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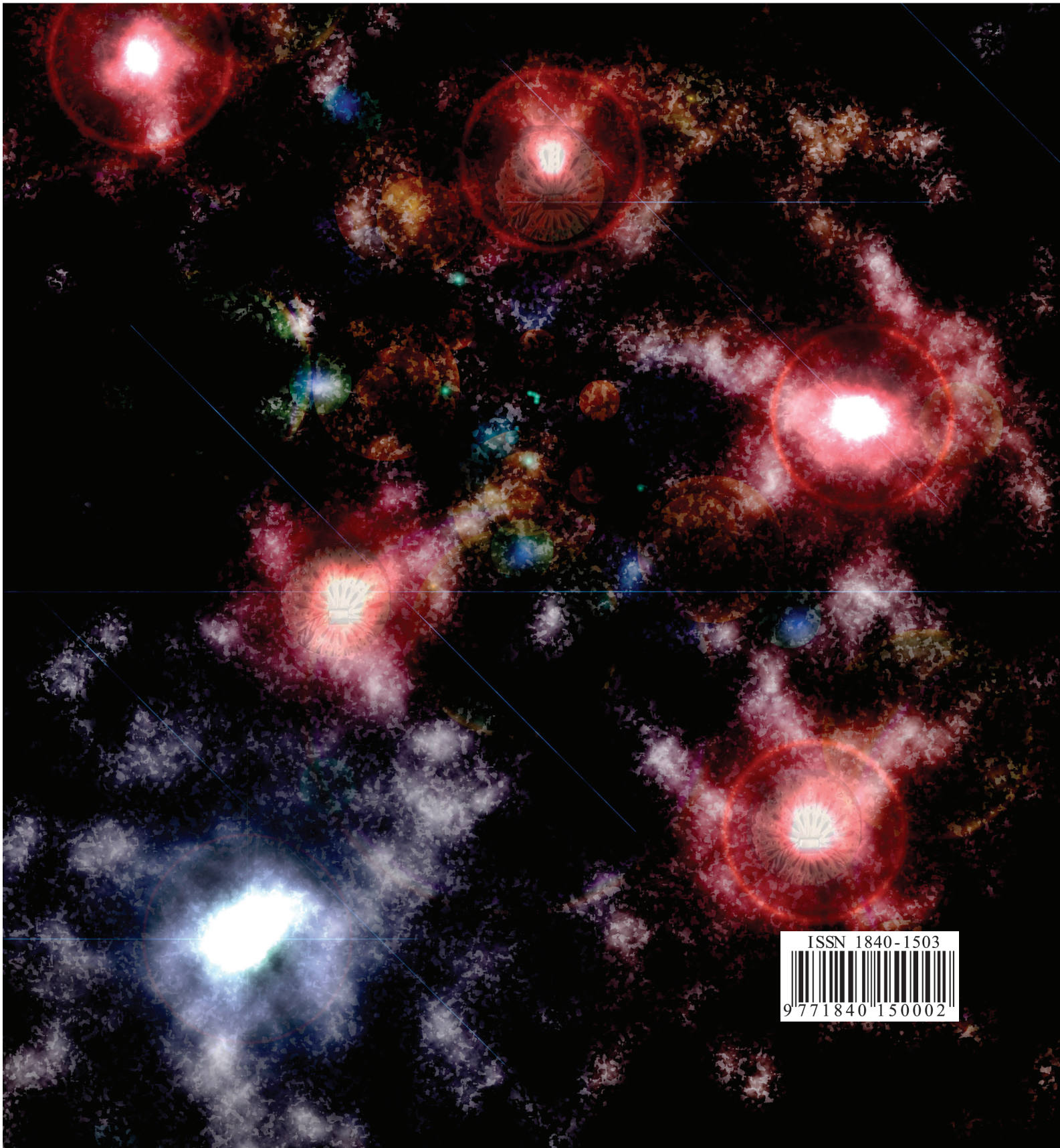

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Frequency of remission of symptoms of depressive disorder in the treatment of antidepressants and anxiolytics during six months of monitoring

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Abstract

Objectives: Depression is the most common mental disorder today. The results of the study show that every 5th woman and every 10th man can experience at least one serious depressive episode during their lifetime. Antidepressant therapy is specific because it can cause serious clinical adverse reactions during therapy and immediately after cessation of therapy. The study aims are to show the frequency of remission of depressive symptoms during defined medical treatment.

Materials and methods: The study was designed as a prospective, cross-sectional study. The survey was conducted voluntarily and with respect for anonymity. The study included patients who are being treated for mild to moderate depression without psychological symptoms, and antidepressant drugs are available in pharmacies.

