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Research article

Studies regarding influences of ethanol on hypoxemic stress in neuroblastoma cells

Simona Isabelle Stoica [1,2], Prof Dr Gelu Onose [1,2], Coralia Bleotu [3,4,5], Ana Iulia Neagu [3], Ioana Mădălina Pitica [3], Denisa Dragu [3]

1 University of Medicine and Pharmacy "Carol Davila" (UMPCD), Bucharest, Romania
2 Teaching Emergency Hospital "Bagdasar-Arseni" (TEHBA), Bucharest, Romania
3 Stefan S Nicolau Institute of Virology, Bucharest, Romania
4 Research Institute of The University of Bucharest, Earth, Environment and Life Sciences, Bucharest, Romania
5 Romanian Acadiemy of Scientists, Ilfov 3, Bucharest, Romania

* Correspondence: Simona Isabelle Stoica, e-mail:stoica.simona@umfcd.ro

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Abstract: Introduction In the modern world a pathology with increasing frequency is that of spinal cord injury (SCI), with the risk of dysfunction on multiple levels. Following our clinical experience, we have conducted studies on the effect of hypoxic stress (also present in spinal cord injury) on neuronal cell cultures treated for a long time with ethanol (trying to reproduce chronic alcoholism). In this article we present the behavior of neural cells subacutely exposed to ethanol after hypoxic stress, in order to perform a comparative analysis with chronic exposure to alcohol. Materials and methods We performed subacute treatments with ethanol in neural cell cultures. We evaluated gene expression and protein synthesis in the case of experimentally induced hypoxic stress. Disscusions The complexity of the human body is superior to experimental models. This experiment creates a model of extremely complex changes after spinal cord injury. The results cannot undoubtedly overlap the conditions of the physiopathological reality. Results and conclusions The model of neural hypoxic suffering in cell cultures is similar in the case of cell cultures treated subacutely with ethanol, except: the risk of neurodegeneration, the phenomenon of axonal die-back, proapoptotic tendencies, proinflammatory tendencies. The effect of chronic (more than acute/subacute) ethanolic consumption seems to determine geno-molecular neural changes with a potentially favorable effect regarding the response (immediate and long-term) to spinal cord injury

Keywords: spinal cord injury, alcoholism, hypoxia, neuromuscular recovery

1. Introduction

In the modern world a pathology with increasing frequency is that of spinal cord injury (SCI), which affects all population categories, with the risk of dysfunction on multiple levels [(1) (2)] . Despite the many known harmful effects of ethanol on the central nervous system [(3)], in our long clinical experience **we** were contradicted by of an unexpected observation regarding frequent differences between the objective neuro-dysfunctional state after spinal cord injury between patients known to abuse chronic ethanolic and those who did not have such behavioral disorders, with a better post-traumatic status and a more efficient recovery process for allcoholic patients [(4)]. We tried to explain through paraclinical investigations (including at tissue and intimate molecular level) the positive (beneficial) influence of chronic alcoholism on the evolution (acute and subacute) of the neuro-dysfunctional clinical state in SCI patients [(5)].

After SCI, primary and secondary degenerative changes occur of which we evaluated at the neural level: inflammatory processes (inflammasome), apoaptotic and cellular stress [(5)].

