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Adverse effect of the disturbances of glycemia and insulimemia on model PC12 cells – preliminary report

Niekorzystny wpływ zaburzeń glikemii oraz insulinemii na modelowe komórki PC12 – doniesienie wstępne

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Abstract

Background. One of the most important worldwide health problems of the 21st century is an increasing incidence of diabetes and insulin resistance. Morover, it is indicated that both these disturbances are connected with an increased incidence of Alzheimer's Disease. The literature data indicate that not only disturbed glucose concentration, especially hyperglycemia, is a crucial factor of the development of dementia but those data also emphasize that hyperphysiological concentrations of insulin and insulin resistance of brain tissue is an increasingly significant factor. **The aim of this study** was to evaluate the influence of glucose and insulin concentration reached in human carbohydrate metabolism disorders such as i.e. impaired fasting glucose, impaired glucose tolerance and diabetes state as well as averageand high degree hyperinsulinemia, on the survival of PC12 cell line. **Material and methods.** Because of the close association indicated between diabetes and neurodegenerative diseases, in the experiment we used PC12 cell line derived from a transplantable rat pheochromocytoma, commonly used as an neurotoxicity and neuroprotection model. These cells were incubated in RPMI 1640 with addition of fetal bovine serum, horse serum, antibiotics and appropriate concentrations of glucose(from 84 to 240 mg/mL) and insulin(0.5 to 7 mg/mL) at 37°C in a humidified atmosphere containing 5% of $CO_2$for 24 h and 48 h. Cell viability was expressed as a percentage of survival (PS [%]) against the negative control after MTT assay execution. **Results.** The highest mortality was demonstrated for PC12 lines incubated for 24 h with the glucose level reflecting the condition of diabetes mellitus (DM), while after an incubation period of 48 h, the highest mortality was demonstrated for the incubation with insulin concentration corresponding to high levels of hyperinsulinemia (HH). **Conclutions.** The results suggest the greater susceptibility of PC12 cells to extended hyperinsulinaemia incubation than hyperglycemia,which indicates the increasing importance of insulin disorders in the induction of cell death.The results demonstrated in our experiment are particularly important for the development of a study model for testing the substances with hypoglycaemic, hypoinsulinemic and neuroprotective action.

Key words

hyperinsulinemia, hyperglycaemia, PC12, neurodegenerative diseases

Streszczenie

Wstęp. Jednym z najważniejszych światowych problemów zdrowotnych XXI wieku jest rosnąca liczba przypadków cukrzycy i oporności na insulinę. Jednocześnie wskazuje się, że oba te zaburzenia są związane ze zwiększoną zapadalnością na chorobę Alzheimera. Dane literatury potwierdzają, że nie tylko zaburzenia metabolizmu glukozy, zwłaszcza hiperglikemia, są istotnym czynnikiem rozwoju chorób otępiennych, ale przypisują temu coraz większy udział ponadfizjologicznych stężeń insuliny i insulinooporności tkanki mózgowej. **Celem pracy** była ocena wpływu stężeń glukozy i insuliny zakwalifikowanych do zaburzeń metabolizmu węglowodanów, takich jak nieprawidłowe stężenie glukozy na czczo, nieprawidłowa tolerancja glukozy oraz stan cukrzycy jak również hiperinsulinemia w średnim i wysokim stopniu, na przeżywalność komórek linii PC12. **Materiał i metody.** Ze względu na wskazywany ścisły związek cukrzycy i chorób neurodegeneracyjnych w eksperymencie wykorzystano linię komórkową PC12 pochodzącą z przeszczepowego szczurzego ogniska chromochłonnego, stosowaną powszechnie w modelu badań neurotoksyczności i neuroprotekcji. Komórki inkubowano w RPMI 1640 z dodatkiem płodowej surowicy bydlęcej, surowicy