Results: Unexpectedly, the therapeutic effect of sertaline is particularly bad, as 70% of the treated subjects suffer from a severe episode of depression, 14% from severe, 4% from moderate, and 12% from mild. Complete remission was not achieved by any of the respondents. There is a statistically significant difference in treatment outcome compared to different choices and suppressive ($\chi 2$ test = 49.943, P = 0.000, P <0.001). Failure to treat depression and inability to achieve remission of the disease was statistically significantly associated with the chosen antidepressant (Speraman correlation factor = -0.141, P = 0.008). The level of suicide was statistically significantly associated with the chosen antidepressant (Speraman correlation factor = -0.141, P = 0.008). Failure to treat depression and frequent recurrence was statistically significantly associated with the chosen antidepressant (Speraman correlation factor = -0.391, P = 0.000; P <0.001).

Conclusion: Subjects included in this study generally have a poor treatment outcome and, despite treatment, suffer from a severe, most often recurrent episode of depression.

Key words: depression, depressive episode, remission, antidepressant therapy

Introduction

Depression is the most common mental disorder today. The results of the study show that every 5th woman and every 10th man can experience at least one serious depressive episode during their lifetime (1). Depression is a disease that is accompanied by low mood, which changes the overall thinking, perception, physical condition, behavior, and social functioning of a person (2-3). It is a disorder that occurs sporadically, and in 50% to 80% of people with the first depressive episode, a new depressive episode will occur. When depressive episodes recur in some people, they are referred to as recurrent depressive disorder. An untreated depressive episode lasts an average of 6 to 13 months, and most are treated for two to three months (4-6). Among patients suffering from depression, the majority of patients are between the ages of 35 and 55 (7-9).

Depression and anxiety are common symptoms that occur when stopping antidepressant treatment. In doing so, adverse drug reactions develop in which the clinical picture is characterized as more severe. Unfortunately, these symptoms are usually misjudged as recurrent episodes of depression and the patient continues to take an antidepressant thus creating a vicious circle. Depression

is accompanied by symptoms of recent discontinuation of antidepressants: anxiety, restlessness, febrile fever, irritability and aggression, insomnia with nightmares, nausea and vomiting, dizziness, loss of coordination, gastric pain, and tremor (10-11)**.**

There is a danger that antidepressant treatment will cause an increase in the serious clinical picture of depression in some people instead of a decrease in depression and recurrent episodes. In this regard, the risk of suicide is increased. *There is a particularly high risk during the first two months of antidepressant treatment.*

Materials and methods

The study was designed as a prospective, cross-sectional study. The survey was conducted voluntarily and with respect for anonymity. The study included patients who are being treated for mild to moderate depression without psychological symptoms, and antidepressant drugs are available in pharmacies. Before accessing the survey, respondents were provided with relevant information, which clarified the research's purpose, objectives, and importance.

Treatment success and assessment of treatment efficacy were stratified through the standardized "Hamilton Depression Scale" (12)**,** and 2-6 months after treatment with a specific antidepressant.

The total number of respondents included in this study is 349, and they were selected according to the following criteria:

- that they have been diagnosed with a depressive disorder by a doctor without psychotic symptoms;
- that the respondents are aged 19-65;
- that they have been treated with one of the five most commonly prescribed antidepressants and anxiolytics (lasting at least 2-6 months), and that data on the treatment of these patients are available, including possible complications during treatment;
- that available patient indicators are clear in terms of gender, age, and anamnestic.

The diagnosis was based on two typical and two other common symptoms **(DSM-IV) (13)**. *Typical symptoms are: depressed mood, loss of interest and pleasure, decreased energy, and increased fatigue. Other common symptoms are: disturbed sleep, decreased appetite, decreased concentration and attention, decreased self-esteem and self-confidence, ideas of guilt and worthlessness, a gloomy and pessimistic view of the future, and ideas of self-harm and injury*.

The research was approved by the Commission for Ethical Issues of the Pharmaceutical Chamber.

The standard Statistical Package for Social Research (SPSS) version 19.0 was used to analyze the results. Statistical processing of results was performed using standard methods of descriptive statistics. The $\chi 2$-test and t-test were used to test the statistical significance of the difference between the selected variables. Non-parametric Sperman correlation test and multivariate analysis of variance with multiple regression analysis - ANOVA were used for multivariate correlation analyses.

Results

The failure of antidepressant treatment for 2-6 months is unexpectedly high. The lowest incidence of delayed severe and severe episodes of depression was (unexpectedly) recorded during combination antidepressant therapy with bromazepam. The most severe remission of depressive episode symptoms is achieved by treating the most commonly used antidepressants:

i. paroxetine (Seroxat) in 48 patients out of a total of 89 treated, and
ii. sertraline (Zoloft) in 24 patients out of a total of 50 treated.

