

ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Effect of alcohol on insulin secretion and viability of human pancreatic islets

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Alcohol consumption is a part of the tradition and customs of many communities worldwide. It is customary to take a glass of drink as an aperitif, and wine or beer during or after meals. Alcohol is taken primarily for good mood or for better digestion. The question is how and to what extent the amount of consumed alcohol affects insulin secretion of human adult pancreatic islets. Perfusion model of the rat pancreas and measure of basal insulin secretion showed that ethanol reduced glucose-induced insulin secretion by means of dose-related effect [1].

Dembele et al. [2] examined the effects of ethanol and fatty acids on β -cell (cell line from rats) metabolism and survival. It was observed that both substances generate cellular oxidative stress, and affect mitochondrial function. Ethanol causes β -cell death by apoptosis, while fatty acids cause cell death predominantly by necrosis. Pancreatic β -cell dysfunction is a prerequisite for the development of type 2 diabetes. Alcoholism is a risk factor and ethanol increases oxidative stress in β -cells. Cells in such a state increase expression of PHB (poly3-hydroxybutyric acid) synthase genes, to protect them from the harmful effects of ethanol [3].

Studies in rats *in vivo* showed that long-term alcohol feeding suppresses apoptosis in the pancreas; however, it increases the sensitivity of acinar cells to endotoxin-induced injury that may cause pancreatitis in alcoholics [4]. Alcohol causes reactive hypoglycemia by attenuating the release of counter regulatory hormones, redistribution of pancreatic blood flow and direct stimulation of insulin secretion. Signaling of ethanol-induced insulin secretion from rat insulin-secreting cell lines (INS-1 and INS-1E) bypasses calcium and protein kinase C (PKC) involving steps. An extra pool of secretory vesicles not available for glucose is exploited for exocytosis after ethanol stimulation [5]. Study of an *in-vitro* isolated rat pancreas perfusion system showed that ethanol decreases glucose-stimulated insulin secretion. Second phase secretion (30–60 minutes) was inhibited at both low (100 mg/dl) and high (1,000 mg/dl) alcohol concentration [6]. Several studies have shown that ethanol causes insulin resistance in the liver and skeletal muscle by interfering with insulin signaling [7, 8]. One study, examining the influence of alcohol, ethanol concentration of 20 mM or 80 mM for 24 or 48 hours in β -cell lines and isolated murine islets showed that chronic exposure to ethanol causes β -cell dysfunction by re-

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ducing not only insulin secretion but also β -cell insulin content by interfering with muscarinic signaling and PKC activation but not the K-ATP channels [9]. Light-to-moderate alcohol consumption (for women < 28, 28–64, > 64 g/week) for healthy women was associated with enhanced insulin sensitivity, reduced basal insulin secretion rate, and lower fasting plasma glucagon concentration, providing consistent mechanisms for the reduced risk of type 2 diabetes [10]. Data for the effect of alcohol consumption on insulin secretion are conflicting; some studies show the absence of an association in men [11, 12]. The final product of ethanol oxidation is acetate, which is converted into acetyl-CoA in peripheral tissue. Acetate may modulate fat oxidation and decrease lipolysis. Therefore, an increase in skeletal muscle oxidative capacity after alcohol intake has been suggested even though evidence is limited in humans [13, 14].

The aim of the present study is to examine effect of ethanol on viability and insulin secretion of human adult pancreatic islets in the culture during short-term incubation period (seven days) to solve the controversy present in the literature.

METHODS

Human adult pancreatic tissue was procured from the Institute for Gastrointestinal Diseases, Clinical Center of Serbia, Belgrade. Tissue samples were collected from live donors, after total or subtotal pancreatectomy for cysts or tumors [15]. In tumors, healthy tissue was obtained near the line of the resection. Histopathological analysis of the tissue samples showed no tumor cells. All procedures were performed in accordance with the rules of Ethical Committee of the School of Medicine in Belgrade. Written consent was obtained from all patients.

Pancreatic tissue was transported in physiological solution from the Institute for Gastrointestinal Diseases to the Laboratory for Cell Culture at the Clinic for Endocrinology, Diabetes and Metabolic Diseases. The material was kept in a refrigerator at 4°C (cold ischemia). Biometric data of the material (tissue weight, cold and warm ischemia) are given in Table 1.