The inflammasome is a protein cytosolic complex that acts as an intracellular receptor for environmental and cellular stress [6] mediating the innate immune response, causing tissue damage when is overactivated (Fig.1) [7] [8] [5]. Events in SCI lead to Damage-Associated Molecular Pattern (DAMPs) and Pathogen Associated Molecular Pattern (PAMPs) (in the intercellular space), which interact with Pattern Recognition Receptor (PRRs), C-Type Lectin (CTLs), Retinoic acid– inducible gene (RIG)-I-Like Receptor (RLRs), The Nucleotide binding domain Leucine-rich Repeat (NLRs) and Toll-Like Receptor (TLRs) (whose activity is also controlled by Heat Shock Protein70 - HSP70), then activating of Apoptosis-associated Speck-like protein containing a CARD (ASCs) (and the Caspase Activation and Recruitment Domain - CARD of its interior), followed by caspase interaction: caspase-1 (with inflammation produced by IL-1β and IL-18, via the canonical inflammasome activation pathway) or caspases -4 and -5 (stimulating Gasdermin - GSDM and pyroptosis on the non-canonical inflammasome activation pathway). The stress produced by SCI can also activate membrane Mitogen-Activated Protein Kinases (MAPK), with the activation of p38 (and stimulation of Inducible Nitric Oxide Synthase- iNOS leading to neurotoxicity) and Big MAPK (BMK) (stimulated also by TNFR-Associated Factor 2 - TRAF2) and c-Jun N-terminal protein Kinase (JNK) (activated by TRAF2, which stimulates *cjun* and Activating Transcription Factor2 - ATF2) pathways. The stress produced after SCI can also act at the nuclear level, via RAD17 (which is phosphorylated at serines S635 and S645, inhibiting mitotic activity). Also, SCI events can lead to the transcriptional activation of Indoleamine 2,3-DiOxygenase-1 (IDO-1) which can inhibit the synthesis of serotonin or stimulate the quineric acid pathway (with the risk of producing neurotoxicity and neurodegeneration). But SCI stress can also activate cytoplasmic clusterin which can inhibit ROS. ROS can stimulate thioreduxin1, which can activate JNK and p38 proteins in the MAPK pathway. SCI can stimulate the synthesis of HSP27 (with a cytoprotective role), claspin and p27 (which inhibits DNA activation). Hypoxia following SCI influences DNA (at nuclear and mitochondrial level) by stimulating Hemeoxygenase 1 (HMOX1) and increasing levels of Hypoxia Inducible Factor-1α (HIF-1α) and HIF-2α, which then activates Hypoxic Response Element (HRE), causing synthesis of ALDOlase, fructose-bisphosphate A (ALDOA) (with stimulation of neurofilament light chain mRNA), of Glucose transporter1 (GLUT1) (which favors the entry of glucose inside the cell) and Fetuin B (FETUB) (producing resistance to the action of glucose), of Carboic Anhidrase 9 (CA9). At the mitochondrial level, SCI stimulates HSP60, and hypoxia stimulate HRE that controls cellular energy metabolism by Adenosine DiPhosphat (ADP) phosphorylation (stimulating Adenilat Kinase3 - AK3), ADP phosphorylation (stimulated by PhosphoGlycerate Kinase1 - PGK1), by lactic acid production (through PhosphoFructoKinase, Liver Type -S PFK-L synthesis), by bilateral lactate-pyruvate conversion (through the synthesis of Lactate DeHydrogenase A and B - LDHA and LDHB). Mitochondrial hypoxia stimulates Epidermal-Growth Factor - EGF with influnces in vascular remodeling. HRE stimulates Paroxonase2 - PON2, activating Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NF-κB) (by inhibiting Nuclear Factor Kappa-light-chain-enhancer of activated B cells α anb β - IKKα and IKKβ). SCI can inhibit ROS by stimulating SuperOxide Dismutase1 (SOD1) and SOD2, Catalase (CAT). In the cytosol, SCI can stimulate Tyrosine kinase with immunoglobulin like and EGF like domains 1 (TIE1) (with vascular remodeling). In the endoplasmic reticulum, SCI events increase the production of Brain-Derived Neurotrophic Factor (BNDF) and Nerve Growth Factor (NGF) (via Proprotein Convertase Subtilisin/Kexin - PCSK1).