Figure 1. shows a comparative presentation of the structure of failure to achieve remission of depression with the selected drug expressed by severe or severe levels of depression during continuous treatment for 2-6 months.

During treatment for 2-6 months with the chosen drug, success in withdrawing symptoms and achieving complete remission is achieved with the following frequency: 8% during treatment with paroxetine (Seroxat); 0% during treatment with sertraline (Zoloft); 6% during flusetin treatment; 0% during treatment with escitalopram and 7% during treatment with an antidepressant in combination with bromazepam. *(Figure 2.)*

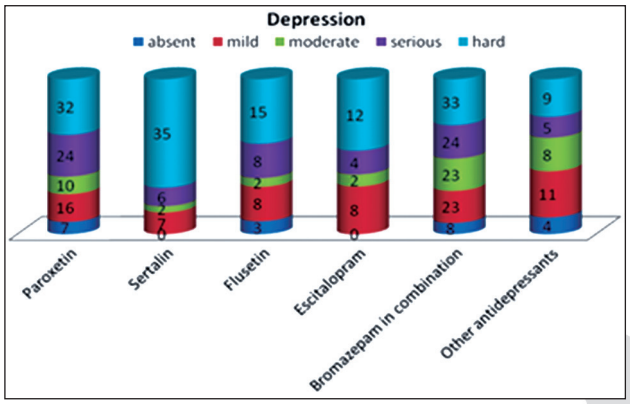


Figure 1. The Comparative presentation of the structure of failure to achieve remission of depression with the selected drug expressed by severe or severe levels of depression during continuous treatment for 2-6 months.

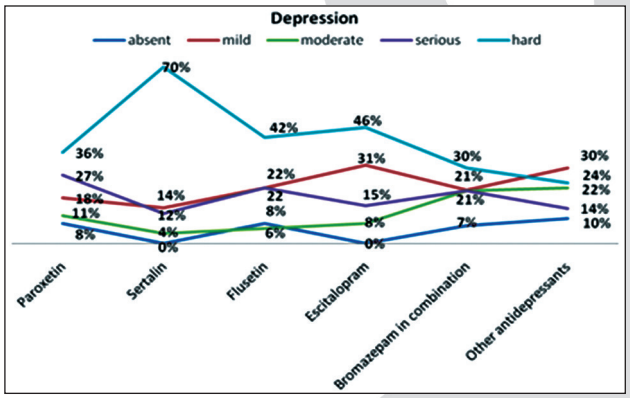
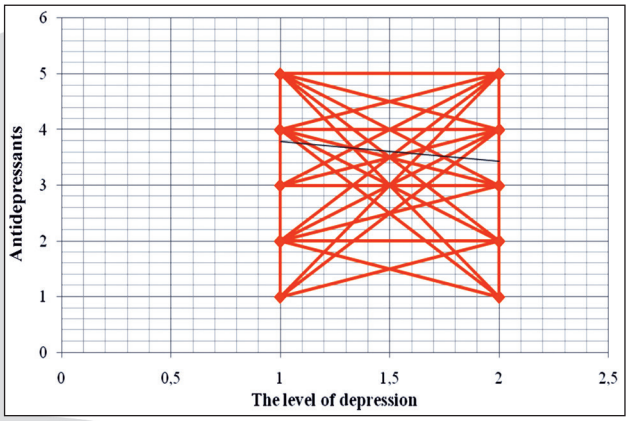


Figure 2. The Comparative prevalence rate of failure to establish remission of depression expressed by the prevalence of severe and severe depression during the continuous treatment with selected antidepressants for 2-6 months

A severe episode of depression is the most severe clinical form of the disease and the greatest sign of treatment failure was found in 36% of paroxetine-treated subjects, 70% of sertraline-treated subjects, 42% of flusetin-treated subjects, 46% of escitalopram-treated subjects, and only 24% bromazepam-treated patients in combination with antidepressants. It is also similar in prevalence to severe episodes of depression serious clinical form of the disease, and an important sign of treatment failure. We found this in 37% of paroxetine-treated subjects, 14% of sertraline-treated subjects, 22% of flusetin-treated subjects, 15% of escitalopram-treated subjects, and only 21% of bromazepam-treated antidepressant-treated patients. Unexpectedly, the therapeutic effect of sertraline (Zoloft) is particularly bad, as 70% of the treated subjects suffer from a severe episode of depression, 14% from severe, 4% from moderate, and 12% from mild. Complete remission was not achieved by any of the respondents. There is a statistically significant difference in treatment outcome compared to different choices and suppressive ($\chi 2$ test = 49.943, P = 0.000, P <0.001).