Table 1. Biometric data of samples

Parameters	Tissue weight (g) mean \pm SE	Warm ischemia (min.) mean \pm SE	Cold ischemia (min.) mean \pm SE
Control group	5.03 \pm 0.57	117.2 \pm 17.14	70.2 \pm 28.33
Group with alcohol	2.26 \pm 0.48	128.63 \pm 20.06	112.13 \pm 35.70
Statistical significance (p \leq 0.05)	0.195	0.061	0.1

Digestion of pancreatic tissue

For pancreatic tissue digestion we used Collagenase XI – product number C7657 (Sigma-Aldrich, St. Louis, MO, USA), activity > 1,200 collagen digestion units per mg

solid; 2–5 FALGPA hydrolysis units per mg solid). This enzyme preparation also contained clostripain, nonspecific neutral protease and tryptic activities. Isolation of pancreatic islets was performed in aseptic conditions in a laminar flow hood by the non-automatic method [16]. The tissue was transferred to Hanks solution (Sigma-Aldrich) and mechanically chopped. This material was collected with a pipette and put in test tubes containing the Collagenase solution (5 mg/ml concentration). The duration of incubation was 30 minutes at 37°C with occasional mechanical stirring. After the incubation, the content of the test tube was centrifuged at 400 \times g for 10 minutes at 15°C. Supernatant was decanted and the remaining islets were rinsed several times with Hanks solution to eliminate excess of lipids and collagenase. After rinsing, islets were resuspended in the final RPMI 1640 medium (Sigma-Aldrich) supplemented with 0.1% L-glutamine, 5.5 mM glucose, 25 mM hepes, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% fetal calf serum (FCS, Sigma-Aldrich). The islets were incubated in Falcon 3013 plastic flasks, volume 50 cc (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a 5% CO₂ and 95% humidity atmosphere for seven days. RPMI medium was changed every 24 hours of incubation. Warm ischemia time is the time measured from the beginning of the mechanical mincing of the tissue, including the isolation procedure, to the moment when the islets were placed in the culture medium. A total of 20 cultures were incubated in the standard medium (control group), and 27 cultures were incubated in the medium with ethanol (test group) in standard conditions for seven days. In the test group, a certain concentration of ethanol was added to the culture medium using Widmark formula. It was taken under the assumption that a man of an average weight of 75 kg drinks two glasses (2 \times 0.5 dl) of 40% alcoholic drink per day, which would make his blood alcohol concentration 0.62‰. The authors have chosen this formula so that the influence of alcohol in *in vitro* researches would be the closest to *in vivo* conditions. In order to achieve specified concentrations, 10 μ l of pure ethanol was added to 100 ml RPMI medium to give 0.00165 M (0.16 mM) solution of alcohol. Ethanol was added to the medium during the incubation every 24 hours when replacing RPMI medium, and before the start of glucose stimulation.

Determination of the viability of isolated human adult islets

Viability of the islets was determined by dithizone (DTZ) staining on the first, third, and seventh day after their isolation.

Preparation of the dithizone solution

Fifty milligrams of DTZ was dissolved in 10 ml of DMSO and 10 ml of Hanks solution. The solution was sterilized by passage through nylon filters with pore size of 0.20 μ m. Samples (1 ml of each culture) were stained with 0.2 ml of DTZ solution and placed in an incubator at 37°C in a 5% CO₂ and 95% humidity atmosphere for 30 min. Stained

islets were rinsed in Hanks solution and resuspended in 1 ml of RPMI medium. Islets viability was determined using a stereo-light microscope and special counting microchambers [17].

Determination of the functional capacity and insulin secretion

To determine preservation of the functional capacity of the isolated islets, glucose-stimulated insulin secretion was measured on the first, third and seventh day of cultivation [18, 19]. A static glucose stimulation assay was performed. Samples (approximately 1,000–2,000 islets per culture) were incubated for one hour in low glucose (2.8 mM/L RPMI) medium with ethanol (10 μ l ethanol in 100 ml RPMI), then one hour in high glucose (20 mM/L RPMI) medium and one hour in low glucose medium again. After each step of stimulation, the cultures were centrifuged at $400 \times g$, for 10 minutes at 15°C. Supernatant was decanted and stored at -18°C for insulin quantification. Insulin content was determined by radioimmunoassay (RIA INSULIN PEG). Sensitivity of the assay is 0.60 mIU/L and detection range 0.6–300 mIU/L. Relative insulin release was expressed as insulin stimulation index (SI) and calculated as the ratio of insulin release during high glucose stimulation to insulin release during low glucose stimulation.