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końskiej, antybiotyków i odpowiednich stężeń glukozy (od 84 do 240 mg/mL) i insuliny (od 0,5 do 7 mg/mL) w 37°C w wilgotnej atmosferze zawierającej 5% CO2 przez 24 h i 48 h. Żywotność komórek wyrażono jako procent przeżywalności (PS[%]) względem negatywnej kontroli po wykonaniu testu MTT. **Wyniki.** Najwyższą śmiertelność wykazano w przypadku komórek linii PC12 inkubowanych przez 24 godziny ze stężeniem glukozy odzwierciedlającym stan cukrzycy (DM), zaś po okresie 48-godzinnej inkubacji wykazano największą śmiertelność po inkubacji ze stężeniem insuliny odpowiadającym wysokiemu poziomowi hiperinsulinemii (HH). **Wnioski.** Wyniki sugerują większą wrażliwość komórek PC12 na wydłużoną inkubację w warunkach hiperinsulinemii niż hiperglikemii, co wskazuje na większe znaczenie zaburzeń insulinemii w indukowaniu śmiertelności komórek. Wyniki eksperymentu są szczególnie ważne w opracowaniu modelu badań do testowania substancji o działaniu hipoglikemicznym, hipoinsulinemicznym i neuroprotekcyjnym.

Słowa kluczowe
hiperinsulinemia, hiperglikemia, PC12, choroby neurodegeneracyjne

Introduction

The increasing incidence of diabetes is one of the most important worldwide health and economic problems of the 21st century. Chronic hyperglycemia in diabetes consequently leads to damage, failure and dysfunction of many organs, including kidneys, heart, eyes, blood vessels and neurons. In addition, it affects the immune system, triggering susceptibility to infections, prolonging their maintenance, potential in fungal and bacterial infections [1, 2]. The pathogenesis of diabetes based on two main pathophysiological defects, namely impaired insulin secretion by pancreatic beta cells and reduced peripheral tissue sensitivity to this hormone, what is defined as insulin resistance. The full-blown diabetes isassociated with insulin resistance and it is often preceded by the presence of supraphysiological levels of insulin (hyperinsulinemia) [3]. Hyperglycemia adversely affects the secretion and action of insulin, especially by secondary glucotoxic damage of pancreatic beta cells (this phenomenon is called glucotoxicity) [1]. Insulin physiologically acts through the connection with a transmembrane fragment of insulin receptor with the features of tyrosine kinase presence on almost all cells in different tissues and organs. This induces signal liberation by tyrosine phosphorylation and its transmition into two pivotal intracellular pathways, i.e. caspase of 3-phosphatidylinositol kinase and extracellular regulated kinase, which results in a biological effect onto the cells [4]. The role of insulin and insulin receptor (IR) on peripheral tissues is related to the regulation of glucose metabolism, and in the central nervous system this hormone mediates in many different processes. Insulin resistance and hyperinsulinemia have negative systemic consequences on the organism and also participate in neurological disturbances and pathogenesis of neurodegenerative diseases. Abnormal carbohydrate metabolism is associated with both vascular and neurodegenerative cognitive dysfunction [5,6]. In the course of diabetes, the changes in the structure and physiological functions of neurons and neurotransmitter disorders are observed. Moreover, in people with diabetes, the risk of dementia occurrence is increased [5]. The literature data indicate that not only is disturbed glucose concentration, especially hyperglycemia, a crucial factor of the development of dementia but it is also emphasized that hyperphysiological concentrations of insulin and insulin resistance of brain tissue are increasingly significant factors [7]. Recent studies on type 2 diabetes mellitus (T2DM) and Alzheimer's disease (AD) suggest the existence of the link between these two conditions and indicate the increased risk of development of these two disorders with ageing of patients. Apart the age, insulin resistance is indicated as one of the important risk factor of AD and T2DM. It is believed that people with type 2 diabetes are at 2.5 times highere risk of developing AD in the future [8]. Threfore, it is important toassess the effects of hyperphysiological concentrations of glucose in comparison to the effects of hyperphysiologicalinsulin levels on the nervous system.Due to the problems with estimating their impact on the neuronal cells, its estimation in the model studies is proposed. PC12 cells line, derived from a pheochromocytoma of the rat adrenal medulla, are most commonly used in ex vivo model[9],which was also used in our experiments.

The aim of this study wasto evaluate the influence of the hyperphysiological concentrations of glucose and insulin according to the concentration reached in human carbohydrate metabolism disorders, on the survival of PC12 cell line. In our ex vivo model we examined these substances in concentration reflecting i.e. normal fasting blood glucose, impaired fasting glucoseand diabetes conditionas well as normal insulin level and medium and high degree hyperinsulinemia. PC12 cells were incubated with selected concentration of glucose and insulin for 24 and 48 h and the viability of cell culture was measured using MTT assay assessing cell metabolic activity.