**Antidepressants: 1- Paroxetine; 2- Sertraline; 3- Flusetin; 4- Escitalopram and 5- Bromazepam*

***Depression level (treatment success / failure): 1- depression absent; 2- mild depression; 3- moderate depression; 4. severe depression; 5- severe depression*

Figure 3. Correlation between selected antidepressants and successful/unsuccessful treatment outcomes expressed in different levels of depression (Scater plot)

Failure to treat depression and inability to achieve remission of the disease was statistically significantly associated with the chosen antidepressant (Speraman correlation factor = -0.141, P = 0.008)

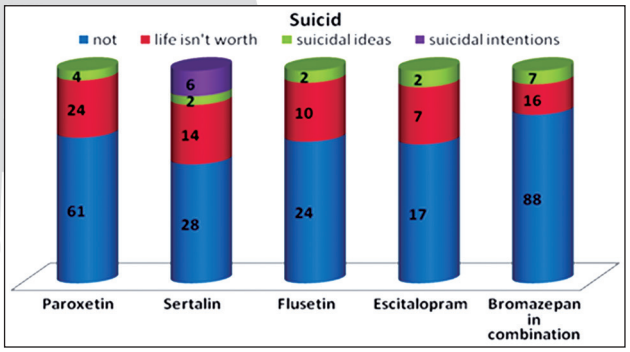


Figure 4. Comparative presentation of the structure of failure to achieve successful therapy of depression expressed by the absence of suicidal ideation and intentions according to the chosen antidepressant / sedative drug during continuous treatment of subjects for 2-6 months

The highest risk of suicide is during treatment with Sertalin (> 10%). (*Figure 5.*)

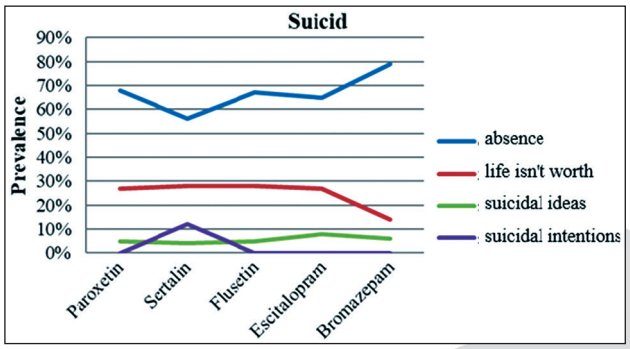


Figure 5. The Comparative structure of treatment failure expressed by the most difficult outcome by the presence of suicidal ideas and intentions in the subjects according to the chosen antidepressant/sedative during treatment for 2-6 months.

The level of suicide was statistically significantly associated with the chosen antidepressant (Speraman correlation factor = -0.141, P = 0.008).

The following table presents a comparative presentation of differences in the intensity of adverse symptoms related to the frequency of anxiety symptoms in subjects treated with the 4 most commonly used antidepressants.

If the success of treatment of depression is observed according to the frequency of previous recurrent episodes of depression and according to the leading drug of choice (antidepressant/sedative), the highest frequency of recurrent episodes of depression had subjects previously treated with paroxetine (Seroxat: 1X- 29 of 89; > 3X- 34 out of 89) *(Figure 6.)*

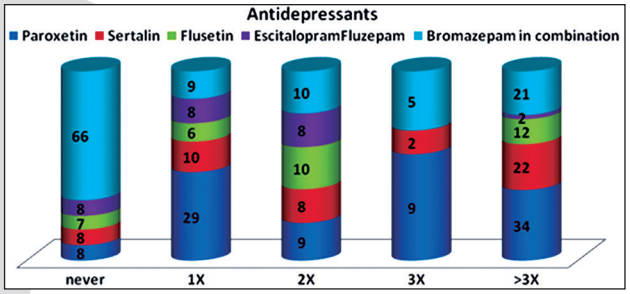


Figure 6. Comparative structure of the frequency of recurrent episodes of depression transiently relative to the choice

Failure to treat depression and frequent recurrence was statistically significantly associated with the chosen antidepressant (Speraman correlation factor = -0.391, P=0.000; P <0.001).

