cell cultures;

- the prospective attempt to explain and identify, through fundamental research approaches, the intimate mechanisms possibly involved in the generation of the (paradoxically) more favorable evolution of myelic tissues under conditions of glial cell aggression from primary (rat) cell cultures.

The work is structured in two sections:

- The general part that reviews current scientific data on the researched fields (spinal cord injury, the influences of chronic ethanol impregnation on the human body in general and on the central nervous system - with a main focus on the spinal cord - in particular and a brief systematization of the main mechanisms related to the molecular biology of after SCI injury events, and their interference with neurofunctional clinical evolution, including recovery in the stages from acute to subacute).
- The personal contributions include a clinical-statistical retrospective analysis of the studied problem and a prospective experimental research of the dynamics at an intimate level in neuronal and gliocyte biology, including from the spinal level in experimental conditions with elements prone to the reproduction of conditions similar/partially similar to those in the spinal cord traumatic lesions.

The first step was to document our clinical observations by evaluating the state of knowledge of the issue at the level of the international scientific community [4]; then we carried out an exhaustive retrospective clinical study of patients hospitalized in the THEBA RNM Department with diagnoses of tetraplegia or paraplegia post-SCI (from the archive of the mentioned Clinic in the period 01.01.2005- 01.06.2022) creating a database that includes 1057 such cases , where we performed the statistical analysis of the objective data extracted from the clinical observation sheets of the respective hospitalized persons, including using the clinical-functional evaluation parameters quantified by the specific measurement scales/grids (AIS, Frankel). The retrospective analysis of the data related to the 1057 patients revealed to us that they do not present a normal distribution (taking into account the limiting selective criteria applied), but there are statistically significant correlations between chronic ethanol abuse and the status, as well as the favorable evolution, from the point of view motor vision, as well as the yield of motor and sensory recovery in such spinal cord injury patients.

Later, we carried out a prospective study on neuronal and glial tumor cell lines (in the Department of Molecular and Cellular Pathology of the "Ștefan S. Nicolau" Institute of Virology) on models of cellular suffering (through mechanical trauma and hypoxemic interventions) in neural cell cultures HTB-11 and U87 MG tumors treated excessively with ethyl alcohol and corresponding in time to alcoholism. We observed morphological differences between neural cells (neurons and gliocytes) grown with and without ethanol treatment, as well as between cells exposed and not exposed to traumatic conditions (through scratching and hypoxia) but without these structural differences jeopardizing cell survival. Then we analyzed (comparing between cells exposed and not exposed to ethanol treatment) the molecular response by studying the chronological post-traumatic cellular events (as known from the literature; initially, after the occurrence of a vertebral-medullary trauma, cellular necrosis processes take place, which - by affecting the membrane excitoconductivity - it causes the efflux of potassium in the extracellular environment - with impact depolarization - and the influx of calcium, followed by the excess production of glutamate, which causes a state of local hyperexcitability, which - along with ischemia through bleeding and vaso- spasticity - causes hypoxic changes, which quickly induce metabolic dysfunctions and local oxidative stress, with the activation of the innate immune response at the inflammasome level, with a defensive role, being followed by the initiation of apoptotic mechanisms - involved in both neuroprotective and neurodegenerative processes - as well as influencing molecules of cellular signaling, with the functional reactivation of some embryonic neural pathways). The molecular biology evaluation was carried out in the proteosome conceptual paradigm, by evaluating gene expression (at messenger RNA level) and protein synthesis, specific to the posttraumatic chronological stages described above. Gene expression was assessed by real time polymerase chain reaction (RT PCR) and protein synthesis was quantified by the DotBlot technique, which used specific antibodies. The cell cycle was also evaluated by flow cytometry and the images of the cell cultures were made under an inversion and fluorescence microscope (after special stainings).

Ethanol-impregnated cells (although having a lower resistance to stress) showed regenerative capacity to refill gaps in the scratch test similar to untreated ethanol-treated cells.

Evaluation of the response to hypoxia (targeted by molecular markers: Hypoxia Inducible Factori-1alfa – HIF-1α, Nuclear Factor Kappa-light-chain-enhancer of activated B cells-beta - NFk-β, Vascular Endothelial Growth Factor- VEGF, Fms related receptor tyrosine kinase -1 – FLT-1, Transforming Growth Factor beta - TGF-β, Protocaderine12 - PCDH12, Hemoxigenase1 - HMOX1, Hemoxigenase2 - HMOX2, Carbonic Anhydrase 9 - CA9, Hypoxia Inducible Factori-2alfa - HIF-2α, Indoleamine 2,3-DiOxygenase - IDO) of neuroblastoma cells exposed to chronic ethanol treatment showed that the following effects of ethyl alcohol occur: reduction of neurodegeneration and neurotoxicity functional improvement of the response/metabolic-functional resilience of neurons, therefore neuroprotective, anti-inflammatory, anti-apoptotic, angiogenesis, of synaptic remodeling/neuroplasticity.

Cell stress study (through the molecules: interleukin-1β - IL-1β, TNF-α - Tumor Necrosis Factor, interleukin-6 -IL-6, interleukin-8 -IL-8, interleukin-4 -IL-4, Catalase, Claspin , Clusterin, Hat Shock Protein27- HSP27, Hat Shock Protein60 - HSP60, Hat Shock Protein70 - HSP70) showed that chronic exposure to ethanol induces (on neural cells treated with hypoxemic agents) the following effects: antioxidant, adaptive regulation of energy metabolism, neuroprotective, anti-inflammatory, antiapoptotic, anti-scarring, nerve regeneration inhibitor as a defensive response, to restore nerve endings, to inhibit axonal dieback phenomena.

Cell stress study (through the molecules: interleukin-1β - IL-1β, TNF-α - Tumor Necrosis Factor, interleukin-6 -IL-6, interleukin-8 -IL-8, interleukin-4 -IL-4, Catalase, Claspin, Clusterin, Hat Shock Protein27- HSP27, Hat Shock Protein60 - HSP60, Hat Shock Protein70 - HSP70) showed that chronic exposure to ethanol induces (on neural cells treated with hypoxemic agents) the following effects: antioxidant, adaptive regulation of energy metabolism, neuroprotective, anti-inflammatory, antiapoptotic, anti-scarring, nerve regeneration inhibitor as a defensive response, to restore nerve endings, to inhibit axonal dieback phenomena.

Chronic ethanol treatment of immortal neuronal cultures (of neuroblastoma), exposed to hypoxemic experimental conditions, produced the following consequences at the level of cellular carbohydrate metabolism (through the molecules: Adenylkinase3 - AK3, Fetuin-B - FETU-B, Phosphoglycerate Kinase - PGK1, Proprotein Convertase Subtilisin/Kexin type 1 - PCSK1, PhosphoFructoKinase, Liver Type 1 - PFKL1, High Mobility Group Box 1 - HMGB1, Glucose transporter 1 - GLUT1, Tyrosine kinase with immunoglobulin like and EGF like domains 1- TIE1, Lactate DeHydrogenase B - LDH B): energy depletion and increase in lactic acid level, but also stimulation of the proteolytic activity of insulin, improvement of the yield of glucose use, favoring its intracellular penetration, and (including) vascular remodeling.

Apoptosis study (through the molecules: Caspases -3, -7, -8, -9, -10, HTRA2/Omi, Livin, p21, p27/Kip1, Phospho-p53 (S15), Phospho-p53 (S46), Phospho- p53 (S392), Phospho-Rad17 (S635), Small mitochondria-derived activator of caspases/ direct inhibitor of apoptosis-binding protein with low pI - SMAC/Diablo, Survivin, Tumor Necrosis Factor Receptor - TNF R, X-linked inhibitor of apoptosis protein – XIAP, Bad, Bax, Bcl-2, Bcl-x, Pro-Caspase-3, Cleaved Caspase-3, Catalase, Inhibitor of apoptosis proteins-1 - cIAP-1, Inhibitor of apoptosis proteins 2 - cIAp-2 , Claspin, Clusterin, Cytochrome C, TNF-Related Apoptosis Inducing Ligand1 -1- TRAIL R1, TNF-Related Apoptosis Inducing Ligand2 - TRAIL R2, Fas-Associated Death Domain - FADD, Fas) showed that chronic exposure to ethanol causes antiapoptotic effects (in the extrinsic and intrinsic way), including in conditions of hypoxemic interventions.

Evaluation of intercellular signaling molecules (Laminin4 - LAMA4, Chitinase 3–like protein 1 - CHI3L1, Lipocalina2 - LCN2, Tissue inhibitor of metalloproteinase-1 - TIMP1, Semaphorin3 - SEMA3) and embryonic communication pathways (Sonic hedgehog signaling - SHH, Prominin -1 - CD133, Neurogenic locus notch homolog protein1 - Notch1, Hairy Ears, Y-linked – Hey, Bone morphogenetic proteins 4 - BMP4, Hairy/enhancer of split – Hes, Wingless/Integrated – WNT, Caderina 2 - CDH2, Vimentina – Vim, Nestina – Nestin, Neuronal differentiation 1 - NeuroD1, Glial fibrillary acidic protein - GFAP, Oligodendrocyte transcription factor 2 - Olig2) in neurons (neuroblastoma type) chronically treated with ethyl alcohol revealed the following consequences: reduction of neuraxial scars; stimulation of neurite development, vascular remodeling, neurogenesis, synaptogenesis, neuroregeneration; increasing the ability to differentiate into neurons or glial cells.

As the study on tumor cells presents the great disadvantage of significant genetic and metabolic differences, compared to non-tumor myeloid cells, we carried out (in the RNM SCUBA Research Core) another prospective study on the relationship between ethanol exposure and (spinal-)traumatic heart suffering in cell cultures newborn rat primaries cultured in ethanol exposure (trying to reproduce, as far as possible, the temporal correspondence with chronic alcoholism in humans). The analysis was carried out by evaluating the protein synthesis (using the ELISA test) at the level of the inflammasome (with the evaluation of IL-6) and the apoptosis process (by determining TNF-α) in the cells of primary cerebro-spinal glial cultures from the rat. We observed that, in primary cultures with such glial cells, the stress induced by ethanol treatment, supplemented by hypoxia, accentuates inflammatory intercellular communication, but without leading to marked levels of programmed cell death.

The last aspect of our research concerned the prospective analysis of phenomena at the human biological level, which is why we analyzed the distribution of cytokines (inflammatory and proapoptotic) in the cerebrospinal fluid collected from several patients with damaged dura mater following a SCI (possible collection for bioethical reasons only in such situations, which explains the small number of these cases).

Experimental evaluation of CSF was performed by electrophoresis and ELISA method for TNF-α and IL-6. The analysis of CSF in patients chronically consuming excess ethanol revealed the presence of pro-inflammatory and pro-apoptotic tendencies, possibly including as a mechanism of isolation and protection of the injured medullary area, of course the number of cases studied being insufficient for processing and statistical analysis.

Overall, this doctoral thesis addresses a subject we consider particularly interesting and encountered only tangentially - concretely we did not find it studied from the perspective of our working hypothesis - in the literature, which, especially under the conditions in which, unfortunately, in currently there is still no form of therapeutic intervention able to effectively heal spinal cord injuries, including traumatic ones (their neurodysfunctional consequences are not infrequently devastating due to their huge disabling potential, often for life), opens a way to explore a field which, under the conditions of additional deepening, could lead to the identification of contributory therapeutic-restorative solutions.

I. GENERAL PART

1. General data on spinal cord injury

SCI "represents the cause of a plegic deficit, with temporary or permanent loss of nervous control over a somatic and vegetative territory", caused by damage to the spine and spinal cord (and spinal nerves), after: acts of violence, road accidents, falls, sports accidents[4] [5] [6]. The incidence of TVM is 10.4–83 cases/million patients/year (ie approximately 768 473 annual cases), affecting mostly patients in the age group of 30 years [7], being a complex challenge (through its individual, family consequences and social) [8] [9]. Mortality after SCI is directly proportional to the height of the lesional level (spinal cord), the advanced age of the victims and the force of the traumatic impact [9].

In the body, SCI produces various changes: represented by primary injuries (the consequences of the traumatic impact force on the spine: flexion, extension, rotation, compression; all affecting the spinal cord, causing medullary contusion, with transient or permanent neurological dysfunctions, depending on the severity insult) and secondary (in which the nerve suffering from the initially injured area spreads around, especially through the ischemic phenomena produced by vascular injuries, capillary thrombosis, vasospasm and edema; with the appearance of a significant local metabolic and energetic imbalance) [8] [9].