Material and methods

Cell culture

PC12 cell line, derived from a transplantable rat pheochromocytoma, is indicated to be useful in studies focused on glutamate-induced cytotoxicity leading to neurodegeneration. It has been confirmed that PC12 synthesizes dopamine, glutamate and can be easily differentiated into a sympathetic phenotype expressing neurites using Nerve Growth Factor (NGF). The presence of the responding effectors and pathways in PC12 such as:cystine/glutamate antiport, caspases, cytochrome c, ERK

pathway and PkB/Akt/PI3 pathway, is also confirmed [10]. As it is preliminary report, we used undifferentiated PC12 cell line, grown in RPMI 1640 (Biological Industries) with 10% fetal bovine serum (Biological Industries, USA), 5% horse serum (Biological Industries, USA) and addition of 1% penicillin-streptomycin (Sigma Aldrich, USA). The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in Panasonic MCO-170AIC incubator. Both the passage and setting up of experimental 96-well plates were initiated by the trypsinisation processusing trypsin (0.25% trypsin solution in PBSwith 0.02% EDTA). Figure 1 presents the morphology of PC12 cells under the inverted miscroscope (Delta Optical IB-100).

Glucose and insulin concentrations used in the experiment

In these preliminary studies of the influence of glucose and insulin concentrati on reached in human carbohydrate metabolism disorders on the survival of PC12 cells we used the concentration of both agents based on the diagnostic criteria for dysglycemia published by Polish Diabetes Association (PTD), World Health Organization (WHO) [11], and dysinsulinemia according to laboratory reference range [12], which is shown in Table I.

Negative control consisted of PC12 cells in RPMI 1640 hepes with the addition of horse and bovine serum and antibiotics only. For the purpose of the experiment, it was assumed that the highest value of both insulin and glucose should not result cell mortality higher than 80% compared to negative control. On the basis of these assumptions, the mean concentrations of insulin and glucose representing the individual states of glycemia and insulinemia were experimentally determined.

For the preparation of appropriate concentrations of insulin it was necessary to convert enzyme activity units (μIU) into its mass. A factor proposed by the World Health Organization (WHO) specifies that 1 unit of human insulin activity corresponds to 0.0347 mg [13]. The results of the calculations were also confirmed by the traditional molar mass method of insulin – 5808 g/mol [14].The final value ofglucoseconcentrations used in the experiment ranged from 84 to 240 mg/mL, and insulin concentration rangedfrom 0.5 to 7 mg/mL. The detailed data for glucose and insulin concentration reflected individual glycemia (NFBG, IFG, NGT, IGT, DM) and insulinemia (NFBI, AH, HH) disturbances which are presented in the Table II.

Cell viability assay – MTT

MTT viability assay based on the reduction of a tetrazolium dye (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) to its insoluble formazan, which has a purplecolor. MTT is used to analyze the viability of metabolically active cells by determining the degree of activity of energy metabolism which is reflected bythe activity of NAD(P)H-dependent cellular oxidoreductase enzymes connected with mitochondrial membranes. The amount of color-reduced MTT crystals is proportional to the oxidative activity of the mitochondria of the cell and the more live cells the higher optical density read at 570 nm [15]. The cells growing in culture vessels were detached and seeded on 96-well plates at a density of 4×10^4 cell per well. After the incubation with different glucose and insulin concentrations, the medium of each well was replaced with 100μl of 0.5 mg/mL MTT (Sigma Aldrich, USA) solution in RPMI 1640. After 3 hours of incubation, 100 μL of isopropanol with 0.04 M HClfor each well was added. The absorbance was quantified using a multiwell scanning at 570 nm (SYNERGY HTX, BioTek Instruments). Cell viability was expressed as a percentage of survival (PS%) against the negative control according to the formula: PS [%] = (a-x) / (K-x) * 100%,