Table 1. Comparative presentation of differences in the intensity (size of the perception of the problem) of adverse symptoms related to the frequency of anxiety symptoms in subjects treated with the 4 most commonly used antidepressants

The frequency of symptoms	Paroxetine (Seroxat) n=89 (%)	Sertaline (Zoloft) n=50 (%)	Flusetine n=36 (%)	Escitalopram n=26 (%)	P
Nervousness					χ^2=61.878 (df =20)
absence	10 (11%)	2 (4%)	4 (11%)	1 (4%)	0.000 <0.001
low level	12 (13%)	7 (14%)	8 (22%)	7 (27%)	
moderate level	39 (44%)	10 (20%)	9 (25%)	4 (15%)	
high level	**22 (25%)**	**28 (56%)**	**15 (42%)**	**14 (54%)**	
serious level	**6 (7%)**	**3 (6%)**	0	0	
Upset, fear					χ^2=56.057 (df =20)
absence	15	2	3	2	0.000 <0.001
low level	14	7	6	4	
moderate level	32	8	13	7	
high level	**26**	**24**	**14**	**13**	
serious level	**2**	**9**	0	0	

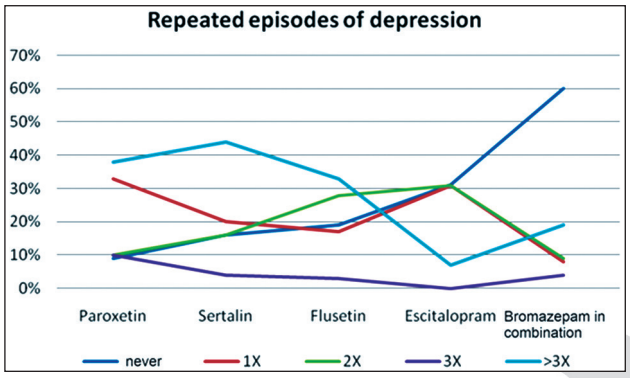


Figure 7. Comparative structure of the frequency of recurrent episodes of depression about treatment with antidepressants and anxiolytics

Discussion

Complete remission should be the goal of antidepressant therapy - everything "below" leaves the patient with residual symptoms and an increased risk of relapse and relapse (14).

The result of the research that only 6% of the subjects achieved remission and the absence of depressive disorder with treatment is very serious. Another worrying fact is that severe depression was recorded in 20% of respondents, and very serious with treatment in as many as 39% of respondents. Thus, it is not surprising that the failure to treat depression is over 94%.

The research noticed that the most severe remission of depressive episode symptoms is achieved by treating the most commonly used antidepressants with paroxetine (Seroxat) and sertraline (Zoloft). There may be a problem with neglecting the etiological factors of depression during treatment, inadequately diagnosed during the initial choice of medication, inadequately evaluating the effects of treatment over time or resistance to medication. *Szegedi and colleagues* in their study stated that rapid response / early improvement to paroxetine therapy is a very sensitive predictor of a later more stable response or stable remission. Namely, the authors concluded that after 3 weeks of paroxetine treatment, in patients who did not improve the clinical picture in that period, in the further observation period, there was no stable response to therapy or stable remitter at a later course (15). Furthermore, studies have shown that patients treated with SSRI antidepressants continuously without interruption for the first 3 months experience a lower risk of relapse and relapse (risk ratio: OR = 0.42, 95% CI, 0.40 to 0.44). The patients who have three or more follow-up visits to a psychiatrist or chosen doctor in the first 3 months reduce the risk of relapse / recurring. Factors associated with a significant increase in relapse/relapse are comorbid chronic diseases, anxiety disorder, and alcohol consumption (16-23).

It is known that people with mental disorders commit about 90% of all suicides. Affective diseases are the most common diagnoses among perpetrators and account for 60 to 70% of suicides, and the lifetime risk of suicide in patients with depression is 15%. As suicide is not a mental disorder or a psychiatric diagnostic category, the success of its prevention depends on the treatment of the depressive disorder itself and the control of side effects during therapy and its cessation (24). The results obtained in the study show that in general the best success was shown by the absence of suicide during combination therapy with antidepressants with bromazepam and this was observed in 88 subjects out of 111 who were on that therapy. Paroxetine (61 out of 89 treated), flusetin (24 out of 36), and escitalopram (17 out of 26 subjects) showed far worse results. The most common side effects (nervousness, tension, and anxiety) are most intense in subjects treated with paroxetine (high in 25% of cases, severe in 7% of cases) and sertraline (high in 56% of cases, severe in 6% of cases). Anxiety and agitation can significantly increase the severity of suicidal thoughts, as suicidal people may be more likely to carry out their suicidal thoughts when they become less depressed, and patients on selective serotonin reuptake inhibitor therapy should be closely monitored for the first few weeks (24-25).

According to the results of the study, the failure to treat depression and the frequent recurrence of relapses is statistically significantly associated with the chosen antidepressant. When there is no favorable response to antidepressant therapy of first choice and different mechanisms of action in monotherapy, it is recommended to try second-line therapy in combination with antidepressant therapy of different mechanisms of action with lithium or atypical antipsychotic or lamotrigine or quetiapine in monotherapy (26-28).