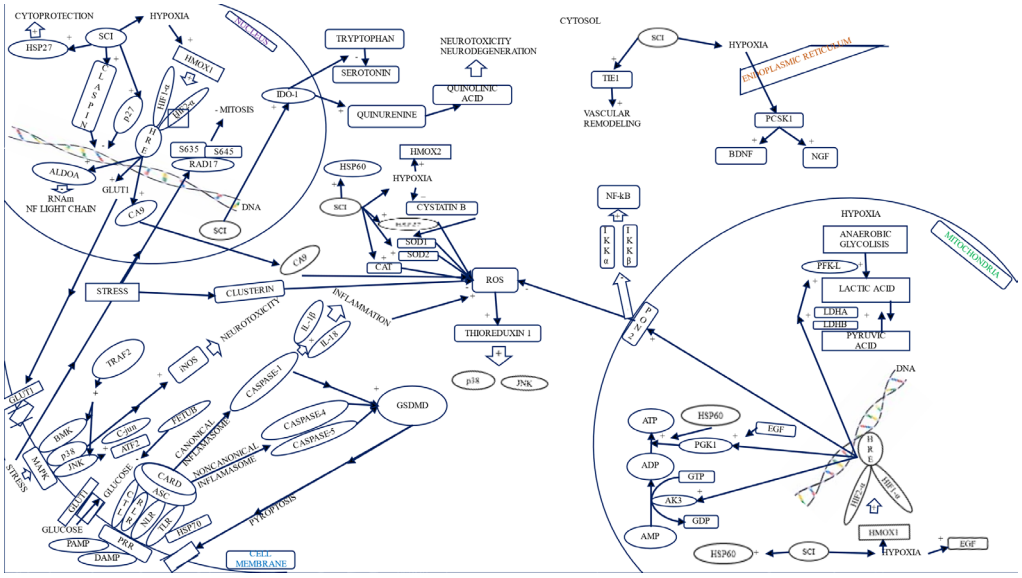

Fig. 1. Metabolic and inflammatory changes after SCI. [5]

Cell death, (according to Nomenclature Committee on Cell Death - NCCD) can be: accidental (accidental cell death - ACD) and programmed (regulated cell death - RCD) [9][10][5]. ACD is a form of instantaneous cell death produced by the disintegration of the cell membrane through the action of many factors (physical, chemical or mechanical), and RCD is the consequence of the activation of one or more cellular signaling pathways [10][9][5].

After SCI, cell death occurs through ACD and RCD [9]. Neural cell death is produced by: apoptosis, necrosis, necroptosis, ferroptosis, pyroptosis, autophagy, paratantosis, oncosis [11][12] [13] [14] [10][9][15][5]. Apoptosis is a regulated, non-inflammatory process of programmed death essential for permanent renewal (at the cellular and whole-organism level) [16] [15] [17][5].

Fas ligand (FASL) and TNF-Related Apoptosis Inducing Ligand (TRAIL) act on the DISC -Death-Inducing Signalling Complex (FAS, TNF-Related Apoptosis Inducing Ligand 1 and 2 -TRAILR1 and TRAILR2 that interact with Fas-Associated Death Domain - FADD, activating procaspase-8); similar to Tumor Necrosis Factor α (TNFα) acting on the DISC (TNF Receptor1 - TNFR1, TNFR2 interacting with TNF Receptor-Associated Death Domain - TRADD, activating procaspase-8) (Fig.2). DISC then activates caspase-8 which stimulates effector caspases (-3, -6, -7), which act on Poly (ADP-Ribose) Polymerase (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, beside Mitochondrial Permeability Transition pore (MPT) (stimulated by hypoxia, ROS and DNA damage) activates Mitochondria Outer Membrane Permeabilisation (MOMP) which (mediated by Small mitochondria-derived activator of caspases/ direct inhibitor of apoptosis-binding protein with low pI - pi-SMAC/ DIABLO and OMI/ HTRA2) triggers the intrinsic pathway of apoptosis and inhibits X-linked inhibitor of apoptosis protein (XIAP) which (with survivin) inhibits Apoptosis Protease Activating Factor-1 (APAF-1) (which forms the apaoptosome together with cytochrome C). SCI, via ROS, stimulates ferroptosis. The apoptosome activates necroptosis (mediated by p53) and stimulates the activation of pocaspase-9 to caspase-9 which stimulates the effector caspases of the extrinsic apoptotic pathway (-3, -6, -7) that activate MOMP (involved in the intrinsic apoptotic pathway). The balance in the intrinsic apoptotic pathway is maintained by 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 inflammasome complex 1 (consisting of TNFR1, TNFR2, TRADD, Receptor Interacting Protein1 - RIP1, CIAP1, Cellular Inhibitor of

Apoptosis Protein 2 -CIAP2) which (together with RIPKinase1 - RIPK1 and RIPK3) acts on inflammasome complex 2 (consisting of RIP1, RIP3, FADD, TRADD and caspase-8). Inflammasome complex1, via Transforming growth factor-β-activated kinase-1 - TAK-1, TAB2, IKK (inhibited by CYLD) stimulates NF-kB triggering inflammation. Inflammasome complex2, by permeabilizing the lysosomal membrane and Apoptosis-inducing factors - AIF, PARP triggers necroptosis. Mitochondrial AIF causes activation of cytoplasmic Nuclear Localization Signal - NLS (which causes DNA damage). The action of AIF is inhibited by calpain. SCI can produce nuclear DNA damage that stimulates p53 expression (activating DNA repair and the extrinsic apoptotic pathway) and phosphorylation of p53 at serine 46 (activating 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 DNA action of p300-CBP (cAMP response element-binding protein-binding protein) complex (stimulated by cited-2). The endoplasmic reticulum can also be involved in apoptosis, attaching caspase-12 and caspase-7, triggering the autophagy process[5].