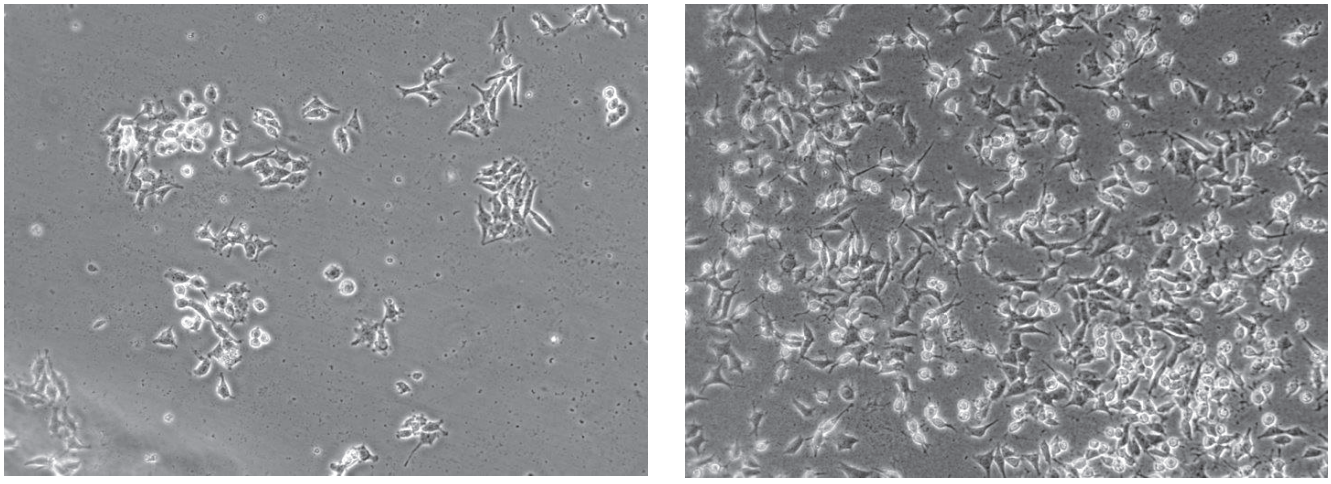

Fig. 1. PC12 cell culture under a microscope (100x) at low confluence (A) and before the trypsynization process – confluence about 80% (B)

Ryc. 1. *Hodowla komórek PC12 pod mikroskopem (100x) przy niskiej konfluencji (A) i przed procesem trypsynizacji – konfluencja około 80% (B)*

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Table I. Characteristics of the states reflecting different glycemic and insulinemic disorders and their reference ranges
Tabela I. *Charakterystyka stanów odzwierciedlających różne zaburzenia glikemii i insulinemii oraz ich zakresy referencyjne*

Stan/State	Skrót/Abbreviation	Zakres referencyjny/ Reference Ranges
prawidłowa glikemia na czczo/ Normal Fasting Blood Glucose*	NFBG	70–99 mg/dL
Nieprawidłowa glikemia na czczo/Impaired Fasting Glucose*	IFG	100–125 mg/dL
Prawidlowa tolerancja glukozy/Normal Glucose Tolerance*	NGT	<140 mg/dL
Nieprawidłowa tolerancja glukozy/Impaired Glucose tolerance *	IGT	140–199 mg/dL
Cukrzyca/Diabetes Mellitus *	DM	>200 mg/dL
Prawidłowa insulinemia na czczo/Normal Fasting Blood Insulin**	NFBI	<24.9 μIU/mL
Hiperinsulinemia średniego stopnia/Average Hyperinsulinemia ***	AH	25–150 μIU/mL
Hiperinsulinemia wysokiego stopnia/High Hyperinsulinemia ***	HH	>150 μIU/mL

*2017 Guidelines on the management of diabetic patients, The Polish Diabetes Association 2017, Vol. 6, Supplement A/WHO recommendation ** Laboratory reference range [Mayo Medical Laboratories, 2017] *** The division of hiperinsulinemia for our experimental model, due to the great dyspertion of insulin concentration in human

Table II. The average glucose and insulin concentrations used in the experiments, for specific glycemic and insulinemic disturbances
Tabela II. *Średnie stężenia glukozy i insuliny stosowane w eksperymentach dla wybranych zaburzeń glikemii i insulinemii*

Stan/State	Średnie stęże nieglukozy/Average glucose concentration [mg/dL]	Średnie stężenie insuliny/ Average value of insulin [μIU/mL]	Średnie stężenie insulin/Average value of insulin [mg/mL]
NFBG	84	-	-
IFG	120	-	-
NGT	120	-	-
IGT	168	-	-
DM	240	-	-
NI	-	15	0.5
AH	-	55	2
HH	-	200	7

where: A – average value of absorbance of PC12 cells exposed to the glucose/insulin, X – average absorbance of the blank reagent containing MTT and isopropanol with HCl, K – average value of absorbance negative control.