Conclusion

Subjects included in this study generally have a poor treatment outcome and, despite treatment, suffer from a severe, most often recurrent episode of depression. Recurrent depression with recurrent episodes has a high prevalence and poses a risk of a "silent epidemic of depression."

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SGLT2 inhibitors in the treatment of type 2 diabetes

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Abstract

Sodium-glucose co-transporter 2 (SGLT2) inhibitors are a newer class of drugs for the treatment of type 2 diabetes. SGLT2 is used in combination with diet and exercise in patients with type 2 diabetes, alone or in combination with other drugs to treat diabetes. SGLT2 inhibitors, or glyflosins, are drugs that inhibit the sodium and glucose 2 transporters, the major transmembrane protein responsible for glucose resorption in the proximal renal tubules.

Currently, four SGLT2 inhibitors have been approved in the EU, as a single substance agent and as a fixed-dose combination with metformin: canagliflozin (Inovkana and Vokanamet), dapagliflozin (Forxiga, Edistride, Ebymect, Xigduo, Qtern), empagliflozin (Jardiance, Synjyrdy, Glyksambi) and ertuglyflozin (Steglatro, Segluromet, Steglian).

The Agency for Medicinal Products and Medical Devices of Bosnia and Herzegovina has registered the drug Jardiance 10 and 25 mg film-coated tablets, manufactured by Boehringer Ingelheim RCV GmbH & amp; Co.KG, Austria. Of the approved drugs, empagliflozin (Jardiance) has the highest selectivity for SGLT2 compared to SGLT1, while canagliflozin is the least selective.

Key words: sodium-glucose-2 (SGLT2) inhibitors, type 2 diabetes mellitus (DMT2), heart failure, empagliflozin, dapagliflozin, canagliflozin.

1. Introduction

SGLT 2 inhibitors (such as empagliflozin, dapagliflozin, and canagliflozin) are blood glucose-lowering drugs, which they achieve by increasing the renal excretion of glucose into the urine.

Diabetes (lat. *Diabetes mellitus*) is a chronic disease that occurs when the level of sugar, or glucose, is too high. According to the guidelines of the American Diabetes Association (ADA), diabetes is defined as a fasting glucose level greater than or equal to 7.0 mmol/L, or an oral glucose tolerance test (OGTT test) greater than or equal to 11.1 mmol/L, or as a finding of glycosylated hemoglobin (HbA1c) greater than or equal to 6.5%, or in patients with classic symptoms of hyperglycemia or hyperglycemic crisis, a random finding of plasma glucose greater than or equal to 11.1 mmol/L. Insulin, a hormone secreted by the endocrine part of the pancreas, helps glucose from the food enter cells and be used for energy. The most pronounced effect of insulin on carbohydrate metabolism is the hypoglycemic effect. This effect is caused by the effect of insulin on accelerating the transfer of glucose through the membranes of tissue cells sensitive to insulin, by stimulating glycogenesis in the liver and muscle, and by stimulating lipogenesis in the liver and fatty tissue. A lack of insulin or the absence of its effect will have the opposite metabolic consequences. Reduced utilization of glucose in peripheral tissues and increased production in the liver due to increased glycogenolysis and activated gluconeogenesis will increase the concentration of glucose in the blood. The biological effect of insulin depends not only on its normal synthesis and secretion but also on other factors. There are conditions with normal or even increased concentration of the circulating hormone with simultaneous signs of lack of its effect. Over time, too much glucose in the blood can cause health problems, especially in the nerves, blood vessels, kidneys, and eyes (1,2).

1.1 Therapeutic indications of SGLT2

Type 2 diabetes mellitus: Treatment of adults with poorly controlled type 2 diabetes as an adjunct to diet and exercise:

- as monotherapy when metformin is not considered appropriate due to intolerance;
- as an addition to other medicines for the treatment of diabetes

Heart failure: In adults for the treatment of symptomatic chronic heart failure with reduced ejection fraction.

Chronic kidney disease: In adult patients for the treatment of chronic kidney disease.

/SmPC for dapagliflozin has an approved indicated indication/

2. Pharmacological characteristics

2.1 Clinical efficacy and safety SGLT2

The sodium-glucose cotransporter-2 is a protein expressed in the proximal renal tubules and is responsible for most of the reabsorption of filtered glucose from the tubular lumen. By inhibiting the action of SGLT2, drugs of the sodium-glucose-2 cotransporter inhibitor class cause the removal of a greater amount of glucose through the urine and thus, using a mechanism independent of insulin, lower the blood glucose level. SGLT2 inhibition by empagliflozin, dapagliflozin, and canagliflozin reduces glucose reabsorption from the glomerular filtrate in the proximal renal tubule, simultaneously reducing sodium reabsorption, which leads to glucose excretion through urine and osmotic diuresis.