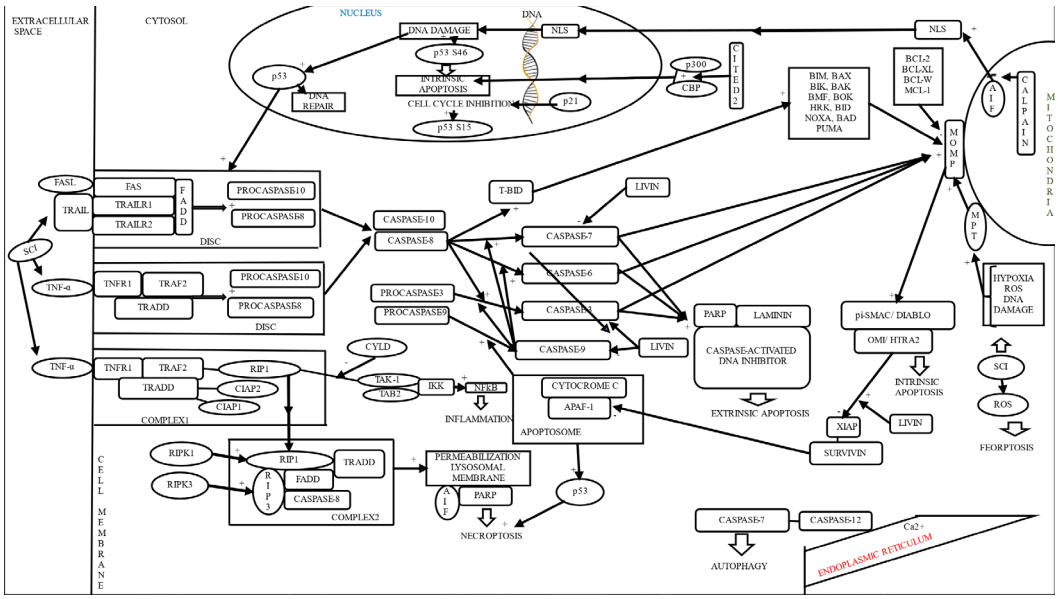

Fig. 2. Pathways of cell death in SCI [5]

Ethanol is one of the cheapest and most used antidepressant by many people over time (Fig.3) [5]. 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) [5] [18] [19].

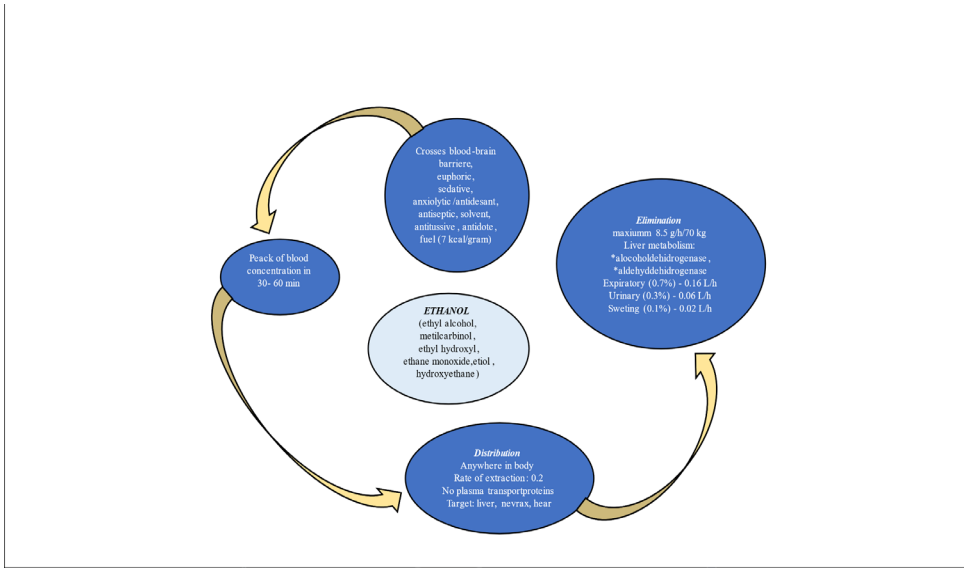

Fig.3. Pharmacokinetics and biological effects of ethanol (5)

We carried out a prospective study on a neuronal tumor cell line (in the Department of Molecular and Cellular Pathology of the "Ștefan S. Nicolau" Institute of Virology) on models of cellular suffering (through hypoxemic interventions) in HTB-11 cell cultures treated excessively with ethyl alcohol and corresponding in time to alcoholism [20].