The etiological treatment of traumatic vertebral-medullary disorders is still in the research stage, without known therapeutic molecules capable of inducing the disappearance of motor deficits and sensitivity disorders following SCI.

Recovery after vertebral-medullary traumas is an arduous process (based on the spinal resources of the reorganization of the nervous structures that have survived post-traumatic), of long duration most of the time; being the result of the work of the specialized team (consisting of: recovery doctor, physiotherapist, clinical psychologist, medical assistant, nurse, stretcher bearer, occupational therapist, joined by the patient's family members) by promoting the patient's survival, with the adoption of a lifestyle adapted to the new conditions [11] [12].

2. Chronic alcoholism and its physiopathological consequences, respectively complex, in spinal cord injuriy

Alcohol (ethyl) is perhaps the cheapest and most used antidepressant by many people over time. The consequences of this fact are multiple, taking on the dimensions of a societal problem. Our brains have reward and tolerance pathways that quickly turn occasional ethanol consumption into a vicious lifestyle. Ethyl alcohol affects the development of the human body in all its stages, starting from intrauterine life, influencing individuals of all age categories, sex, lifestyle or other comorbidities.

We define chronic alcoholism as a long exposure (greater than 7 days/two weeks) to high doses of ethanol: more than 8 standard daily ethanol units (standard drink per day = 10 mg ethanol)[26] [27].

From a molecular point of view, the structures of ethanol and polyethylene glycol (a molecule researched in the treatment of SCI) have many physicochemical properties in common, as they are part of homologous series of the class of alcohols.

Tabel.1. 1. Physico-chemical and structural characteristics of ethanol and PEG (taken from own article [3]) [42,43]

ALCOHOLS/ POLYOLS	**ETHANOL (ethyl alcohol, metilcarbinol, ethyl hydroxyl, ethane monoxide, etiolte, hydroxyethane)**	**POLYETHYLEN GLYCOL (oxid de polyethylen oxide, polyoxyetilene)**
Method of production	Fermentation of cereallo/ fruits/ some plants	Industrial biosynthesis procedures
Physical propreties	Liquid, volatile, flammable, characteristic smell	Molecular weight variables: liquid/ solid
Structure	CH3-CH2-OH	H-(O-CH2-CH2)n-OH
Molecular weight	46,076844 g/mol	18.02 + 44.05 ng/mol (300-10000000 g/ mol)
Density	0.7894 g/cm (at 25 Celsius degrees)	Variable
Boiling point	78,37 Celsius degrees	182 – 287 Celsius degrees
Freezing point	-114 ,4 Celsius degrees	Variable
Chemical properties	Water-soluble alcohol	Water-soluble alcohol, methanol, ethanol, acetonitrite, benzene, dichlormetane, acetone. Insoluble in diethyleter, hexane
Biological Effects	It crosses the blood-brain barriere, euphoric, sedative, anxiolytic/ antidepressant, antiseptic, solvent, antitussive, antidote, fuel (7 kcal/gram)	Osmotic laxative, coating film for drug molecules, vector in genetic therapy

Tabel.1.2. Pharmacokinetics of ethanol and PEG (taken from own article [3]) [43–47]

ALCOHOLS/ POLYOLS	**ETHANOL**	**POLYETHYLEN GLYCOL (PEG 400/2000 / 5000)**

Absorbption	Stomach, small intestine Peack of blood concentration in 30- 60 min	Intravenous use
Distribution	Anywhere in the body Rate of extraction = 0.2 There are no plasma transport proteins Target: liver, nevrax, heart	0.07% per gram of neural tissue
Elimination	Limited to the maxiumm 8.5 g/h/70 kg Liver metabolism: *alocoholdehidrogenase, *aldehyddehidrogenase Expiratory (0.7%) - 0.16 L/h Urinary (0.3%) - 0.06 L/h Sweting (0.1%) - 0.02 L/h	PEG 2000- 1.4 ml/ min/ kg PEG 5000- 0.4 ml/min/kg

Tabel. 1.3. Pharmacodynamics of ethanol and PEG (taken from own article [2]) [43,47]

ALCOHOLS/ POLYOLS	ETHANOL	POLYETHYLEN GLYCOL
Mechanism of action	NMDA receptor antagonist (Decrease of neurotoxcity due to NMDA receptor antagonist) GABA- A receptor agonist 5HT3 agonist Stimulate opiate receptors and production of dopamine	Decreases intracelluarrelease of oxygene free radicals; stabilizes cell membranes against lipid peroxidation; limits injury seconday membrane lesions
The acute phase is subdivided into an immediate phase (in the first 0-2 hours) and an early acute phase (at 2-48 hours posttraumatic).	The acute phase is subdivided into an immediate phase (in the first 0-2 hours) and an early acute phase (at 2-48 hours posttraumatic).	The acute phase is subdivided into an immediate phase (in the first 0-2 hours) and an early acute phase (at 2-48 hours posttraumatic).

3. Molecular biology mechanisms in spinal cord injury and data on current therapeutic possibilities

Spinal cord injury is defined as an acute injury event, occurring at the level of the spinal cord and its adjacent structures. Post-traumatic changes are manifested from the initial stage, which extends between 2-48 hours post-injury [48]. The following stages after SCI are described: acute phase (0-48 hours after SCI), subacute (between 2 days and 2 weeks post-traumatic) and chronic (following the subacute phase) [49]. In the immediate acute phase, axonal ruptures, hemorrhages in the gray matter, local ischemia, neuronal death occur. Now the microglia are activated, with the release of TNF-α, IL-1β (even from the first minutes after the injury) and with the production of glutamate in cytotoxic amounts [50].

The structural and functional changes induced by SCI originate in nerve cell dysfunctions at different levels, starting with the process of cell division (in the case of glocytes and neural stem cells).

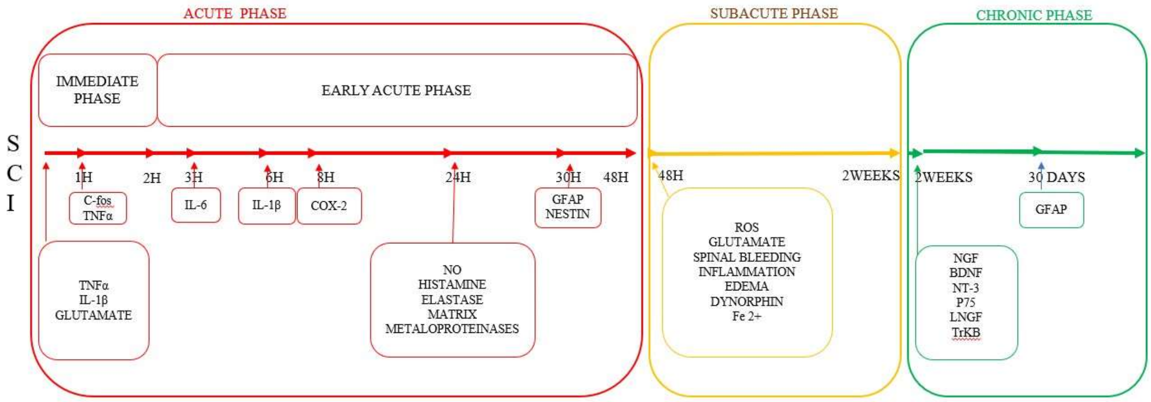


Fig. 1.1. Evolutionary phases after SCI and molecular changes produced in nervous tissue. The following stages are distinguished according to SCIS: the acute phase (in the first 48 hours post-traumatic) consists of the immediate phases (which happens in the first 2 hours after SCI) and the early acute phase (between 2 hours and 48 hours post-traumatic), the subacute phase (with a duration of 2-14 days after SCI and the chronic phase (which begins after the first 2 post-traumatic weeks). At the time of TVM production, the following are released (in the nerve tissue): TNFα, IL-1β, glutamate; one hour post-traumatic, the following are released: c-fos, TNFα; 3 hours after TVM: IL-6 is released; at 6 hours IL-1β is released; at 8 hours: COX-2 is produced; and at 24 hours: nitric oxide, histamine, elastase are synthesized , matrix metalloproteinases; while 30 hours post-traumatic: GFAP and nestin are produced; 48 hours after TVM are produced (on the background of spinal bleeding and local edema): reactive oxygen species, dynorphin and ferric ions; after 28 days posttraumatic are produced: NGF, BDNF, NT-3, LNFG, TrKB, and 30 days after the SCI, GFAP is synthesized.

Hypoxia is defined as the decrease or deprivation of oxygen at the level of organs, tissues, cells by decreasing the oxygen supply (due to damage to the vascular network, anemia) or by increasing oxygen consumption (as in the sudden increase in the rate of cell proliferation)[58].

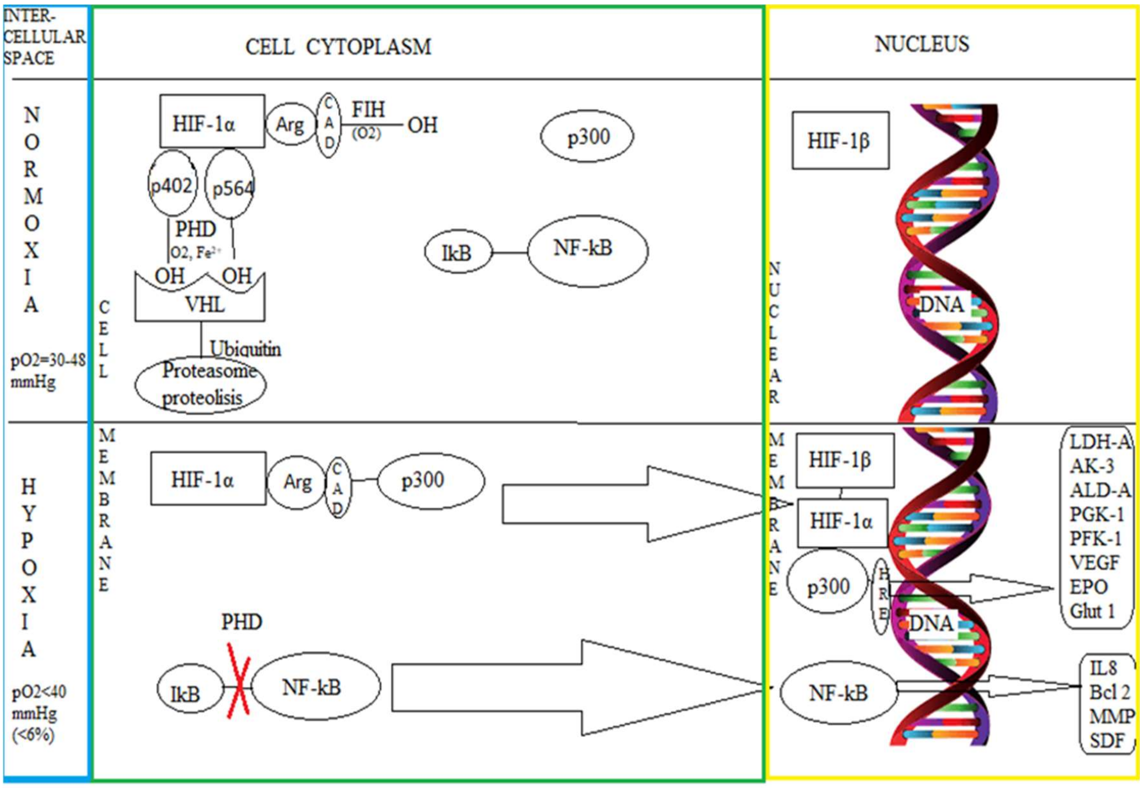


Fig. 4.1. Hypoxic cellular mechanisms with the influence of oxygen partial pressure, pO2, on deoxyribonucleic acid, DNA, HIF, with participation, FIH; ARG; CAD; protein 300, p300, VHL and PHD. We observe the activation (on the atypical pathway) of NF-kB (with the involvement of I-kb), of HRE with the stimulation of gene expression: LDH-A; AK-3; ALD-A; PGK-1; PFK-L; VEGF; EPO; Glut-1; IL-8; Bcl-2; MMP; SDF (drawing taken from own article[209])

The inflammasome is a multiprotein cytosolic complex that functions as an intracellular receptor for cellular and environmental stress [98] mediating the innate immune response, which can cause tissue damage when overactivated [99] [100].