Results

The effects of various metabolic states related to different degrees of glycemia and insulinemia were investigated in model of PC12 cell line. The results of the evaluation of their survival after adequate incubation times are shown and

illustrated in the following figures. Figures 2 and 3 present the survival of PC12 cells for individual metabolic states, both for glycemic and insulinemicdisorders, and fasting normal blood level of glucose and insulin, after 24 and 48 hours of incubation, respectively.

For all metabolic conditions: NFBG – normal fasting glucose (84 mg/mL), IFG – abnormal fasting glucose (120 mg/mL), NGT – normal glucose tolerance (120 mg/mL), IGT – abnormal glucose tolerance (168 mg/mL), DM – diabetes (240 mg/mL), NFBI – normal insulinemia (0.5 mg/mlL, AH – average hyperinsulinemia (5 mg/mL) and HH – very high hyperinsulinemia (7 mg/mL), PC12 cell survival has decreased after 24 hours of incubation compared tonegative control. The lowest descrease of percentage of survival cells compared to negative control was observed for NFBG (almost 17%). For IFG and NGT states, the percentage survival of PC12 cell line has decreased by almost half (about 45%) in comparison to the negative control. IGT state demonstrated a greater decrease in the percentage of the survival of PC12 cells by approximately 62% compared to the control. In the case of diabetes state (DM), the percentage of survival was decreased similarly, by approximately 65%. Normal fasting blood insulin state is characterized by a decrease in PC12 cell survival of approximately 43% compared to control, while AH and HH demonstrated almost 60% reduction of PC12 survival.

In case of48 h of incubation, a decrease in the survival of PC12 cells for almostall metabolic states compared to the negative control, was also observed. In addition, for DM, AH and HH states, a decrease in cell survival was greater than after the 24 h period of incubation. For a state reflecting diabetes and average hiperinsulinemia after the 48 h incubation, the percentage of survival was similar to the 24 h of incubation, whereas HH state after longer time of incubation demonstrated a significant decrease in cell survival (approximately 20% compared to an incubation period of 24 h). The most noticeable was a decrease in SP% seen with very high hyperinsulinemia (HH) which was almost 80%.

Disscusion

There are many important articles in the current scientific literature focused on the role of insulin and glucose on human neuronal cells. This topic is widely discussed by researchers because of the increasing incidence of both type 2 diabetes and Alzheimer's disease, which are considered to be civilization diseases [16]. Glycemia and insulinemia disturbances, and particularly insulin resistance of peripheral tissues, are obviously associated with the development of diabetes and its complications. On the other hand, attention is being paid to the phenomenon of insulin resistance of the brain in the condition of hyperglycemia and hyperinsulinemia, which, according to indicatons, trigger an increase ofthe development of neurodegenerative disease [17]. That is why it seems to be interesting to assess the influenceof different degrees of hyperinsulinemia (low, middle, and high) and various states of glycemia disturbances in humans, such as impaired fasting glucose, impaired glucose tolerance or diabetes-associated