Cellular stress produced by experimental neuronal traumatic models (on cells chronically exposed to ethanol) determined the following effects: *antioxidant* (by decreasing SOD_2 synthesis), *adaptive regulation of energy metabolism* (by decreasing HSP_{60} synthesis), *neuroprotective* (by stimulating HSP_{27} phosphorylation), *anti-inflammatory* (by inhibiting IL-1β, IL-8 gene expression), *antiapoptotic* (by decreasing the protein synthesis of HSP_{70} and thioredoxin-1), *restoring nerve endings* (by improving the protein synthesis of sirtuin), *inhibiting of axonal dieback phenomena* (by stimulating JNK phosphorylation) [5].

Materials and methods

In this work we have studied a cellular traumatic model under conditions of subacute ethanol consumption to see if there are differences in response to cellular stress (compared to chronic alcohol exposure).

We worked on Cell line - SK-N-SH (ATCC ATCC HTB-11, 581 Rockville, MD, USA. The cells were maintained in Dulbecco's Modified Essential Medium: F12 582 (Sigma-Aldrich, USA) supplemented with 10% inactivated fetal bovine serum (Sigma-583 Aldrich, USA), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich, USA). The cells were kept in the incubatore in a humidified atmosphere, at 37°C, containing 5% CO2.

We evaluated, according to the proteomic concept, gene expression (through RT-PCR using taqman assays (Thermo Fisher Scientific, USA) for the following genes: Casp3 (Hs00234387_m1), Casp8 (Hs00154256_m1), Casp7 (Hs00169152_m1), Casp9 (Hs00154261_m1), IL-18 (Hs01038788_m1), IL-1β (Hs01555410_m1), GasderminD (CAT#: HP214995), RIPK1(CAT#: HP207257), and protein synthesis (through Dot Blot analysis using Proteome Profiler Human Stress array kit (ARY 018, R&D 673Int. J. Mol. Sci. 2022, 23, x FOR PEER REVIEW 22 of 27systems, Minneapolis, MN, USA) under conditions of hypoxic stress (produced after treatment with deferoxamine, DFX, and cobalt chloride, CoCl2, in concentrations of 100mM each) in neural cells from HTB-11 cultures exposed to concentrations of 50 mM ethanol, for 7 days. The relative fold differences in gene expression were calculated based on ΔΔCq method.

Results

We observe (in Fig.4) how the ethanolic treatment has an anti-inflammatory effect by decreasing the expression of gasdermin and NLPR3 genes. And the hypoxemic conditions induced by Deferoxamine treatment increase the expression of genes involved in pyroptosis, especially in cells treated subacutely with ethanol. Hypoxemia experimentally induced by treatment with cobalt chloride has a pro-inflammatory effect in neuroblastoma cell cultures and shows an anti-inflammatory effect in the case of ethanol treatment (with decreased expression of GASD, NLRP3, RIPK1, IL18, IL1 genes).