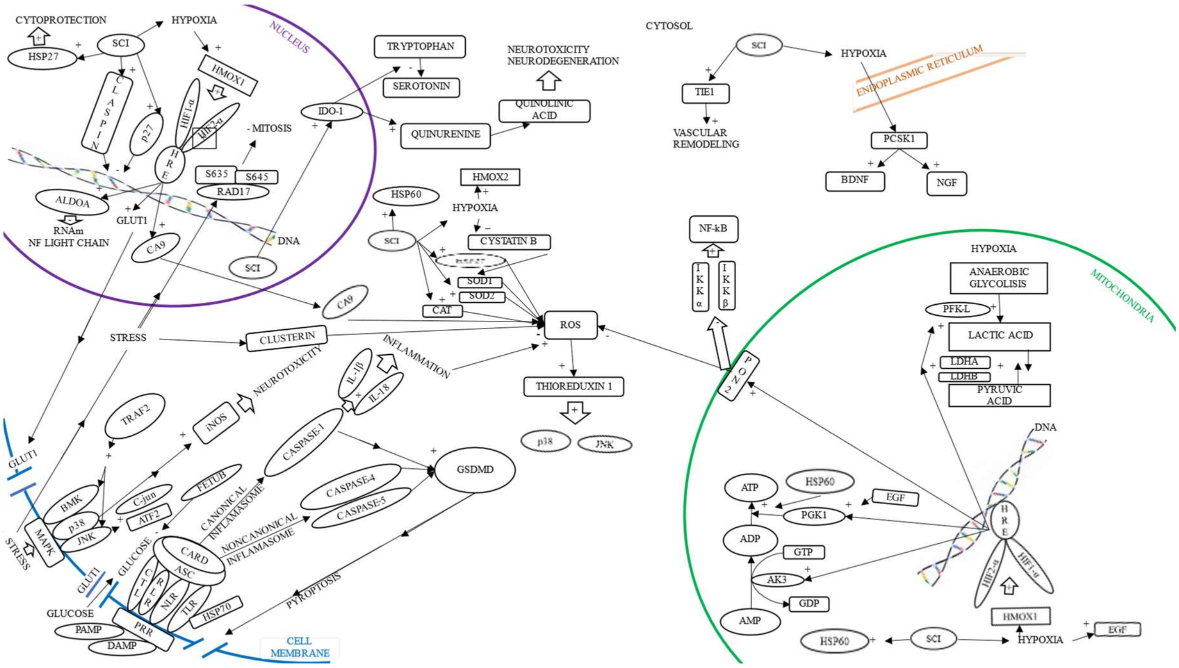


Fig. 1.2. Baseline metabolic and inflammatory changes after SCI. Events in SCI lead to the appearance of DAMPs and PAMPs (in the intercellular space), which interact with PRRs, then with CTLs, RLRs, NLRs and TLRs (whose activity is also controlled by HSP70), which causes the activation of ASCs (and the CARD of its interior), followed by caspase interaction: with caspase-1 (which leads to inflammation by stimulating IL-1β and IL-18, via the canonical inflammasome activation pathway) or with caspases -4 and -5 (which stimulate GSDM and pyroptosis on the non-canonical inflammasome activation pathway). The stress produced by SCI can also activate membrane MAPK, which then initiates the p38 (with stimulation of iNOS leading to neurotoxicity) and BMK (which is stimulated by TRAF2) and JNK (activated by TRAF2, which stimulates cjun and ATF2) pathways. The stress produced after SCI can also act at the nuclear level, via RAD17 (which is phosphorylated at S635 and S645, inhibiting mitotic activity). Also at the nuclear level, SCI can lead to the transcriptional activation of IDO-1 which can inhibit the synthesis of serotonin from tryptophan or stimulate the quineric acid pathway (with the risk of producing neurotoxicity and neurodegeneration). But stress can also activate cytoplasmic clusterin which can inhibit ROS (with an antioxidant role). Activated ROS can stimulate thioreduxin1, which can stimulate JNK and p38 proteins in the MAPK pathway. SCI at the nuclear level can also stimulate the synthesis of HSP27 (with a cytoprotective role), claspin and p27 (which inhibits DNA activation). And hypoxia following SCI influences DNA (nuclear and mitochondrial) by stimulating HMOX1, which causes increased levels of HIF-1α and HIF-2α, which then activates HRE, which then causes synthesis of ALDOA (with stimulation of neurofilament light chain mRNA), of GLUT1 (which favors the entry of glucose inside the cell, and FETUB produces resistance to the action of glucose), of CA9. At the mitochondrial level, SCI stimulates HSP60, and the subsequent hypoxia leads to the stimulation of HRE that controls cellular energy metabolism by being involved in ADP phosphorylation (stimulating AK3), in ADP phosphorylation (stimulating PGK1), in lactic acid production (through PFK-L synthesis), in bilateral lactate-pyruvate conversion (through the synthesis of LDHA and LDHB). Mitochondrial hypoxia also stimulates EGF with possible consequences in vascular remodeling. And HRE also stimulates PON2, resulting in NF-κB activation (by inhibiting IKKα and IKKβ). On the other hand, SCI can inhibit ROS by stimulating SOD1 and SOD2, CAT. At the cytosolic level, SCI can stimulate TIE1 (involved in stimulating vascular remodeling); and at the level of the endoplasmic reticulum, it increases the production of BNDF and NGF (via PCSK1).

Cell death, according to the classification of the Nomenclature Committee on Cell Death (NCCD) from 2018, can be of two types: accidental (accidental cell death - ACD) and programmed/regulated (regulated cell death - RCD)[120][9]. ACD is a form of instantaneous cell death produced by the disintegration of the cell membrane through the action of aggressive factors (physical, chemical or mechanical), and RCD is the consequence of the activation of one or more cellular signaling pathways (which can be influenced pharmacologically) [9][120].

After SCI, cell death occurs through both pathways: ACD and RCD [120]. Neural cell death is achieved by: apoptosis, necrosis, necroptosis, ferroptosis, pyroptosis, autophagy, paratantosis, oncosis [121][122] [123] [55] [9][120][124].

Apoptosis is a highly regulated, non-inflammatory process of programmed cell death essential for permanent renewal (at the cellular and whole-organism level)[125] [124] [126].

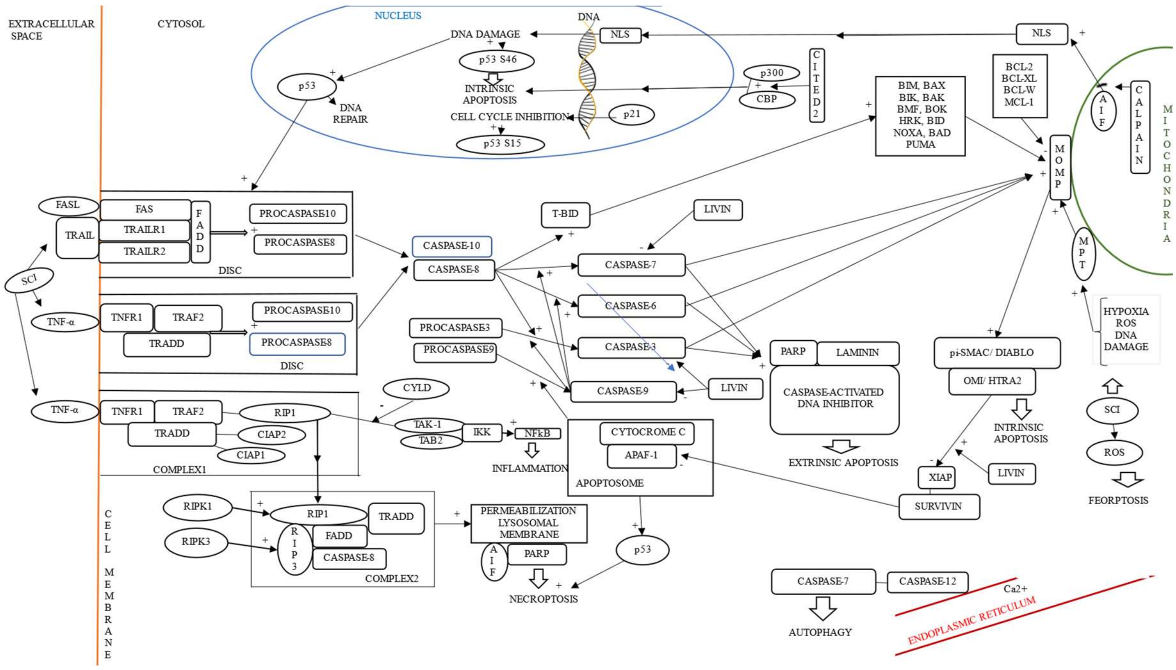


Fig. 1.3. Pathways of cell death in SCI. SCI activates apoptosis through the extrinsic and intrinsic pathway. Thus, FASL and TRAIL act on the DISC (consisting of FAS, TRAILR1 and TRAIL2 that interact with FADD, activating procaspase-8); similar to TNFα acting on the DISC (consisting of TNFR1, TNFR2 interacting with TRADD, activating procaspase-8). DISC then activates caspase-8 which stimulates effector caspases (-3, -6, -7), which act on PARP, laminin, and caspase-activated DNA inhibitor, triggering apoptosis via the extrinsic pathway. Caspase-8 (via T-BID) stimulates proapoptotic genes (BIM, BAX, BIK, BAK, BMF, BOK, HRK, BID, NOXA, BAD, PUMA) which, together with MPT (whose expression is stimulated by hypoxia, ROS and DNA damage) activates MOMP which (mediated by pi-SMAC/ DIABLO and OMI/ HTRA2) triggers the intrinsic pathway of apoptosis and inhibits XIAP which (together with survivin) inhibits APAF-1 (which makes up the apaoptosome together with cytochrome C). SCI, via ROS, also stimulates ferroptosis. The apoptosome activates necroptosis (mediated by p53) and stimulates the transformation of pocaspase-9 to caspase-9 which stimulates the effector caspases of the extrinsic apoptotic pathway (-3, -6, -7) that also activate MOMP (being also involved in the intrinsic apoptotic pathway). The balance in the intrinsic apoptotic pathway is maintained with the help of antiapoptotic genes (BCL-2, BCL-XL, BCL-W, MCL-1) and livin protein (which inhibits caspases -3, -7, -9 and stimulates XIAP). TNFα

also activates inflammation by acting on inflammasome complex 1 (consisting of TNFR1, TNFR2, TRADD, RIP1, CIAP1, CIAP2) which (together with RIPK1 and RIPK3) acts on inflammasome complex 2 (consisting of RIP1, RIP3, FADD, TRADD and caspase-8). Inflammasome complex1, via TAK-1, TAB2, IKK (inhibited by CYLD) stimulates NF-kB triggering inflammation. Inflammasome complex2, by permeabilizing the lysosomal membrane and AIF, PARP triggers necroptosis. Mitochondrial AIF causes activation of cytoplasmic NLS (which causes nuclear DNA damage); and the action of AIF is inhibited by calpain. SCI can produce nuclear DNA damage that stimulates p53 expression (with activation of DNA repair but also with activation of the extrinsic apoptotic pathway) and phosphorylation of p53 at serine 46 (with activation of apoptosis via the intrinsic pathway). Phosphorylation of p53 at serine 15 can be caused by cell cycle inhibition under the action of p21. The intrinsic apoptotic pathway is also activated by the action at the DNA level of the p300-CBP complex (stimulated by cited-2). The endoplasmic reticulum can also be involved in apoptosis, which can attach caspase-12 and caspase-7, triggering the autophagy process.

Looking at posttraumatic neuraxial structural changes, at a microscopic level, the central nervous system is a complex structure made up of cells that are embedded in a soft network of polysaccharides (consisting of proteins and glycosaminoglycans, the best known being: laminin, fibronectin and collagen IV)[138].