Thus, sodium delivery to the distal tubule is increased, which increases tubuloglomerular feedback and decreases intraglomerular pressure. In combination with osmotic diuresis, this leads to a decrease in volume overload, a decrease in blood pressure, and a decrease in preload and afterload, which could have beneficial effects on cardiac remodeling and preservation of renal function. Other effects include an increase in hematocrit values and a decrease in body weight (3, 4, 5, 6).

2.2 Clinical efficacy and safety

2.2.1 Empagliflozin

Diabetes mellitus tipa 2

Improving glycemic control and reducing cardiovascular morbidity and mortality is an integral part of the treatment of type 2 diabetes.

Glycemic efficacy and cardiovascular outcomes were evaluated in a total of 14,663 patients with type 2 diabetes treated in 12 double-blind, placebo-controlled, active-controlled clinical trials of which 9,295 received empagliflozin (empagliflozin 10 mg: 4,165 patients; empagliflozin 25 mg: 5130 patients). The use of empagliflozin 25 mg resulted in a higher proportion of patients with an achieved target value of HbA1c lower than 7% and a smaller number of patients who needed additional treatment to achieve satisfactory glycemic, compared to the use of empagliflozin 10 mg and placebo. In addition to the above, empagliflozin, as an addition to standard treatment, reduced cardiovascular mortality in patients with type 2 diabetes and established cardiovascular disease (7). During the conducted, placebo-controlled trial of empagliflozin monotherapy, data analysis revealed significant reductions in HbA1c values, as well as glucose in fasting plasma (8).

Also, the EMPA-REG H2H-SU trial was conducted comparing the efficacy and safety of empagliflozin 25 mg compared to glimepiride (up to 4 mg per day) in patients on metformin therapy. Daily treatment with empagliflozin in addition to metformin for up to 208 weeks resulted in superior HbA1c reduction with a significantly lower risk of hypoglycemia compared to glimepiride (9).

The efficacy and safety of empagliflozin as an add-on to multiple-daily insulin with or without concomitant metformin therapy were evaluated in the 52-week, double-blind, placebo-controlled EASE trial. In the last week of the trial, this type of combined treatment resulted in a statistically significant reduction in HbA1c and insulin savings compared to placebo, as well as a reduction in fasting plasma glucose and body weight (11). Treatment with empagliflozin resulted in statistically significant reductions in HbA1c and clinically significant reductions in fasting plasma glucose compared to placebo at 24 weeks of study duration (11).

Cardiovascular and metabolic effects of empagliflozin

The double-blind, placebo-controlled EMPA-REG OUTCOME trial compared the combined doses of empagliflozin 10 mg and 25 mg with placebo, as an adjunct to standard treatment in patients with type 2 diabetes and established cardiovascular disease. A total of 7020 patients were treated (empagliflozin 10 mg: 2345, empagliflozin 25 mg: 2342, placebo: 2333) and followed for a median of 3.1 years. The median age was 63 years, median HbA1c was 8.1%, and 71.5% were men.

Compared with placebo, empagliflozin was superior in preventing the primary composite outcome measure of cardiovascular death, nonfatal myocardial infarction, or nonfatal stroke. In the EMPA-REG OUTCOME trial, the effect of empagliflo-