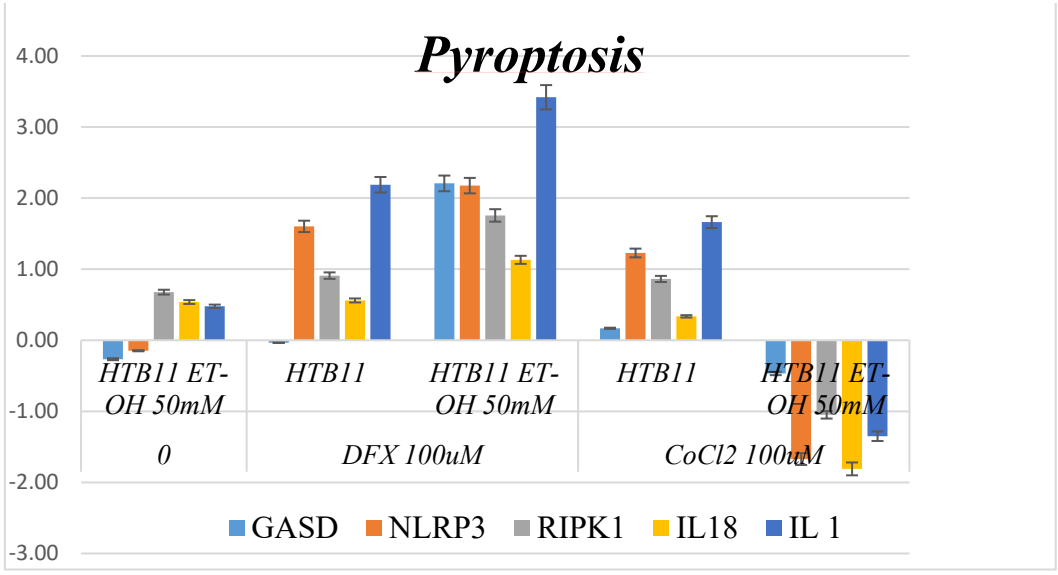

Fig.4. Gene expresion for pyroptosis

A situation similar to the dynamics of the expression of inflammatory genes (pyroptosis) also occurs in the case of the expression of apoptosis genes (Fig.5). Thus, hypoxemic treatments (with deferoxamine and cobalt chloride) increase the expression of apoptotic genes, even under ethanol treatment conditions, with the exception of hypoxemia induced by cobalt chloride in cell cultures subacutely exposed to ethanol (where gene expression is inhibited: caspases -3, - 8, -9, -10).

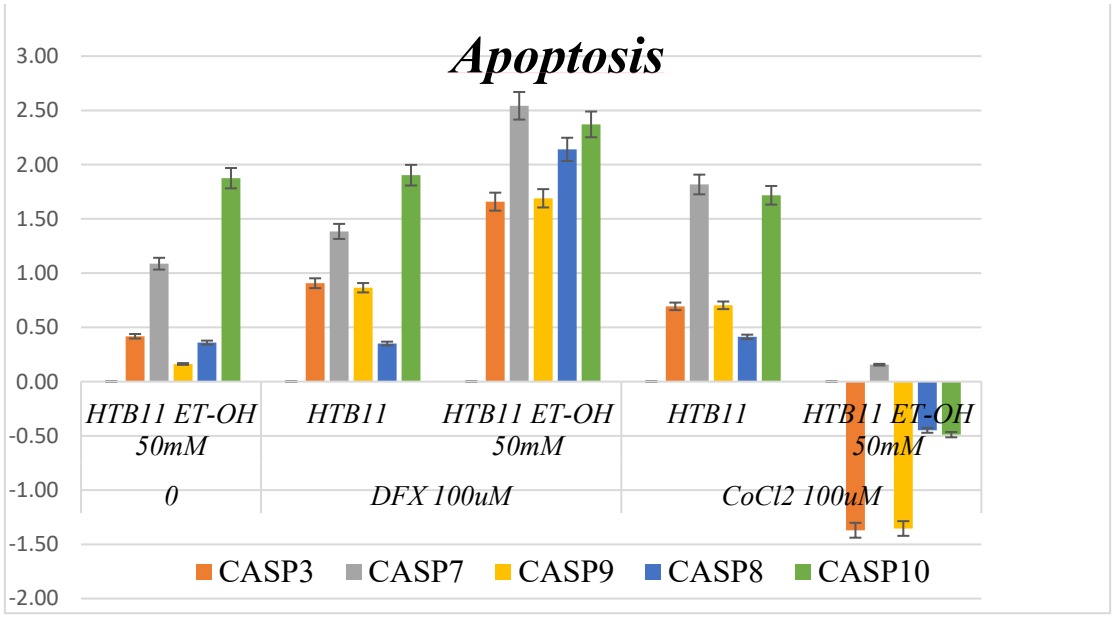

Fig.5. Gene expesion for apoptosis

Protein synthesis from the stressful conditions produced by deferoxamine and cobalt chloride in neuroblastoma cultures treated with ethanol was best represented in the case of molecules involved in the hypoxemic response as carbonic anhydrase (SD= 35.04 in the case of DFX and SD= 34 in the case of CoCl2) (Fig.6) . The synthesis of HIF 1α and 2α is increased especially in the case of CoCl2 treatments (SD 20.25 for HIF-1α and 13.42 for HIF-2α); and the lower values (SD= 17.15 for HIF-1α, SD =3.21 for HIF-2α) of HIF synthesis after DFX treatment can be explained by increased protein consumption in hypoxic conditions. The increase in the synthesis of p53 phosphorylation shows proapoptotic tendencies after treatment with DFX (SD=38.21) and CoCl2 (SD=33.53). The decrease in DKK synthesis may show a neurodegenerative effect when treated with DFX (SD=12.5) and CoCl2 (SD=14.57).