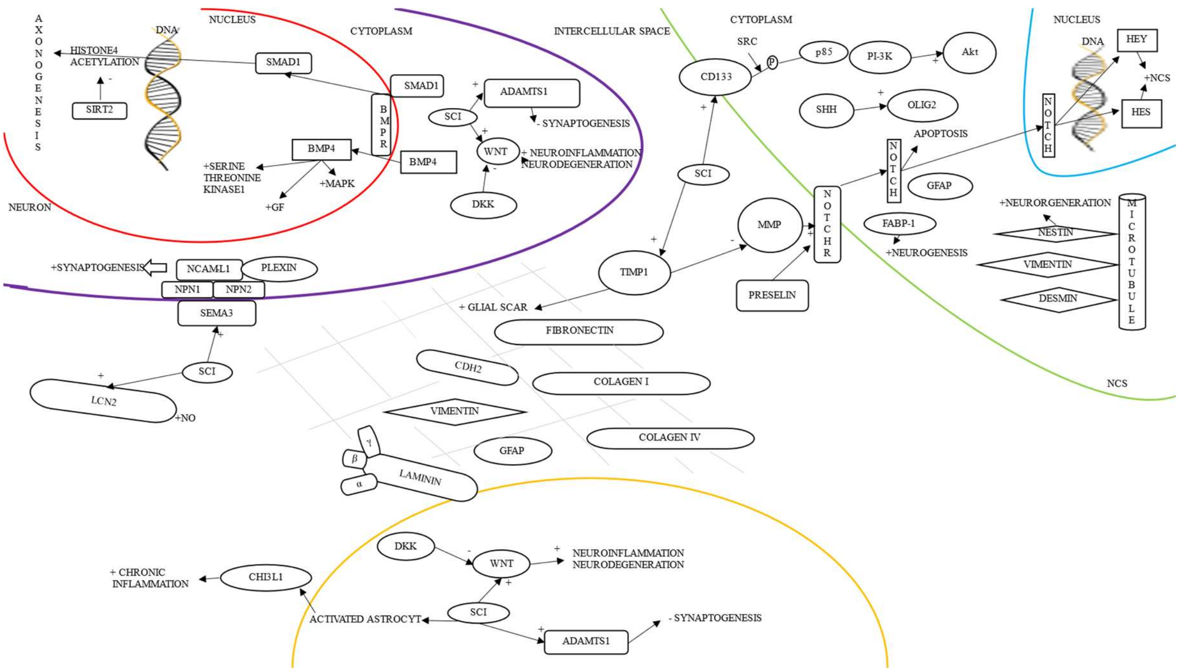


Fig. 1.4. Intercellular signaling after SCI. SCI activates the astrocytic cell which: synthesizes CHI3L1 (promoting chronic inflammation in the intercellular space), stimulates ADAMST1 (with inhibition of synaptogenesis), activates the WNT pathway (leading to neuroinflammation and neurodegeneration, being inhibited under the action of DKK). At the neuronal level, SCI activates the WNT pathway, stimulates ADAMST1, reactivates the SEMA3 pathway (which binds to NPN1, NPN2, NCAM-1 and PLEXIN receptors, stimulating synaptogenesis), acts on BMP receptors via BMP4 (with stimulation of MAPK, increase of serine threonine kinase1), of SMAD1 (with histone 4 acetylation that determines axonogenesis, a process inhibited by SIRT2). SCI also influences neural stem cells by activating: membrane CD133 (which, together with SRC, phosphorylates p53 and PI-3K which activates Akt), TIMP1 (which inhibits MMP, which together with preselin acts on NOTCHR activating the NOTCH pathway which causes apoptosis or activation of Hes and Hey genes causing further activation of NCS), microtubule interaction with vimentin, desmin and nestin (promoting neurodegeneration), FABP-1 (stimulating neurogenesis), SHH (activating OLIG2) and

GFAP. At the level of the intercellular space, SCI stimulates the formation of glial scars (under the action of TIMP1) which are composed of: laminin (with α, β, γ chains), GFAP, collagen I and IV, fibronectin, CDH2 and vimentin; scar that limits post-traumatic nerve regeneration, but also diminishes the local inflammatory process.

Different medicinal substances and therapeutic methods are being studied with a potentially beneficial role in ameliorating the deficits that occur after the occurrence of SCI. Substances such as: non-steroidal anti-inflammatory drugs, group B vitamins, lithium, antioxidant molecules (vitamins A, E, C, coenzyme Q10), iron chelating molecules (deferoxamine), statins, inhibitors are considered to favor the neurorecovery process (including after brain trauma) of phosphodiesterase (methylxanthines - theophylline), acrolein antagonists (acetylcysteine acid), neurite growth promoters (Keltican), neurotrophic molecules (Cerebrolisyn, Actovegin), erythropietin, neuronal antiexcitatory molecules (Memantine, Riluzole), calcium channel blockers [20] [6]. At the same time, research is being done on a class of therapeutic molecules with a positive effect in the recovery of S(C)-I: that of polyethylene glycol (PEG). PEG applied to the site of spinal injury (immediately and 8 hours post-injury) in severe S(C)I – produced by spinal cord compression [195] – in adult guinea pigs determined very good neuro-functional consequences (in 23 out of 25 treated animals intralesional, intravenous and subcutaneous with posttraumatic PEG), by a protective effect against damage to axonal membranes in spinal neurons of experimental animals[196–198].

II. PERSONAL CONTRIBUTIONS

4. Working hypothesis and general objectives

The clinical experience acquired after long periods of work with vertebral-medullary traumatized patients led us to note clinical-evolutionary differences after SCI (between acquaintances with chronic ethanol abuse and those with a normal diet, which led us to initiate a research detailed description of the issue (in a doctoral study).

The working hypothesis of this doctoral thesis refers to the positive (beneficial) influence of chronic alcoholism on the evolution (acute and subacute) of the clinical condition in patients with SCI, because chronic ethanol abuse causes structural and functional impairments in the nervous tissue.

The general objective of this doctoral study is to analyze and demonstrate the validity of the Working Hypotheses.

The specific objectives are:

- demonstration of the statistical significance of the working hypothesis (through the retrospective analysis of the cases of patients with SCI);
- prospective demonstration of the working hypothesis at the level of neuronal cells from (immortal) tumor cell cultures;
- prospective demonstration of the working hypothesis by studying glial cells from primary (rat) cell cultures.

5. General research methodology

Initially, we performed a retrospective study of the data from the archive of the RNM SCUBA Clinic and processed the data through statistical analysis, using the IBM SPSS Statistics v22 program. Some graphics were made with the help of the MS Office 365 Excel program.

The evaluation of the working hypothesis was performed prospectively starting from experimental models that intended to reproduce the neural distress caused by SCI. Thus, considering the post-traumatic damage (in various evolutionary moments) of the vasculature of the spinal cord structures (with the occurrence of tissue ischemic phenomena), we considered the role of cellular hypoxia in the neural lesion evolution to be very important. Hypoxia influences both neural cell metabolism and structure, as well as neuraxial morpho-physiology. Therefore, we chose to reproduce (for prospective studies on cell cultures) posttraumatic suffering by inducing hypoxia (through treatments with specific molecules: deferoxamine and cobalt chloride). The prospective study was performed on neonatal rat primary neural tumor and neuraxial cell cultures. Molecular biology determinations and CSF samples of operated SCI patients were performed.

6. Study 1: Retrospective data analysis

We researched the archive of the Neuro-Muscular Recovery Clinic of the "Bagdasar Arseni" Hospital between 01.01.2005- 01.06.2022 and obtained data for a number of 1057, admitted and treated on the ward for acute and subacute status after SCI.

The study showed (with statistical significance) that the motor status after SCI is superior for chronic ethanol consuming patients, as is the performance of sensory-motor recovery processes.

Table 6.48. The result of statistical comparisons of independent numerical data AIS score according to chronic ethanol consumption

		Consum cronic etanolic	N	Media	Diferența mediilor	Valoare p
Scor AIS motor	la internare	Nu	816	41.1691	8.3869	<0.0001
		Da	241	49.556		
	la externare	Nu	816	43.0294	9.3772	<0.0001
		Da	241	52.4066		
	Delta	Nu	780	4.364	3.3386	<0.0001
		Da	232	7.7026		
Scor AIS senzitiv	la internare	Nu	816	119.7868	4.1966	0.738
		Da	241	123.9834		
	la externare	Nu	816	125.6814	6.3062	0.104
		Da	241	131.9876		
	Delta	Nu	814	7.8958	1.9036	<0.0001
		Da	241	9.7994		

7. Study 2: Experimental evaluation of neurons from tumor cell cultures

The desire to clarify the relationship of the nervous system with ethyl alcohol (especially in traumatic conditions), also directed us to the study of the behavior of neuronal cells, which we exposed for a long time (chronically) to ethanol. Experimentally, we created a model of neural suffering (also present in traumatic conditions due to the ischemic phenomena that occur) by inducing cellular hypoxia.

We subsequently evaluated comparatively (between neuronal cells exposed and not exposed to ethanol) cell behavior and characteristic molecular biology changes. Research was conducted on the ethanol-exposed neuroblastoma cell line HTB-11 (hereafter referred to as SK-N-SH). The viability study showed that a concentration of 50mM ethanol does not significantly affect cell viability (98.7% cells being viable) and for this reason it was used as a chronic treatment with ethanol (and carrying out treatments for 2 weeks and for more than 9 weeks of cell cultures). The hypoxemia treatment used hypoxia inducers: cobalt chloride ($CoCl_2$) and deferoxamine (DFX) in concentrations of 50 μM and 100 μM. The scratch test evaluation is shown in Fig. 7.2.

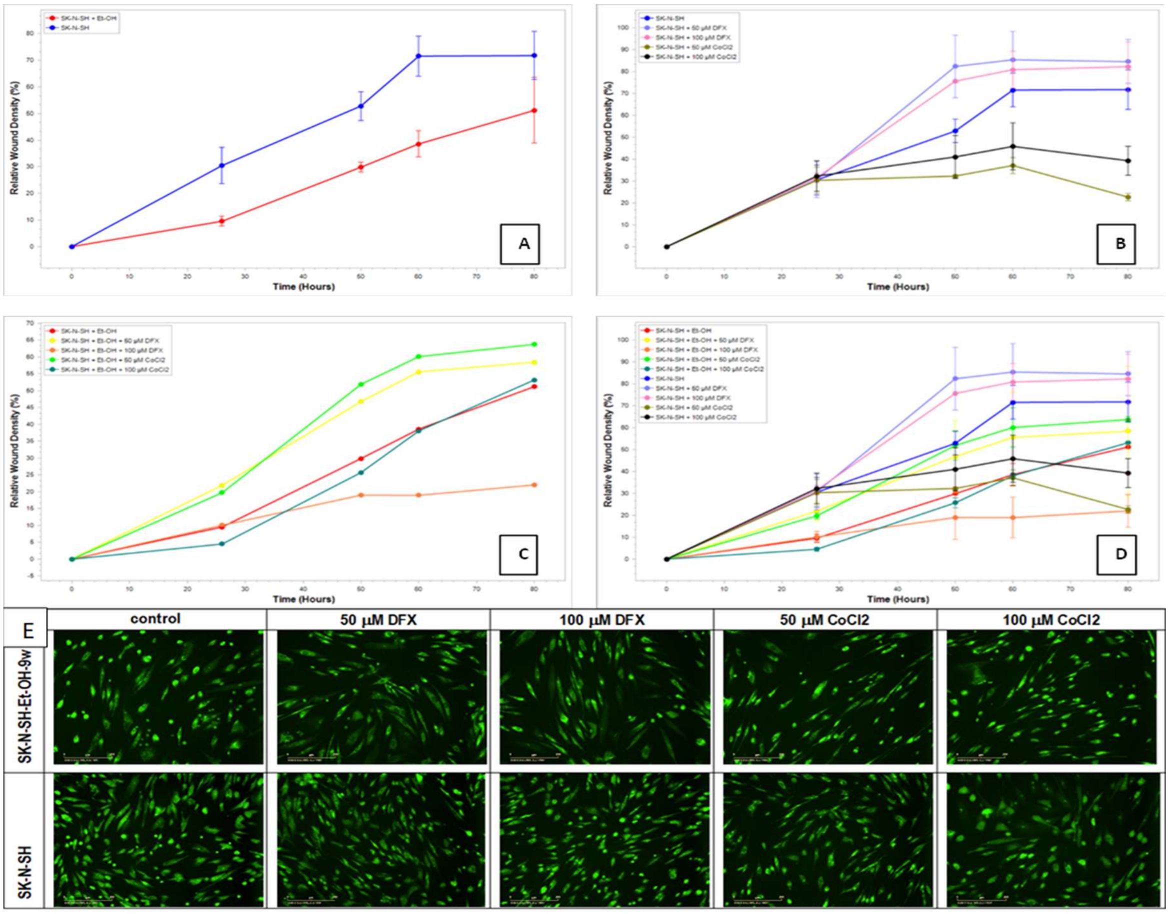


Fig. 7.2. Proliferation and migration of SK-N-SH cells: A. treated (9w) and untreated with ethanol; B. treated (for 24 hours) with DFX and CoCl2 50/100 µM after scratching; C. exposed 9w to ethanol, then treated (for 24 hours) with DFX and CoCl2 50/100µM; D. treated 9w with ethanol, scratched, then treated (24h) with DFX and CoCl2. E. Acridine Orange/Propidium Iodide Stain

The production of reactive oxygen species (ROS) was also analyzed, which at low concentrations of hypoxic inducers (50µM CoCl2 and DFX) had minimal values for cells maintained in ethanol for more than 9 weeks.