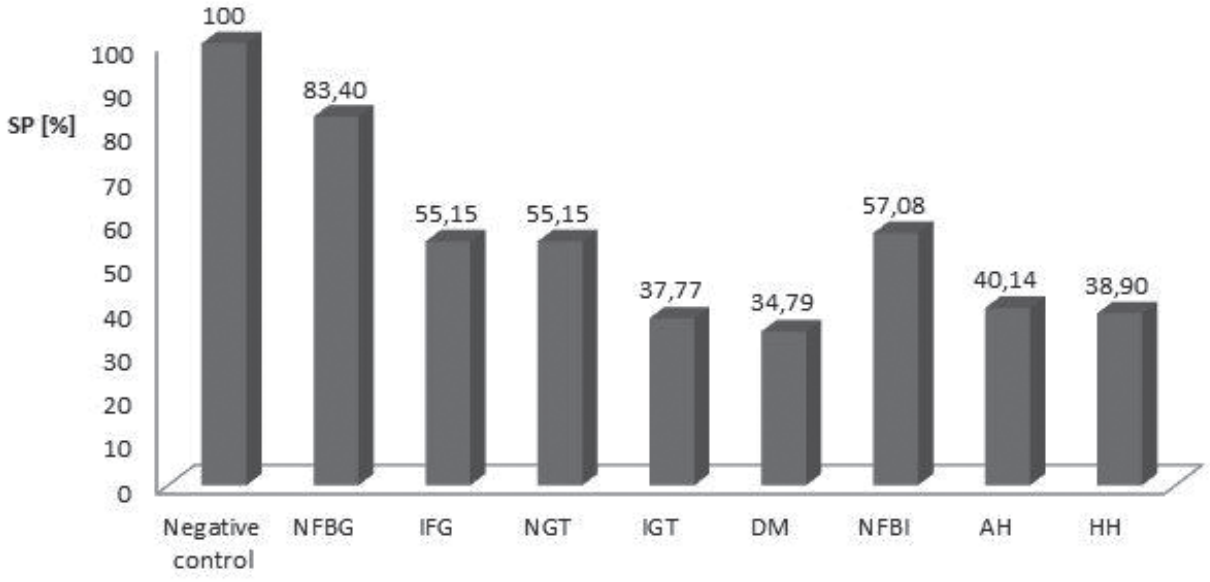

Fig. 2. Comparison of percentage of survival – PS [%] of PC12 cells for different metabolic states after 24 h incubation with appropriate glucose(84–240 mg/mL) and insulin (0.5–7 mg/mL) concentrations
Ryc. 2. *Porównanie procentu przeżywalności – PS [%] komórek PC12 dla różnych stanów metabolicznych po 24 godzinach inkubacji z odpowiednimi stężeniami glukozy (84–240 mg/mL) i insuliny (0.5–7 mg/mL)*

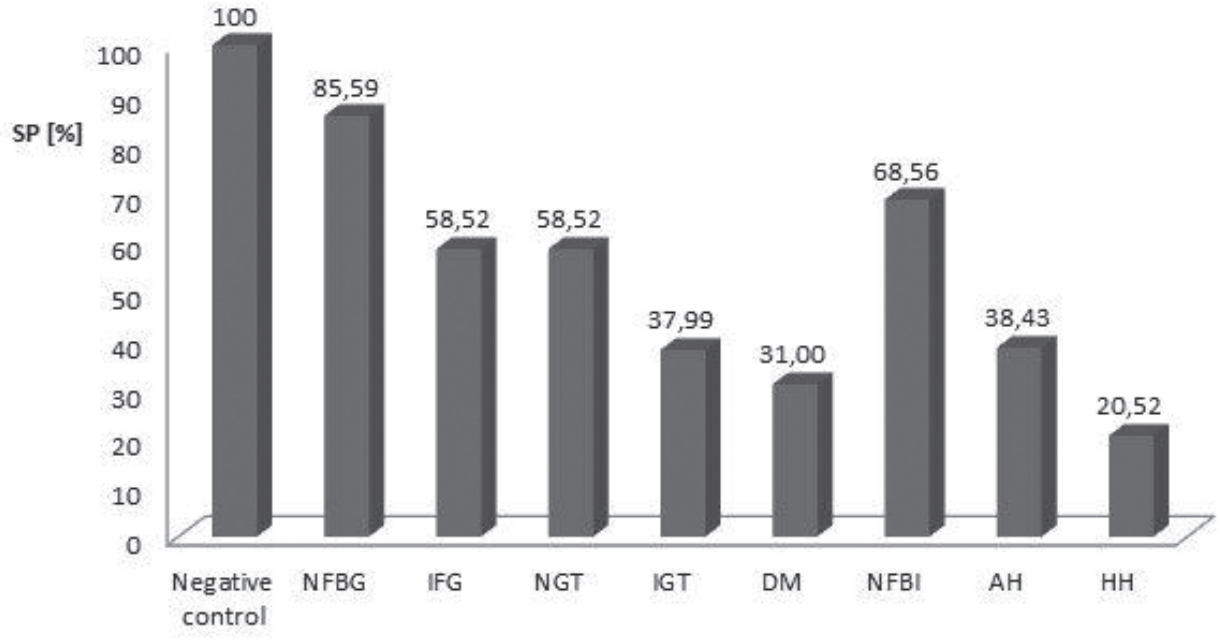

Fig. 3. Comparison of percentage of survival – SP [%] of PC12 cells for different metabolic states after 48 h incubation with appropriate glucose (84–240 mg/mL) and insulin(0.5–7 mg/mL) concentrations
Ryc. 3. *Porównanie procentu przeżywalności – SP [%] komórek PC12 dla różnych stanów metabolicznych po 48 h inkubacji z odpowiednimi stężeniami glukozy (84–240 mg/ml) i insuliny (0.5–7 mg/mL)*

hyperglycemia, on PC12 lines. In the scientific literature there is information about separate influence of glucose or insulin on the PC12 cell line, but there are no studies on the effects of both, insulin and glucose corresponding with human physiological and supraphysiological concentration on PC12 cells.