Statistical analysis

All results are expressed as mean \pm standard error. P-value of less than 0.05 was considered to be statistically significant. For statistical analysis of the data, analysis of variance (ANOVA, Fisher) was used.

RESULTS

Islet viability

The percentage of viable islets in the cultures was determined on the first, third and seventh day after isolation. Viability of the islets was determined by DTZ staining and

results are presented in Table 2. Immediately after isolation (day 1) percentage of viable islets incubated in normal medium, without alcohol (control group) and in medium containing alcohol (test group) was 51.58 ± 2.16 and $64.68 \pm 5.99\%$, respectively, and the difference was not statistically significant ($p = 0.090$). On the third day of cultivation, numbers of distinctly stained islets in control and test groups were similar (57.99 ± 6.49 and $54.23 \pm 4.58\%$, respectively, $p = 0.654$). On the seventh day of incubation, viability was higher in the control group ($39.99 \pm 6.27\%$) compared to the test group ($37.79 \pm 5.64\%$), and the difference was statistically not significant ($p = 0.800$).

Comparison of viability by days of incubation (first and third, first and seventh, third and seventh) in the control group showed no statistically significant difference ($p > 0.05$). In the test group, there is statistically significant difference between the first and the seventh day ($p = 0.011$).

Insulin secretion

To determine the functional capacity of the isolated islets, a static glucose test was performed on the first, third and seventh day of cultivation. SI values calculated for both groups and days of cultivation are presented in Table 3. On the first day of stimulation, SI for the control group was 0.60 ± 0.13 and for the test group SI was 1.22 ± 0.27 . The difference was not statistically significant ($p = 0.257$). On the third day of stimulation, SI value for the control group was 0.80 ± 0.16 , and for the test group it was 1.01 ± 0.13 ($p = 0.215$). On the seventh day of stimulation, SI for the control group was 0.80 ± 0.11 , and for the test group SI = 0.60 ± 0.16 ($p = 0.547$).

Comparison of functional capacity by days of incubation (first and third, first and seventh, third and seventh) in the control group showed no statistically significant difference ($p > 0.05$). In the test group, there is a marginal statistical significance between the first and the seventh day ($p = 0.058$). Between the third and the seventh day of cultivation there is a statistically significant difference ($p = 0.028$), while between the first and the third day there is no statistically significant difference ($p = 0.626$).

Table 2. Percentage of colored islets by culture, viability of the human adult pancreatic islets in the culture, for the control group and the group incubated with alcohol during seven days of cultivation

Parameters	Time of cultivation % of viability (mean \pm SE)			Comparison by days of incubation (p-value)		
	1st day	3rd day	7th day	1st and 3rd day	1st and 7th day	3rd and 7th day
Control group	51.60 ± 2.16	58.00 ± 6.49	40.0 ± 6.27	0.376	0.119	0.081
Test group with ethanol	64.70 ± 5.99	54.30 ± 4.58	37.80 ± 5.64	0.216	0.011*	0.053
p-value	0.090	0.654	0.800			

Table 3. Insulin stimulation index in the control group and cultures with ethanol (the test group) on the first, third and seventh day of incubation

Parameters	Time of cultivation Stimulation index (mean \pm SE)			Comparison by days of incubation (p-value)		
	1st day	3rd day	7th day	1st and 3rd day	1st and 7th day	3rd and 7th day
Control group	0.60 ± 0.13	0.80 ± 0.16	0.80 ± 0.11	0.616	0.506	0.966
Test group with ethanol	1.20 ± 0.27	1.00 ± 0.13	0.60 ± 0.16	0.626	0.058	0.028*
p-value	0.257	0.215	0.547			

DISCUSSION

The percentage of distinctly stained cells in the cultures containing ethanol (test group) on the first day of incubation was higher by 25% compared to the control group (medium without ethanol), but on the third and seventh day of incubation, the percentage of stained cells was lower in the test group by 6.38% and 5.5%, respectively. Since the difference is not statistically significant, we can conclude that there is no difference in cell viability between the control and the test group for all days of incubation (first, third, and seventh). Viability in the control group showed no statistical significance by days of incubation. However, in the test group, viability was the highest on the first day of cultivation and declined on the third and seventh day by 16% and 42%, respectively. The difference was statistically significant ($p = 0.011$). These results are acceptable because islet viability is determined by DTZ, which binds to zinc in insulin. On the first day of incubation, β -cells contain the highest concentration of insulin. In test group, alcohol increases the permeability of the membrane and facilitates degranulation and the release of insulin, so depot of insulin is gradually reduced by the length of incubation (from the first to the seventh day).