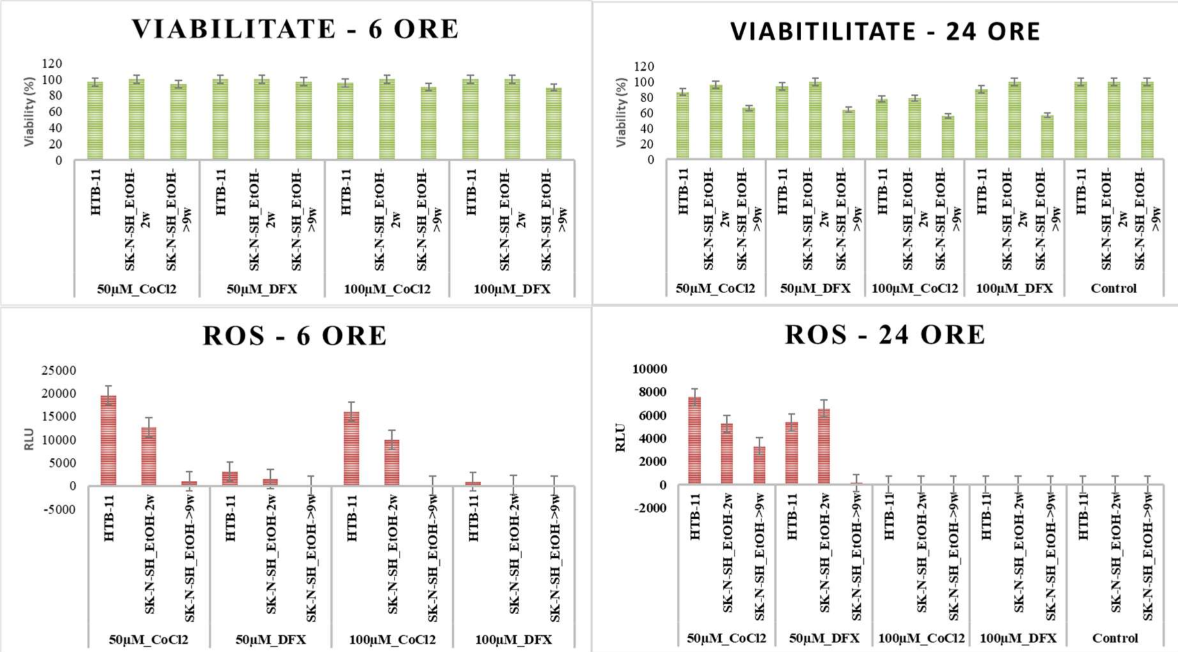


Fig. 7.6. (a, b, c, d) Quantification of cell viability and ROS at 6 and 24 hours

Gene expression of proteins involved in the hypoxic response is shown in Fig. 7.7.

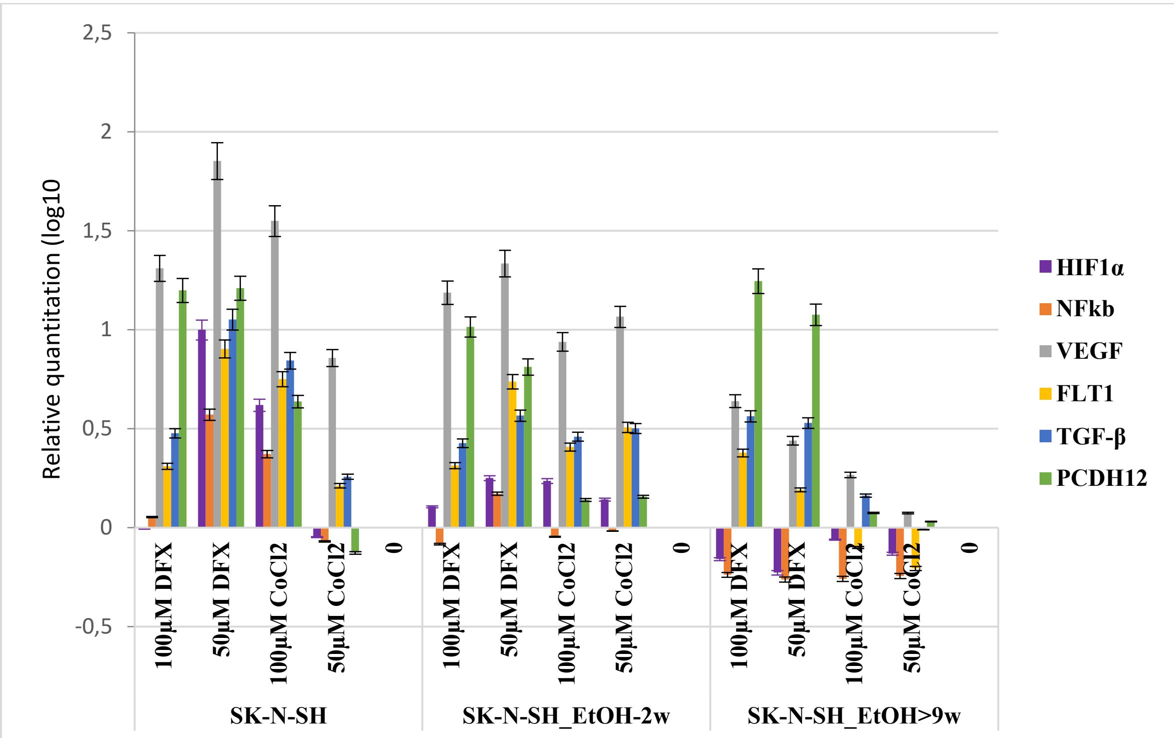


Fig. 7.7. RT PCR Hipoxya

Gene expression of cellular stress proteins is shown in Fig. 7.10.

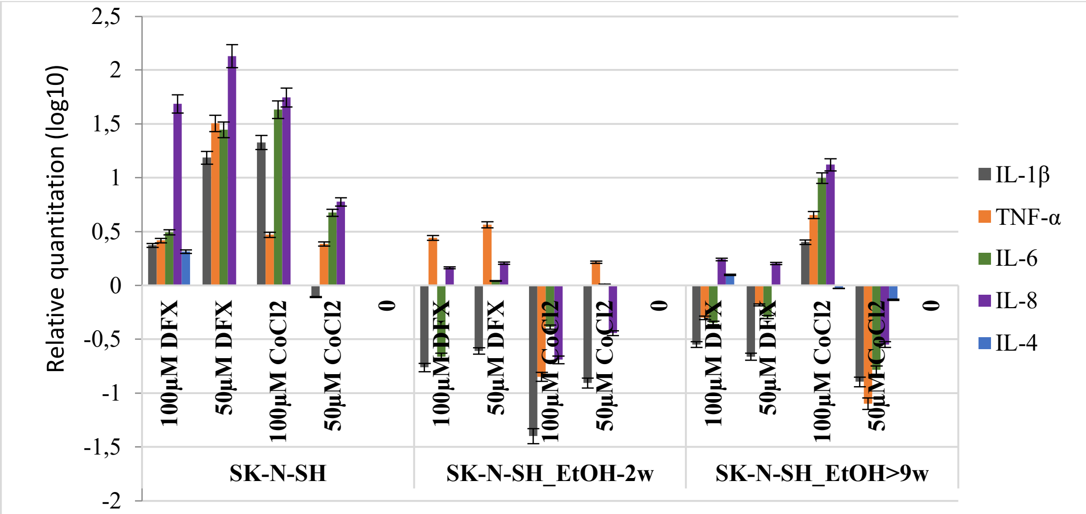


Fig. 7.10. RT PCR Cell stress

The synthesis of cellular stress proteins is shown in Fig. 7.12.

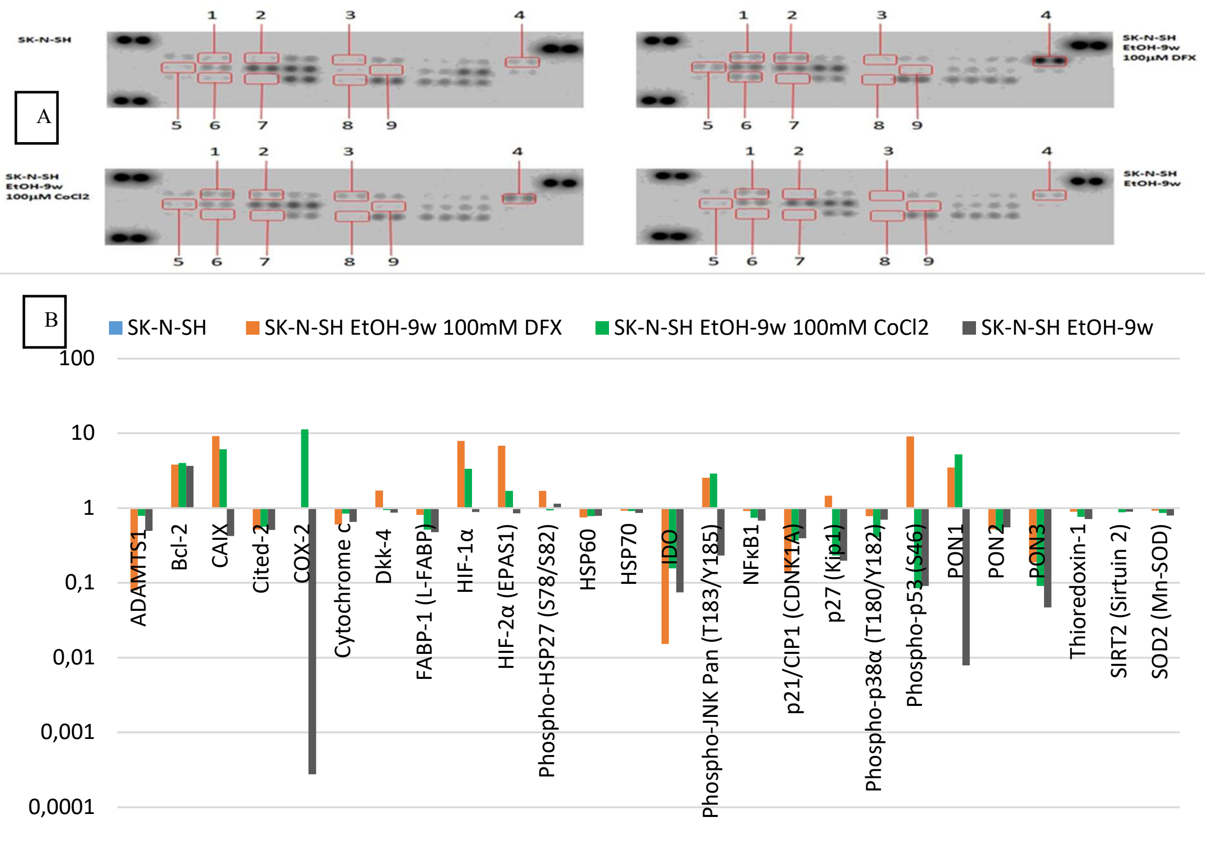


Fig. 7.12. Synthesis of cellular stress proteins under conditions of ethanol exposure and hypoxia (A-view of DotBlot gel and B-graphical representation of logarithmic results)

The gene expressions of the proteins involved in the inflammasome (pyroptosis) are shown in Fig. 7.13.