Our research has been carried out on PC12 cell lines used in neurotoxicity and neuroprotection model studies and it is the first study to assess the effects of insulin and glucose concentrations achievable in humans in certain states of carbohydrate metabolism disorders on PC12 cell line. We adopted the diagnostic criteria for dysglycemia published by Polish Diabetes Association (PTD), World Health Organization (WHO) [11], and dysinsulinemia according to laboratory reference range [12]. In our study, the highest survivability of PC12 lines in the study model was observed, both after 24 h and 48 h, for fasting blood glucose (NFBG), where SP% was 83.40 – 85.58%. For normal fasting blood insulin, we observed roughly 20% lower survival (SP% = 57.08 – 68.56%) for both incubation times, indicating a greater insulin uptake in apoptosis induction in the experimental model we used. The highest mortality, approximately 65%, was demonstrated for PC12 lines incubated for 24 h with glucose levels reflecting the condition of diabetes mellitus. However, after an incubation period of 48 h, the highest mortality was demonstrated for incubated cells with insulin levels corresponding to high levels of hyperinsulinemia (HH) – 80% mortality, suggesting greater susceptibility of PC12 cells to extended hyperinsulinaemia incubation than hyperglycemia.

Hyperglycemia plays a key role in the development of diabetic neuropathy, which can be associated with neuronal cell death caused by high glucose levels [18]. Last year, Chen et al. [19] conducted a study where PC12 lines were treated with high glucose concentrations of 75 mM. The results confirmed significantly less viability compared to the control group where the cells were treated with lower glucose concentrations of 25 mM. Nuclear magnetic resonance (NMR) was used to investigate metabolic changes in PC12 cells treated with high glucose concentrations. Results showed that the death of PC12 cells caused by high glucose levels may be related to changes in energy metabolism, amino acid metabolism, osmoregulation, and membrane metabolism. This year, Jiang et al. [20] conducted the studies of the mediation of insulin-like growth factor-1 (IGF-1) in Alzheimer's disease, the mechanism of gene expression of a human gene encoding a prion protein P (Prion Protein – PRNP), APP protein level, and PI3K/Akt signaling pathway.The model of AD represented by PC12 with A_{25-35} treatment were incubated with different doses of IGF-1 (0, 20, 40 and 80 ng/ml). After 24 hours of incubation mRNA levels PRNP were evaluated using quantitative PCR and western blotting, respectively. With the increase of the dose of IGF-1, the expression of PRNP mRNA and APP protein were more highly expressed in comparison to groups treated with lower IGF-1 concentrations. Furthermore, with regard to the Akt protein, the level of expression of PRNP mRNA, APP protein and pAkt protein in IGF-1 treated groups was significantly higher than

in control and investigated groups. This study demonstrated that IGF-I may mediate the expression of the PRNP gene and APP protein via the PI3K / Akt signaling pathway, in the AD model. As some researchers suggest, insulin receptors and their signaling pathways occur in certain areas of the brain that mediate important physiological effects of that organ such as neuronal development, cognitive processes, including learning and memory. However, it remains unclear and is still being investigated whether insulin is produced in the brain [21]. Resent studies have demonstrated that high levels of insulin are present in human brain, but its origins are still a matter of controversy. There is a hypothesis that part of "brain insulin" is produced in the central nervous system (CNS). Other researchers are contemplating the peripheral origins of insulin, and then crossing the blood-brain barrier [7].

The results of our study are particularly important for the development of a research model for the substances with hypoglycaemic, hypoinsulinemic and neuroprotective action. A good example of the application of our research model would be to re-examine ghrelin for particular states of metabolic disorders not only for glucose but also for insulin [22]. This is especially interesting in the light of indicatedassociation between diabetes and neurodegenerative diseases.