SI was higher in the test group compared to the control group by 50% and 25% on the first and third day of incubation, respectively, while on the seventh day SI was lower by 25% in the test group. There is no statistically significant difference in SI between control and test groups. However, in the test group there was a decline in insulin secretion during the cultivation by 17% and 50% on the third and seventh day, respectively, compared to the first day ($p = 0.058$). There is also statistically significant decline in insulin secretion by 40% between the third and seventh day ($p = 0.028$).

Similar results are presented in another paper, where insulin secretion in cell culture increases after 24 hours of incubation with lower alcohol concentration (20 mM) [9]. However, higher concentration of alcohol (80 mM) has the opposite effect. After 48 hours of incubation, concentration of alcohol reduces the level of insulin secretion compared to the control group. Results of other authors confirm that ethanol reduces insulin secretion [1, 6]. It should be emphasized that reduction in the number of stimulated β -cells during the incubation due to the effects of natural processes of apoptosis, and necrosis does not affect the obtained values of insulin secretion, expressed in the form of SI, which represents the ratio between the values of insulin secretion after stimulation with high glucose concentration and values after low glucose stimulation. Hence, secretory capacity does not depend of the total number of stimulated cells. Although in laboratories worldwide there is a practice to associate SI values with a strict number of stimulated cells [19].

Some authors try to explain how alcohol causes increased or decreased insulin secretion in response to glucose stimulation. Stimulation of insulin secretion with diacylglycerol is explained by the release of Ca from endoplasmic reticulum [20]. Response to high glucose stimula-

tion decreases due to reduced levels of cyclic adenosine monophosphate (cAMP) caused by reduced levels of adenosine triphosphate (ATP) [2, 3, 21].

In standard conditions, glucose-stimulated insulin secretion increases oxygen consumption and ATP. This is associated with potassium (K^+)-induced membrane depolarization, leading to rapid entry of Ca^{2+} ions into the cell through voltage-dependent channels. Fusion of secretory granules containing insulin with the cell membrane depends of calcium ions. cAMP emphasizes action of glucose and amino acids, and stimulates the release of Ca^{2+} ions from intracellular organelles, or may activate a kinase that phosphorylates one of the components of microfilaments/tubules system, so that this structure becomes contractile and sensitive to Ca^{2+} ions. If the Na^+ outside the cell is replaced with other monovalent cation, then the effects of glucose and other secretagogues are reduced. Na^+ concentration may regulate the intracellular Ca^{2+} using a co-transportation system [21]. It is possible that the effect of certain concentrations of ethanol somehow bypasses the dependence of Ca^{2+} release from the synthesis of cAMP.

Higher SI values in the test group (SI higher by 50% on the first day of incubation) mean increased insulin secretion of pancreatic islets in response to high glucose stimulation (see chapter Methods). It is probable that alcohol consumed immediately before or during ingestion can have a protective effect in type 2 diabetes because increased insulin release reduces the harmful effects of postprandial hyperglycemia. It is known that hyperglycemia has a toxic effect on β -cells because it increases the percentage of their apoptosis [22]. The body is struggling to maintain a normal blood sugar level, so a chronic state of hypoglycemia occurred after chronic alcohol consumption, especially if alcohol is taken without food, can lead to insulin resistance, which exists in the pathogenesis of type 2 diabetes [23]. Acute alcohol consumption increases insulin secretory capacity of islets, which could lead to hypoglycemia in patients with normal glycemic control. In vivo researches have shown that ethanol increased insulin secretion during glucose tolerance test [24, 25].