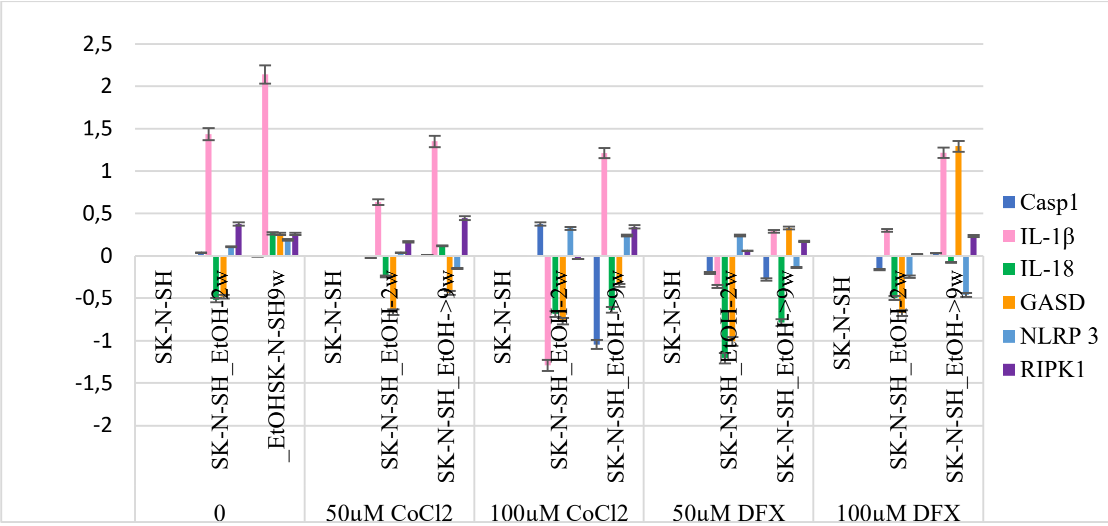


Fig. 7.13. Expression of genes involved in pyroptosis

Gene expressions of carbohydrate metabolism proteins are shown in Fig. 7.16

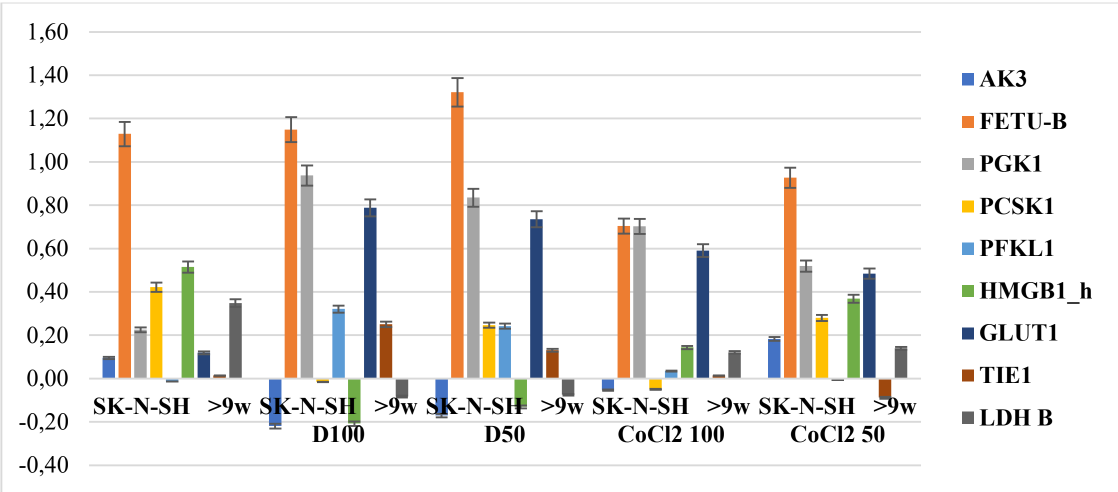
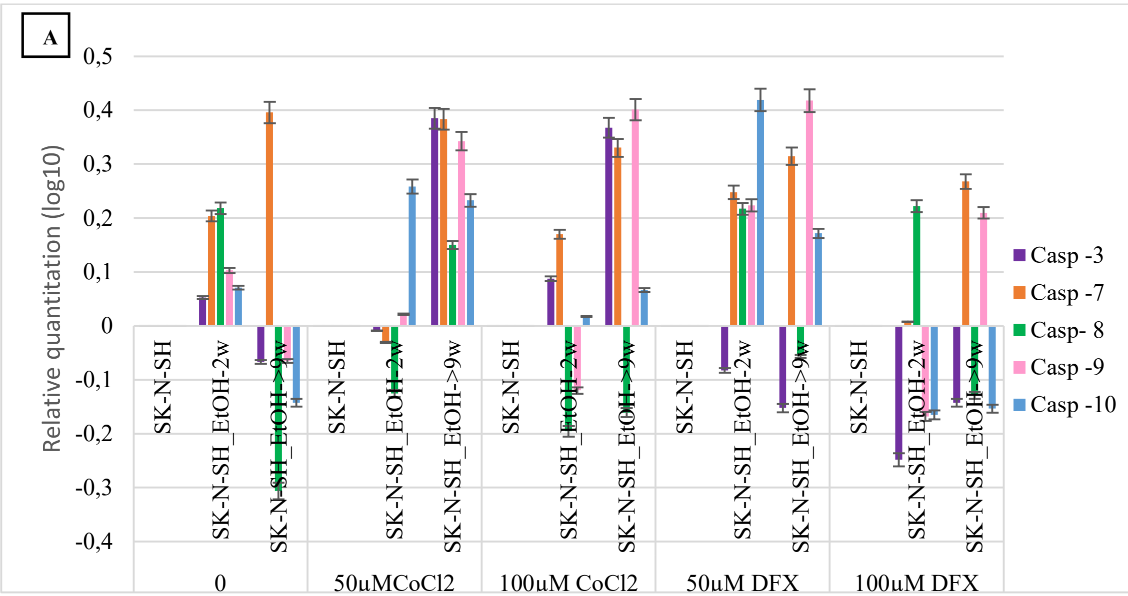


Fig. 7.16. RT PCR Carbohydrate metabolism

Gene expression of apoptotic proteins is shown in Fig. 7.17.



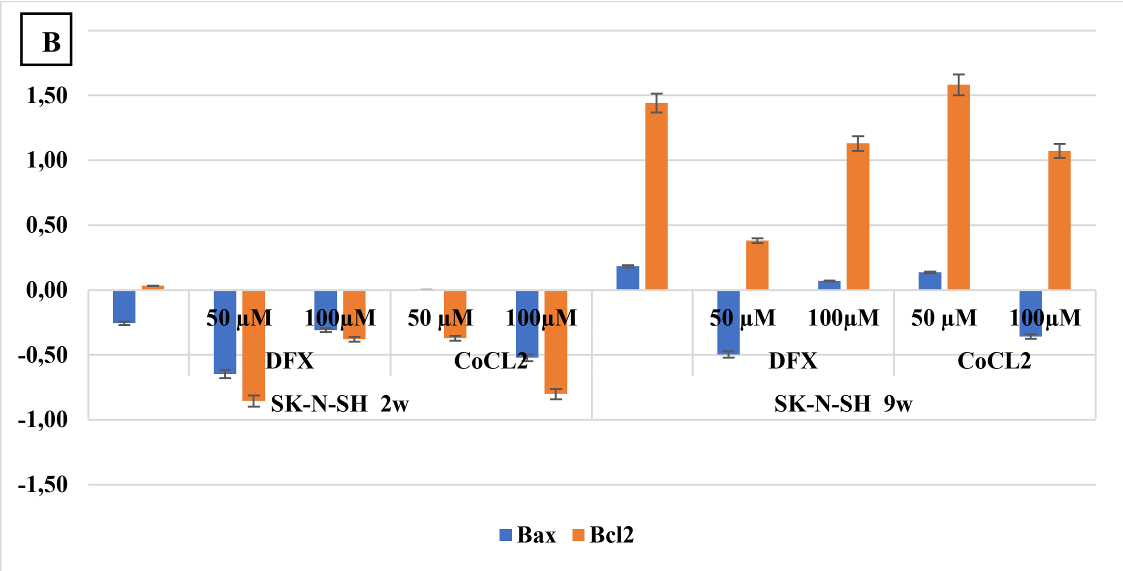


Fig. 7.17. Analysis of gene expression of apoptotic caspases -3, -7, -8, -9, -10 (A) and variation of BAX/Bcl-2 genes (proapoptotic/antiapoptotic) (B)

Apoptotic protein synthesis is depicted in Fig. 7.18.

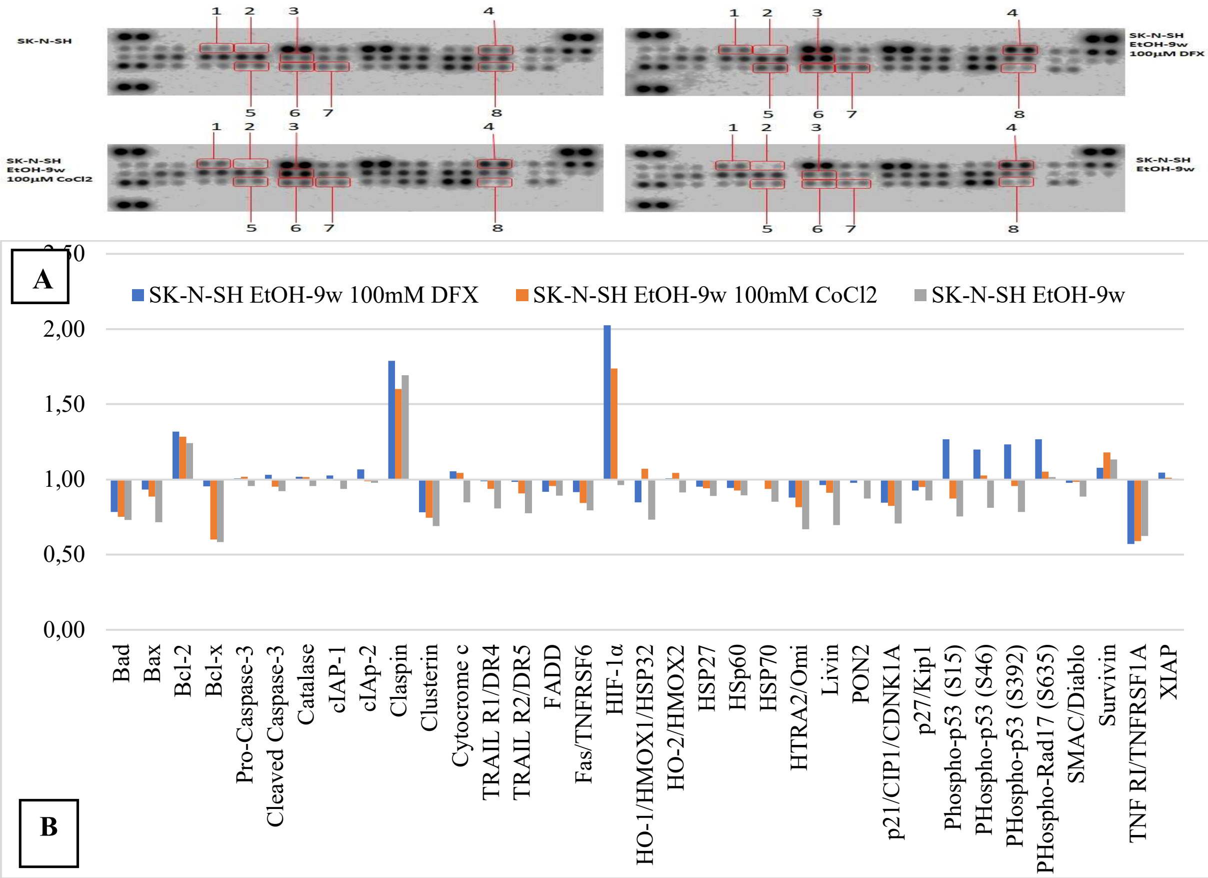


Fig. 7.18. Protein synthesis in apoptosis: A- visualized on experimental DotBlot gel and B- plotted

Gene expression of proteins involved in embryonic signaling pathways is shown in Fig. 7.21.

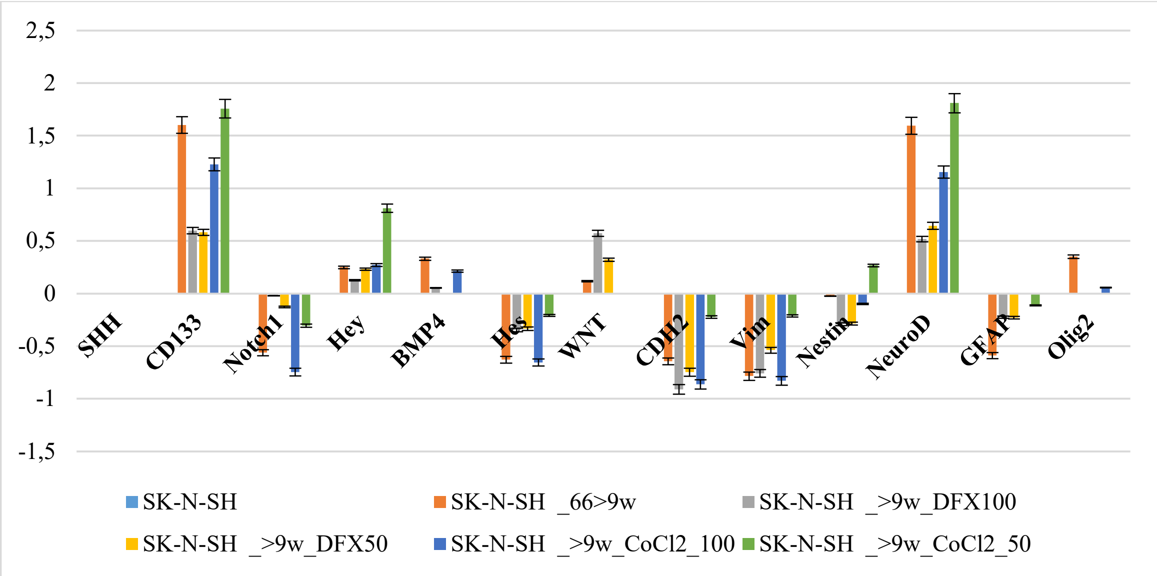


Fig. 7.21. Gene expression analysis of proteins involved in embryonic signaling pathways

Flow cytometry assessment of the cell cycle is shown in Fig. 7.23.