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References

1. Korzeniowska K, Jabłecka A. *Cukrzyca* (Część I). Farm Współ. 2008;1:231-235.
2. Małecki MT. *Otyłość – insulinooporność – cukrzyca typu 2.* Kardiol Pol. 2006;64:561-566.
3. Kinalska I. *Znaczenie upośledzenia wydzielania insuliny w patogenezie cukrzycy typu 2 – implikacje kliniczne.* Diabetol Prakt. 2001;2:7-9.
4. Marchewka Z, Guzy K, Rorbach-Dolata A, Piwowar A. *Leki jako czynniki ryzyka rozwoju hiperglikemii oraz mechanizmy ich działania diabetogennego.* Farm Pol. 2017;73:105-107.
5. Kurowska E, Majkutewicz I. *Zaburzenia sygnalizacji insulinowej w kontekście choroby Alzheimera. Zagadnienia aktualnie poruszane przez młodych naukowców.* 2015;3:347-350.
6. De la Monte S. *Brain Insulin Resistance and Deficiency as Therapeutic Targets in Alzheimer's Disease.* Curr Alzheimer Res. 2012;9:35-66.
7. Blázquez E, Velázquez E, Hurtado-Carneiro V, Miguel Ruiz-Albusac J. *Insulin in the brain: its pathophysiological implications for states related with central insulin resistance, type 2 diabetes and Alzheimer's disease.* Front Endocrinol. 2014;5:161.
8. Nicolls R. *The clinical and biological relationship between Type II diabetes mellitus and Alzheimer's disease.* Curr. Alzheimer Res. 2004;1:47-54.
9. Greene L, Tischler A. *Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor.* Proc Natl Acad Sci. 1976;73:2424-2428.
10. Kritis A, Stamoula E, Paniskaki K, Vavilis T. *Researching glutamate - induced cytotoxicity in different cell lines: a comparative/collective analysis/study.* Front Cell Neurosci. 2015:9-91.
11. *Zalecenia kliniczne dotyczące postępowania u chorych na cukrzycę 2017. Stanowisko Polskiego Towarzystwa Diabetologicznego.* Diabetol Prakt. 2017;6:Supl. A.
12. Mayo Medical Laboratories, accessed: 10.08.2017 (http://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/8664)
13. Burns C, Morris T, Jones B, Koch W et al. *Proposal to initiate a project to evaluate a candidate International Standard for Human Recombinant Insulin.* WHO/BS/10.2143 – Working document QAS/10.381.
14. Marcovina S, Bowsher R, Miller W, Staten M et al. *Insulin Standardization Workgroup. Standardization of insulin immunoassays: report of the American Diabetes Association Workgroup.* Clin Chem. 2007;53:711-716.
15. Patel N, Oudemans P, Hillman B, Kobayashi D. *Use of the tetrazolium salt MTT to measure cell viability effects of the bacterial antagonist Lysobacterenzymogenes on the filamentous fungus Cryphonectriaparasitica.* Antonie Van Leeuwenhoek. 2013;103:1271-1280.
16. Pardeshi R, Bolshette N, Gadhave K, Ahire A et al. *Insulin signaling: An opportunistic target to minify risk of Alzheimer's disease.* Psychoneuroendocrino. 2017;30:159-171.
17. De Felice F, Lourenco M., Ferreira S. *How does brain insulin resistance develop in Alzheimer's disease?* Alzheimers Dement. 2014;10:26-32.
18. Okouchi M, Okayama N, Aw TY. *Hyperglycemia potentiates carbonyl stress-induced apoptosis in naïve PC-12 cells: relationship to cellular redox and activator protease factor-1 expression.* Curr Neurovasc Res. 2005;2:375-386.
19. Chen M, Zheng H, Wei T, Wang D et al. *High Glucose-Induced PC12 Cell Death by Increasing Glutamate Production and Decreasing Methyl Group Metabolism.* Biomed Res Int. 2016:4125731.
20. Jiang G, Wang C, Zhang J, Liu H. *Mediation of insulin growth factor-1 in Alzheimer's disease and the mechanism of PRNP genetic expression and the PI3K/Akt signaling pathway.* Exp Ther Med. 2017;13:2763-2766.
21. Akintola A,van Heemst D. *Insulin, aging, and the brain: mechanisms and implications.* Front Endocrinol. 2015;6:13.
22. Liu X, Xiao Q, Zhao K, Gao Y. *Ghrelin Inhibits High Glucose-Induced PC12 Cell Apoptosis by Regulating TLR4/NF- B Pathway.* Inflammation. 2013;36:1286-1294.