Symptoms of hypoglycemia are very similar to the symptoms of alcohol condition. However, alcohol consumption after meals may improve insulin secretion thereby reducing potential harmful effects of increased concentrations of blood glucose. Consumption of alcohol without food increases insulin secretion and leads to discharge depot of insulin, which can lead to insulin resistance. The effect of alcohol mainly depends on the administered dose in the body. Adverse effects are manifested in the increased consumption of alcohol causing accumulation of nitric oxide (NO) in the organism that has a detrimental effect on the whole body. Increased NO synthesis under the influence of inducible nitric oxide synthase is associated with various cytotoxic damages [26]. Larger amounts of NO produced in macrophages that infiltrate the endocrine pancreas tissue can lead to damage of β -cells and increased apoptosis of these cells [27]. In the initiation of apoptosis, accumulation of calcium ions in the cytosol has particular

importance [26]. Also, long-term alcohol consumption could lead to β -cell sensitivity to the presence of endotoxins in the blood, thereby increasing the percentage of necrosis of pancreatic islets [2, 4]. It is known that the presence of bacteria in the pancreas can also affect insulin secretion and the development of diabetes, and chronic alcohol consumption would only intensify this effect [28].

Our experiment showed that insulin secretion declines during cultivation, especially on the seventh day (Table 3). Inadequate response to glucose stimulation may lead to hyperglycemia and insulin resistance that are prerequisite for development of diabetes type 2. Similar results were obtained by other authors [29–32]. In addition to the impact on carbohydrate metabolism, experiments on rats showed considerable impact of alcohol on lipid metabolism. These results demonstrated that visceral fat is more susceptible to alcohol toxicity compared to subcutaneous fat, and disruption of adipose lipogenesis contributes to the pathogenesis of alcoholic lipodystrophy [33].

Results suggesting a protective role of alcohol consumption in the development of type 2 diabetes [34, 35, 36] should be interpreted with caution. To determine the true impact of alcohol on insulin secretion, results obtained from animal models and animal cell lines should be taken with reserve, because alcohol consumption is characteristic of humans and there are differences in metabolism.

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Considering that authors express alcohol concentration in different units, perhaps there is a need for standardization.

CONCLUSION

Based on the presented results we can conclude that alcohol concentration 0.165 mM slightly increases insulin secretion in the culture on the first and third day of incubation. The longer the cultivation, the greater the decline in insulin secretion in the test group compared to the control group. The effect of alcohol on insulin secretion is most noticeable in the test group, clearly showing the long-term effects of alcohol, which causes a decline in insulin secretion.

Alcohol can increase or decrease insulin secretion of human pancreatic islets, which may result in an inadequate response of pancreatic β -cells to blood glucose, leading to insulin resistance, and increased risk of developing type 2 diabetes.

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Утицај алкохола на инсулинску секрецију и вијабилност хуманих острваца панкреаса

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САЖЕТАК

Увод/Циљ У литератури постоје прилично контрадикторни подаци на тему ефеката алкохола на лучење инсулина, апоптозе и некрозе ендокриног и егзокриног панкреаса. Циљ овог истраживања је да се утврди како алкохол утиче на лучење инсулина и одрживост острваца адултног хуманог панкреаса *in vitro* током седам дана инкубације.

Методе Ткиво хуманог панкреаса је разложено колагенезом XI, користећи неаутоматизовану методу. Културе су биле инкубиране у *RPMI* раствору који садржи алкохол (10 μ l етанола у 100 ml раствора). Инсулински стимулациони индекс и одрживост острваца су одређивани првог, трећег и седмог дана култивације.

Резултати Анализа одрживости острваца показала је да не постоји значајна разлика између контролне и тестира-

не групе. У тестираној групи одрживост култура опала је са временом инкубације. Капацитет инсулинске секреције тестиране групе био је већи у односу на контролну групу за 50% првог и 25% трећег дана култивације. Седмог дана инсулинска секреција се смањила за 25%. Разлика није била статистички значајна ($p > 0,05$). У тестираној групи откривен/пронађен је значајан пад инсулинске секреције трећег и седмог дана инкубације ($p \leq 0,05$).

Закључак Алкохол може повећати или смањити инсулинску секрецију културе острваца, што може довести до неадекватног одзива β -ћелија панкреаса на глукозу у крви, што даље доводи до инсулинске резистенције и повећане могућности за развијање дијабетеса типа 2.

Кључне речи: алкохол; инсулинска резистенција; инсулинска секреција; одрживост; дијабетес тип 2