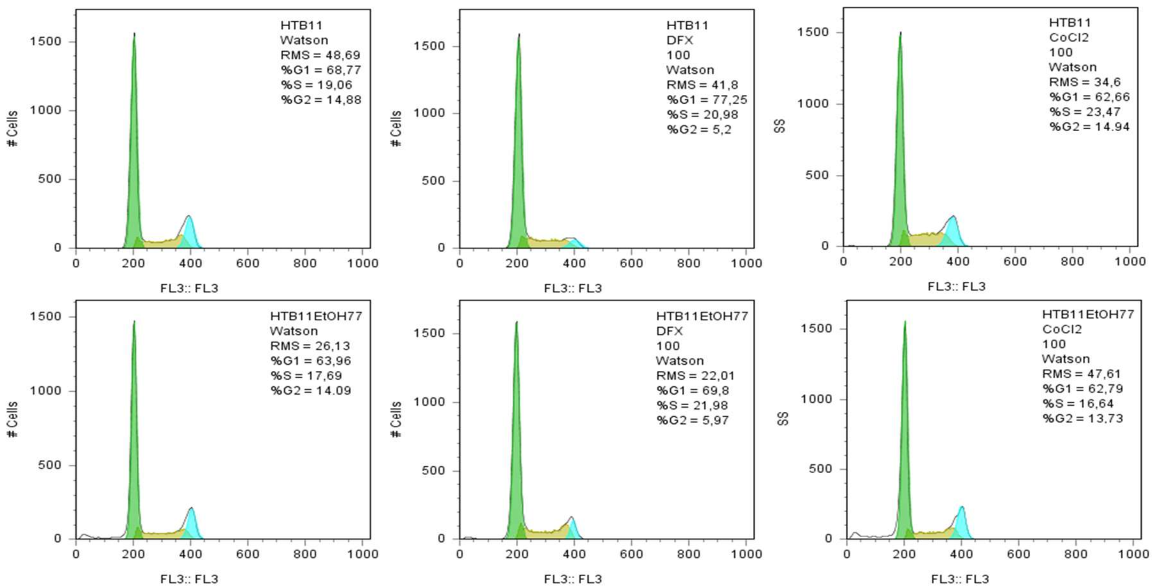


Fig.7.23. Comparative evaluation of the cell cycle

8. Study 3: Evaluation of neural cells from primary cell cultures

An important limitation of this PhD is given by the impossibility of carrying out experiments on normal human nerve cells, which is why we made experimental models on human cells from accredited tumor lines. However, out of the desire to follow the cellular behavior under normal conditions, we performed an experiment on neural (glial) cells collected from a newborn rat (of the Wistar species) (following all the recommendations for working on animals) in which we evaluated murine TNF-α and IL-6 protein synthesis [267].

We note the ELISA results of TNF-α and IL-6 protein synthesis in primary cell cultures in Fig. 8.5.

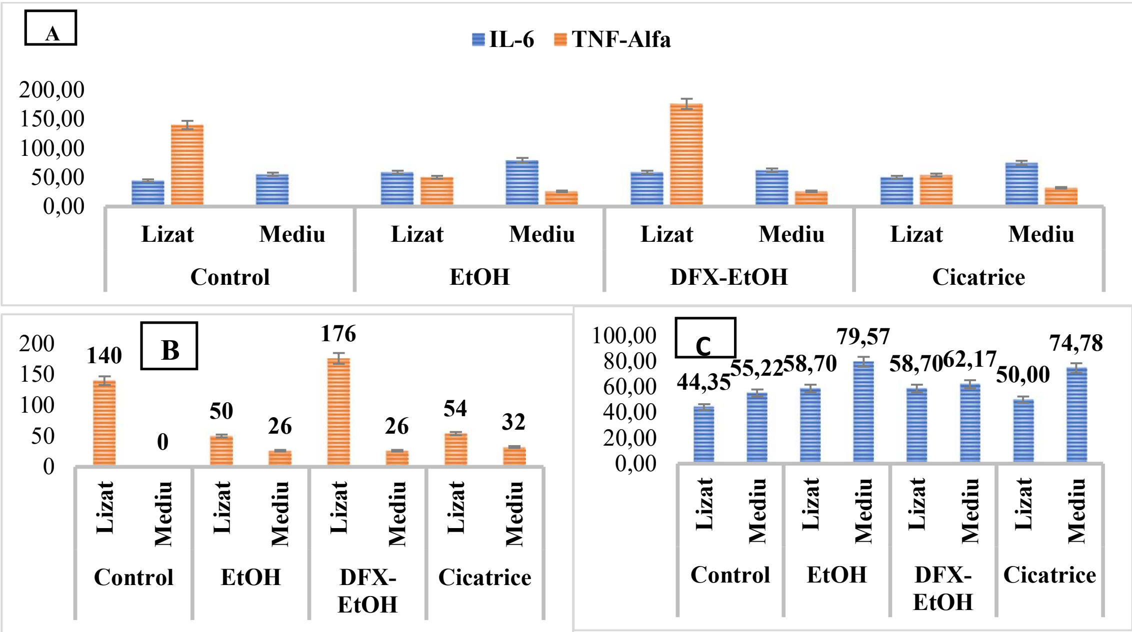


Fig. 8.5. Protein synthesis of TNF-α (B) and IL-6 (C) in gliocytes (in cell lysate and culture medium) (A)

In Fig. 8.6. the values of IL-6 and TNF-α protein synthesis in the studied CSF samples are shown.

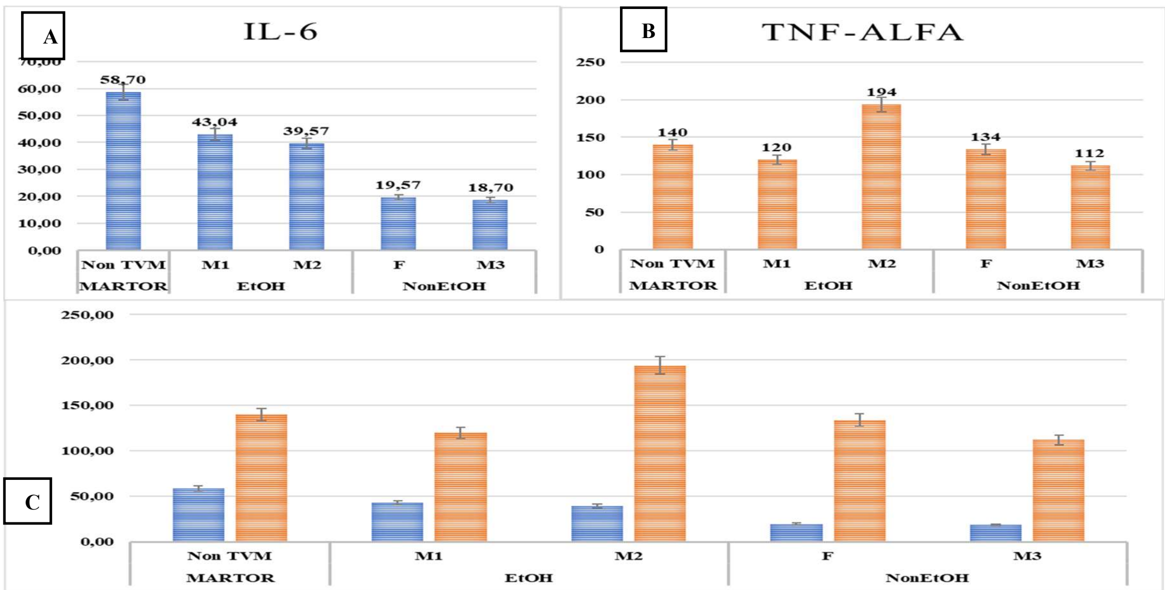


Fig. 8.6. Protein synthesis of TNF-α (B) and IL-6 (A) in CSF (C)

9. Conclusions and personal contributions

The complexity of the body's reactions is based on the variety of molecular biology mechanisms and processes that characterize the cells (including) in the spinal cord.

So, the clinical evolution can sometimes be contradictory and surprising in relation to the level of knowledge we have at a given time. Thus, we can reaffirm that ethyl alcohol is a toxic that should not be neglected, because it affects the human body in its entirety and the relationship between ethanol and the nervous system is complex, taking into account the huge neural capacity for adaptation [278], through a plasticity that is far from to be clarified [279] [280]. As we already showed in the introduction: compared to various aggressive elements, at the level of the neurax, complicated and often inter-conditioned elements are formed (which makes it very often extremely difficult, if not impossible, to draw a clear border between the detrimental and the protective) of defense or/ and repairs. Given the still unsatisfactory results regarding the healing possibilities of spinal cord injuries, spinal cord trauma is still a challenge for the medical world, leaving ample room for research, interpretation and progress. Medical recovery is a specialty that intends to reintegrate (familial/social and professional) the patient affected by dysfunctions of various etiologies; and vertebral-medullary trauma occupies a special place in the patient's efforts to return to the previous SCI state.

For all these reasons, we considered useful the research (both from a retrospective statistical point of view, as well as of the intimate mechanisms (genomolecular and cellular) of observational data and objective evaluation quantified, through our clinical experience, which showed (first) a status less severe neuro-dysfunctional and (respectively) a better motor recovery process (in the acute, subacute and chronic stages) in patients with chronic alcoholism compared to those who do not abuse ethanol. Specifically, the retrospective clinical study of 1057 patients with SCI in the acute, subacute stages admitted to the RNM department of SCUBA, over a period of about 15 years, showed (with statistical significance) a better motor status for patients chronic ethanol users, such as and superior performance in consecutive sensory recovery processes.

In our doctoral approach, considering the working hypothesis, we also included prospective fundamental research elements by evaluating the impact of the direct and hypoxic traumatic model on cells exposed to ethanol impregnation and in primary neural tumor and glial cultures. To the extent of technical possibilities and inevitable related limitations, observational studies were performed on neuroblastoma cell cultures (SK-N-SH) both subjected to chronic ethanol impregnation and without such treatment and (respectively) molecular biology comparisons between reactions of experimental cells (treated or not with ethyl alcohol) to hypoxemic conditions induced by DFX and $CoCl_2$.

We also prospectively evaluated (depending on the economic, inherent limitations) some pro-inflammatory and pro-apoptotic molecules in patients with damaged dura mater after SCI, in whom it was thus possible to collect CSF.

We also followed the molecular dynamics following hypoxia and scratch cell injury model. Microscopic analysis of post-traumatic (scratch) lesion status revealed no difference between resilient and (respectively) hypoxic behavior.

The experimental induction of cellular hypoxia as a model of trauma of the studied neurons caused a decrease in HIF-1α proteolysis, although its gene expression did not increase, followed by trends for: decrease in neurodegeneration and neurotoxicity (by favoring serotonin synthesis produced by inhibition of IDO protein synthesis) , functional improvement of the response/metabolic-functional resilience of neurons (by increasing CA9 and HIF-1β), neuroprotection (by inhibiting NF-kB gene expression), anti-inflammatory action (by stimulating TGF-β gene expression), antiapoptotic (by stimulating the synthesis proteins HMOX1 and HMOX2), vascular regeneration and remodeling (by stimulating VEGF and FLT1 gene expression), synaptic remodeling/neuroplasticity (by stimulating PCDH12 gene expression).

Cellular stress produced by experimental neuronal traumatic models determined the following effects: antioxidant (by stimulating catalase synthesis and by decreasing ROS production, SOD2 and clusterin synthesis), adaptive regulation of energy metabolism (by decreasing HSP60 synthesis), neuroprotective (by stimulating claspin synthesis, HSP27 phosphorylation), anti-inflammatory (by inhibiting IL-1β, TNF-α, IL-6, IL-8 gene expression), antiapoptotic (by inhibiting TNF-α gene expression and decreasing the protein synthesis of HSP70, clusterin, thioredoxin-1), anti-scarring (by inhibiting the expression of the IL-6 gene), inhibitor of nerve regeneration as a defensive response (by inhibiting the expression of the IL-4 gene), restoring nerve endings (by improving the protein synthesis of sirtuin 2), inhibiting of axonal dieback phenomena (by stimulating JNK phosphorylation).