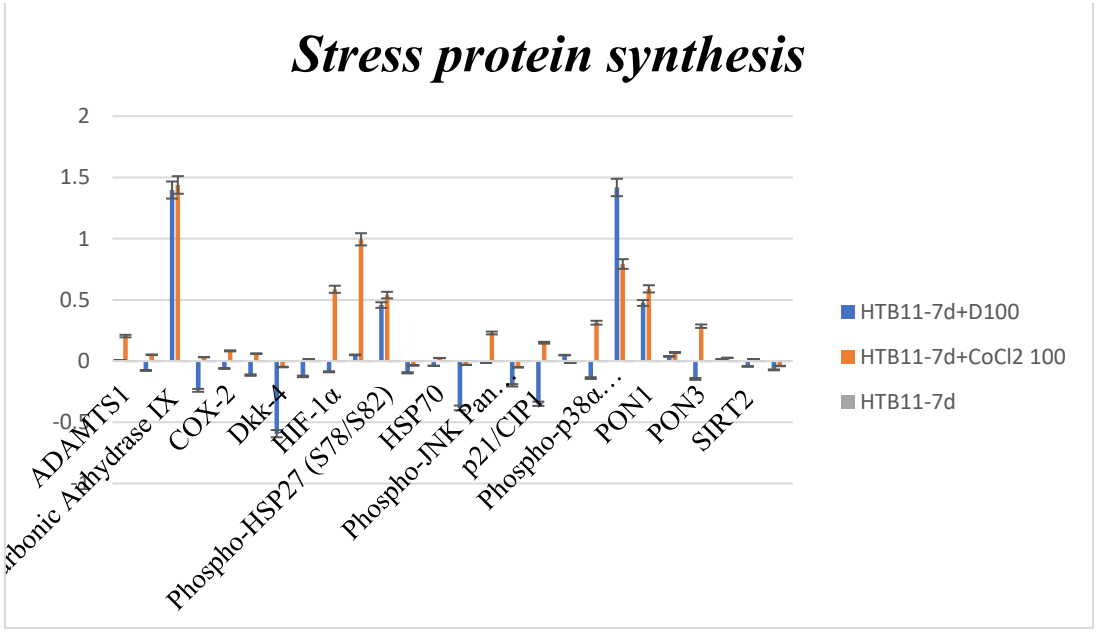

Fig.6. Stress protein synthesis

Disscusions

The complexity of the human body is superior to experimental models. This experiment creates a model of extremely complex changes after spinal cord injury. The results cannot undoubtedly overlap the conditions of the physiopathological reality. Our results evaluate only a diagnostic track in spinal cord injury, with potential therapeutic benefits (which remain to be evaluated).

Conclusions

The model of neural hypoxic suffering in cell cultures is similar in the case of cell cultures treated subacutely with ethanol, except: the risk of *neurodegeneration* (by decreasing DKK4 synthesis), the phenomenon of *axonal die-back* (by decreasing the synthesis of JNK phosphorylation), *proapoptotic tendencies* (by increasing the phosphorylation of p53 at serine 46 and increasing the expression of apoptotic inducing and effector caspases genes, less in the case of CoCl2 treatment), *proinflammatory tendencies* (less in the case of treatment with CoCl2 100 mM). In conclusion, the effect of chronic (more than acute/subacute) ethanolic consumption seems to determine geno-molecular neural changes with a potentially favorable effect regarding the response (immediate and long-term) to spinal cord injury.