The metabolic changes in experimental neural cells exposed to the hypoxic trauma model determined the following effects: energy depletion (through the inhibition of AK3 gene expression) and lactic acid increase (with a decrease in its dehydrogenation to pyruvic acid), increased insulin proteolytic activity (by stimulating the gene expression of FETU-B), by improving the efficiency of glucose utilization (by stimulating the expression of TIE1), by favoring the intracellular penetration of glucose (by stimulating the expression of the GLUT1 gene) and vascular remodeling (by stimulating the expression of TIE1).

The inflammatory response of experimental neurons exposed to the cell trauma model (by hypoxia) determined the following effects (mediated by the inflammasome): antioxidant (by decreasing the protein synthesis of PON2 and PON3), anti-inflammatory (by inhibiting: caspase-1 gene expression, gene expression NLPR3 after treatment with DFX 50/100μM and 50μM ClCo2, of RIPK1 gene expression following treatment with DFX 50/100μM; by decreasing: synthesis of COX2 and NF-kB1, PON2, PON3; of p-38α phosphorylation), decreasing pyroptosis (by inhibiting gasdermin gene expression), cell survival/biological resistance (by inhibiting gasdermin gene expression).

Regarding programmed cell death by apoptosis, treatment of cell cultures with ethanol inhibits the expression of the proapoptotic gene Bax (toxic mitochondrial) in a manner corresponding to the duration of alcohol exposure, and the inhibition of Bcl-2 expression appears to vary inversely with the duration of ethanol exposure . Analysis of apoptotic protein synthesis shows a correspondence between gene expression and protein synthesis of Bax and Bcl-2, with the inhibition of the apoptotic process augmented by additional hypoxic stress (Bcl-2 synthesis being more increased under hypoxemic conditions). Protein synthesis controlled by the Bax gene is most strongly inhibited in SK-N-SH 9w cultures, while Bcl-2 protein synthesis is stimulated under these conditions; fact that demonstrates an antiapoptotic effect of ethyl alcohol in close relation with the duration of the treatment. Dynamics of Bad protein synthesis show how chronic ethanol treatment inhibits apoptosis more than occurs in the presence of cellular hypoxia. The dynamics of Bcl-x protein synthesis show how apoptotic damage (induced by chronic cellular exposure to ethanol) is inhibited (to a lesser extent) also in the case of induced hypoxic suffering, with a possible more pronounced survival tendency in the situation of long-term alcohol treatment ethyl. The experimental values of procaspase-3 show an antiapoptotic tendency manifested in cells grown in culture media with added ethanol, a tendency that is canceled in hypoxic conditions. The cellular response regarding the synthesis of active caspase-3, cIAP-1 and cIAP-2 shows a decrease in apoptosis in cells chronically treated with ethanol, an apoptotic decrease that seems to maintain its trend (but to a lesser extent) also in acute hypoxic conditions through treatment with cobalt chloride. Cytochrome C protein synthesis corresponds to the antiapoptotic effect of chronic exposure to ethyl alcohol, a trend that decreases under superadded hypoxic conditions. Synthesis of TRAIL receptors (TNF-α family members): TRAILR1 (DR4) and TRAILR2 (DR5) is inhibited in SK-N-SH 9w cell cultures, a trend that is also maintained under hypoxic conditions. FADD synthesis is inhibited in SK-N-SH 9w cultures, a fact that is maintained (but to a lesser extent) also in the conditions of hypoxemic stress produced by deferoxamine and cobalt chloride. These experimental findings show that chronic exposure to ethanol has antiapoptotic effects, manifested since the initiation of the extrinsic apoptotic pathway. Fas synthesis is inhibited in SK-N-SH cultures grown with chronic exposure to ethanol, a trend that is maintained (but to a lesser extent) also under acute hypoxic conditions produced by cobalt chloride and deferoxamine. OMI/HTRA2 synthesis is inhibited in cell cultures chronically treated with ethanol, a trend that is maintained (but to a lesser extent) when exposed to hypoxic conditions. Livin, a protein belonging to the inhibitor of apoptosis family, is decreased both in SK-N-SH cultures chronically exposed to ethanol and in additional hypoxic conditions produced by treatment with deferoxamine and cobalt chloride, stimulating the apoptotic process. Related to P21 protein synthesis, it was inhibited in case of chronic exposure to ethanol and increased in hypoxic superadded conditions showing that, in hypoxia of cell cultures treated with ethanol, the cell cycle occurs at a slower rate, while the spatial dynamics of the extensions neuronal cell appears to be more active. The literature states that S15 phosphorylation of p53 shows the tendency for neuronal metabolic improvement, with stimulation of neural stem cells, which was also observed following deferoxamine-induced hypoxia. As we have previously shown, data from the literature state that, under stressful conditions, S46 phosphorylation of p53 proves the antiapoptotic tendency present in experimental neuroblastoma cells exposed to hypoxemic treatments. Phosphorylation at serine 392 of p53 has an inhibitory effect on cell development (according to data from the literature referred to previously), and its analysis in cells chronically treated with ethanol revealed better cell development, inclusively compared to acute hypoxic situations . Phosphorylation at serine 635 of Rad17 shows that the stress of prolonged ethanol exposure, augmented by hypoxic conditions slows cell division, due to a tighter control of the cell cycle to prevent genetic errors. Smac/DIABOLO protein synthesis shows a more pronounced antiapoptotic effect in SK-N-SH cultures chronically treated with ethanol, compared to SK-N-SH cultures developed in culture medium without ethanol; an effect that is also maintained in hypoxic conditions. Protein synthesis of Survivin shows that chronic exposure to ethanol has an antiapoptotic effect on SK-N-SH cultures, an effect that is preserved even in hypoxic conditions. TNFR-1 protein synthesis is inhibited in SK-N-SH cells chronically exposed to ethanol, a trend that is also maintained in hypoxic conditions. XIAP protein synthesis shows an antiapoptotic effect that manifests itself under hypoxic conditions.

Intercellular signaling pathways were investigated at the level of gene expression and protein synthesis for SK-H-SH line neurons treated with ethanol and subjected to the hypoxia cell injury model. From this perspective, neuroprotective trends were observed (by stimulating the synthesis of the Dkk-4 protein, decreasing inflammatory phenomena (by inhibiting the expression of the CHI3L1 and LCN2 genes), reducing neuraxial scars (by inhibiting the expression of the LAMA4, TIMP1, vimentin, nestin, cadherin, Olig2, GAFP, SEMA3, signaling molecules but, at least some of them, also involved in scar development), neurite development and vascular remodeling, neurogenesis (by inhibiting SEMA 3 gene expression and stimulating gene expression BMP4), of synaptogenesis (by decreasing protein synthesis of ADAMTS1), of neuroregeneration (by stimulating Wnt gene expression), of favoring (theoretical) the ability to differentiate into neurons or glial cells in the SK-N-SH line (by stimulating gene expression CD133, NeuroD, FABP-1; by stimulating CD133, NeuroD, FABP-1 gene expression; by decreasing Notch, Hey and Hes gene inhibition). The (theoretical) favorable situation of the ability to differentiate into neurons or glial cells for the SK-N-SH cell line (under the above experimental conditions) was not confirmed by the scratch test (histological finding also valid in connection with the observation of stimulation of experimental neuroregeneration previously described).

Cell cycle analysis did not show significant differences between the cell cultures studied, although P27 protein synthesis shows that prolonged ethanol exposure and added hypoxic conditions cause a favorable response in neuronal cell cycle dynamics. We also reiterate among the conclusions, along with the actual technical and (respectively) related economic limitations of this doctoral work, and the fact that a large part of the steps in the fundamental research were carried out on neuroblastoma lines due to the objective need to be able to work for periods long on immortalized cell cultures; on the other hand, of course, there are also important differences in biological behavior, insculpatory reaction to stress/insults of such tumor cells - and non-neoplastic neurons or gliocytes.

Although ethanol excess negatively influences the nervous system [281] [282] [283] including through a pro-oxidant effect [284][285] the problem of the relationship between ethanol and the human nervous system is very complex. It would seem that the neuraxial suffering from chronic alcoholism causes a decrease in the number of neurons and their biological capacities, with the limitation of hyperexcitability, which can also lead to unsustainable metabolic hyperstimulation with extensive apoptoses - apoptosis like/bad apoptoses, processes followed by the establishment (through severe reduction of neurons at the lesional level and the interruption of the supra/infralesional communication substrate) of the neurofunctional deficit: tetraplegia or paraplegia.

Precisely for this reason, we performed another study on primary glocyte cell cultures from newborn rats, in which inflammation (IL-6) and apoptosis (TNF-α) were evaluated after hypoxic traumatization of the respective cells exposed to ethanol excessively, with the following design: control cells not exposed to ethanol and not exposed to hypoxia, cells excessively exposed to ethanol, hypoxically traumatized cells (deferoxamine only, ethanol-impregnated and non-ethanol-impregnated) and thermally traumatized. In summary, the study on primary neuraxial cultures shows (in the case of ethanol exposure) a very efficient inflammatory intercellular communication, with an apoptotic maximum only in the case of combined treatment with ethanol and deferoxamine, but with a low level of apoptotic intercellular communication of cells treated with ethanol (alone or in combination with deferoxamine). In another study (pilot) with a very limited number of participants - for bioethical reasons (the collection was done only in patients with broken dura mater and CSF leakage) the examination revealed: the presence of pro-inflammatory and pro-apoptotic tendencies. We observe how the stress of ethanol treatment, supplemented by hypoxia, accentuates inflammatory intercellular communication, without leading to the highest levels of programmed cell death. The examination of CSF from patients with SCI resumes the discussion about the effects of inflammation and cell death in the initial phases after SCI, when we observe proinflammatory and proapoptotic statuses better represented in patients with chronic alcoholism, possibly also as a mechanism of isolation and protection of the injured medullary area.

The results of our experiments seem surprising, both in the clinical-statistical retrospective dimension, and in those found experimentally propensity. Briefly recapitulating: chronic medullary impregnation with ethanol, we found that it has the main "protective" results after aggressions: a reduction of neuro-excitotoxic phenomena after SCI (including through the inhibitory action of NMDA receptors [286]), a diminished production of ROS (including, through a possible mitohormesis effect, stimulating the antioxidant effects within the antioxidant response elements: Antioxidant Response Element - ARE [287,288]) so a lower oxidative stress, a decrease in proapoptotic mechanisms. Of course, these results (which try to explain the geno-molecular and cellular support of the status and evolution, clinically and statistically found to be better in chronic alcoholics) do not exhaust, not far from, the vast yet unexplored field in this extreme complex and complicated field of pathology.

We must not forget how important the dose-effect relationship is, even when it comes to molecules with an antioxidant role (an example of this, not related to our topic, is resveratrol: a supportive molecule for cellular resilience to stress and apoptosis , which can become neurotoxic in quantities that exceed certain limits)[289]. At the same time, the discussion is open on the beneficial effects that can be produced by other molecules included in the composition of alcoholic beverages, as well as the influence of the components of these beverages on other physio-pathological molecular pathways at the level of the cells, including the spinal.

The human nervous system possesses endogenous properties (so far very difficult to capitalize) of post-lesion recovery grouped in the modern concept of endogenous defense activity, neuro-biological function systematized in: neurotrophicity, neuroprotection, neuroplasticity, neuro-/(synapto-)genesis [290]. Considering the data from the literature (confirmed also by our current studies) we can say that neuraxial lesions (including SCI) are very complex (with a fragile demarcation between neuroprotective and degenerative ARE processes), without there being (so far) a curative treatment of SCI. In this context, disappointingly decomposed, the chronic ethanol impregnation of the spinal cord tissue deserves to be studied exhaustively in order to try to extract some reactive biological components and pathways from an intimate level that, possibly molecularly separated from the harmful ballast of chronic alcohol ingestion, could find their a potentially valuable therapeutic contribution.

Last but not least, we should reiterate the potential therapeutic effects, in SCI, of molecules structurally similar to ethyl alcohol (for example, polyethylene glycol), which produced in some experimental studies favorable neuroprotective effects [291]: in such situations, research would must persevere to decant the beneficial effects from the toxic ones. The studies in this doctoral thesis have consistent elements of originality, considering that so far we have not encountered research focused on the (including) favorable effects of chronic ethanol consumption on the recuperative, neurofunctional status and evolution of the neuraxial tissue affected by SCI.

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