References

1. Cieza A, Kirchberger I, Biering-Srensen F, Baumberger M, Charlifue S, Post MW, et al. ICF Core Sets for individuals with spinal cord injury in the long-term context. Spinal Cord [Internet]. 2010;48:305–12. Available from: www.nature.com/sc
2. Barclay L, McDonald R, Lentin P. Social and community participation following spinal cord injury: A critical review. Vol. 38, International Journal of Rehabilitation Research. Lippincott Williams and Wilkins; 2015. p. 1–19.
3. Yang F, Luo J. Endoplasmic Reticulum Stress and Ethanol Neurotoxicity. Biomolecules [Internet]. 2015 [cited 2023 Dec 17];5:2538–53. Available from: www.mdpi.com/journal/biomolecules/
4. Stoica SI, Tănase I, Ciobanu V, Onose G. Initial researches on neuro-functional status and evolution in chronic ethanol consumers with recent traumatic spinal cord injury. J Med Life. 2019;12(2).
5. Stoica Simona Isabelle. Research on the consequences of chronic ethanol impregnation in the evolution of myelic lesions in patients with spinal cord injury [Internet] [PHD THESIS SUMMARY]. [Bucharest]: Unviersity of Medicine and Pharmacy "Carol Davila" Bucharest; 2022 [cited 2023 Nov 18]. Available from: https://umfcd.ro/sustinere-teza-drd-stoica-a-simona-isabelle/
6. Dalkilic T, Fallah N, Noonan VK, Salimi Elizei S, Dong K, Belanger L, et al. Predicting Injury Severity and Neurological Recovery after Acute Cervical Spinal Cord Injury: A Comparison of Cerebrospinal Fluid and Magnetic Resonance Imaging Biomarkers. J Neurotrauma. 2018 Feb 1;35(3):435–45.
7. Mortezaee K, Khanlarkhani N, Beyer C, Zendedel A. Inflammasome: Its role in traumatic brain and spinal cord injury. Vol. 233, Journal of Cellular Physiology. 2018.
8. de Vasconcelos NM, Lamkanfi M. Recent insights on inflammasomes, gasdermin pores, and pyroptosis. Cold Spring Harb Perspect Biol. 2020 May 1;12(5).
9. Galluzzi L, Vitale I. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. Cell Death Differ [Internet]. 2018;25:486–541. Available from: https://doi.org/10.1038/s41418-017-0012-4
10. Mei X, Danmeng Z, Wang H, Ru Y, Li F, Wang H, et al. Article 926780 (2022) Mechanism of Ferroptosis and Its Role in. Frontiers in Neurology | www.frontiersin.org [Internet]. 2022;1:926780. Available from: www.frontiersin.org
11. Khoury MK, Gupta K, Franco SR, Liu B. Necroptosis in the Pathophysiology of Disease. Vol. 190, American Journal of Pathology. 2020.
12. Fricker M, Tolkovsky AM, Borutaite V, Coleman M, Brown GC. Neuronal cell death. Vol. 98, Physiological Reviews. 2018.
13. Frank D, Vince JE. Pyroptosis versus necroptosis: similarities, differences, and crosstalk. Vol. 26, Cell Death and Differentiation. 2019.
14. Alizadeh A, Dyck SM, Karimi-Abdolrezaee S. Traumatic Spinal Cord Injury: An Overview of Pathophysiology, Models and Acute Injury Mechanisms. Front Neurol [Internet]. 2019 [cited 2022 May 13];10:282. Available from: /pmc/articles/PMC6439316/
15. Long JS, Ryan KM. New frontiers in promoting tumour cell death: Targeting apoptosis, necroptosis and autophagy. Vol. 31, Oncogene. 2012.
16. Pistritto G, Trisciuoglio D, Ceci C, Alessia Garufi, D'Orazi G. Apoptosis as anticancer mechanism: Function and dysfunction of its modulators and targeted therapeutic strategies. Vol. 8, Aging. 2016.
17. Xu X, Lai Y, Hua ZC. Apoptosis and apoptotic body: Disease message and therapeutic target potentials. Vol. 39, Bioscience Reports. 2019.
18. Harper C. The Neuropathology of Alcohol-Related Brain Damage. Alcohol & Alcoholism [Internet]. 2009 [cited 2022 Jun 3];44(2):136–40. Available from: http://www.braindonors.org
19. Rigler SK. Alcoholism in the Elderly [Internet]. Vol. 61, Am Fam Physician. 2000. Available from: https://www.aafp.org/pubs/afp/issues/2000/0315/p1710.html
20. Stoica SI, Onose G, Pitica IM, Neagu AI, Ion G, Matei L, et al. Molecular Aspects of Hypoxic Stress Effects in Chronic Ethanol Exposure of Neuronal Cells. Curr Issues Mol Biol [Internet]. 2023 Feb 1 [cited 2023 Nov 18];45(2):1655. Available from: /pmc/articles/PMC9955714/