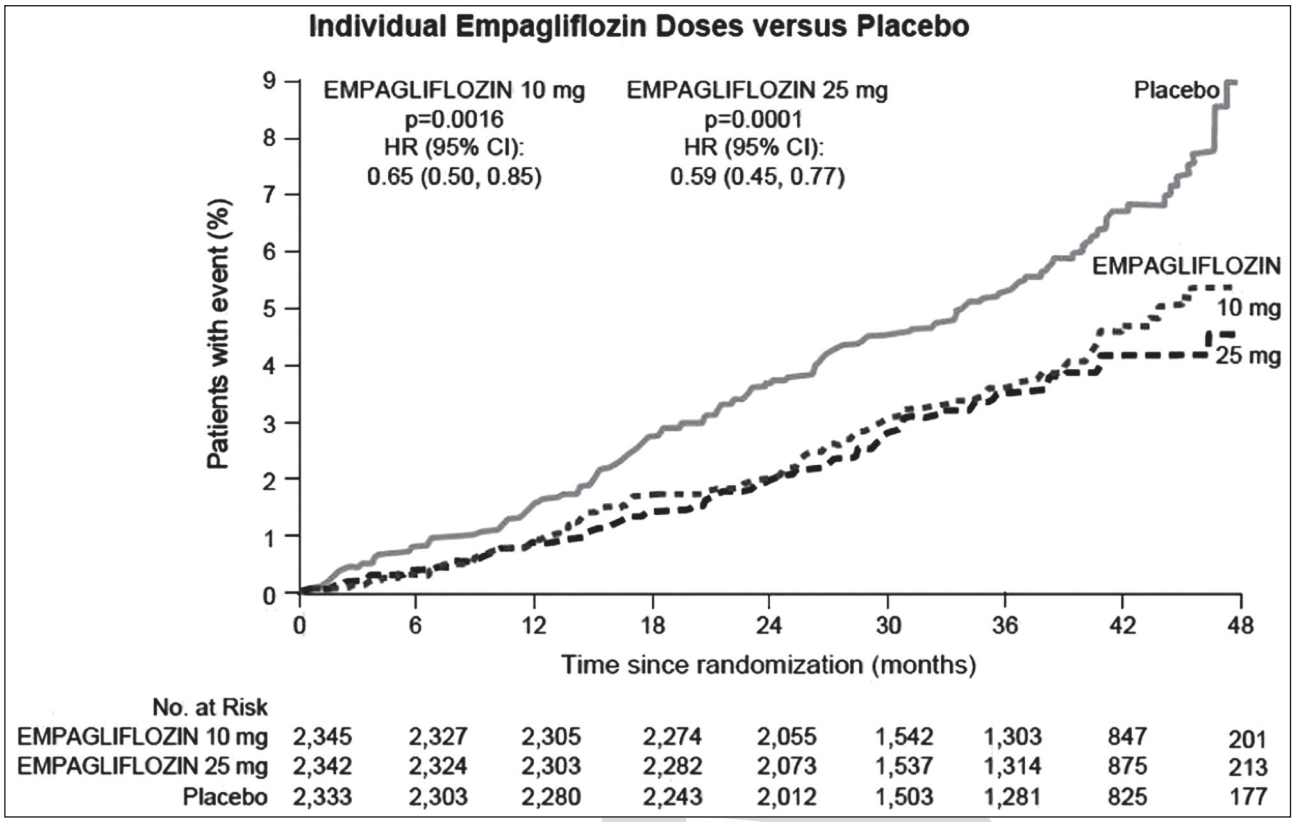

Figure 1. Time to occurrence of cardiovascular death in the trial EMPA-REG OUTCOME

zin on the primary composite outcome measure of cardiovascular death, nonfatal myocardial infarction, or nonfatal stroke was largely independent of glycemic control or renal function (eGFR) and was generally consistent across categories of eGFR up to an eGFR of 30 ml/min/1.73 m2. In the EMPA-REG OUTCOME trial, empagliflozin reduced the risk of heart failure requiring hospitalization compared with placebo (empagliflozin 2.7%; placebo 4,1%; HR 0,65; 95% CI 0,50; 0,85) (12, 6, 13, 14).

Nephropathy

In the EMPA-REG OUTCOME trial, the HR for the time to the first nephropathy event was 0.61 (95% CI 0.53, 0.70) for empagliflozin (12.7%) versus placebo (18.8%). In addition, with empagliflozin, there was a higher frequency (HR 1.82; 95% CI 1.40, 2.37) of persistent normoalbuminuria or microalbuminuria (49.7%) in patients with baseline macroalbuminuria compared with placebo (28.8%), (15).

Body weight

In previously specified pooled analyzes of 4 placebo-controlled studies, treatment with empagliflozin resulted in a reduction in body weight (-0.24 kg for placebo, -2.04 kg for empagliflozin 10 mg, and -2.26 kg for empagliflozin 25 mg) in 24 to 52 weeks (-0.16 kg for placebo, -1.96 kg for empagliflozin 10 mg and -2.25 kg for empagliflozin 25 mg) (16).

Blood pressure

The effects of empagliflozin in patients with and without presumed resistant hypertension (prHT) were analyzed in the EMPA-REG OUTCOME post hoc analysis. Empagliflozin produced clinically significant reductions in SBP as well as improvements in all outcomes regardless of prHT status (13).

EMBRACE-HF is a randomized, multicenter, double-blind, placebo-controlled trial conducted from July 2017 to November 2019, including patients with HF (regardless of ejection fraction, with or without type 2 diabetes) and a previously implanted pressure sensor. in the pulmonary artery (CardioMEMS). At the conclusion of this study, a rapid effect of empagliflozin on reducing pulmonary artery pressure was recorded. This effect of empagliflozin increased over time, apparently independent of treatment with a loop of Henle diuretics (17).

2.2.2 Dapagliflozin

Type 2 diabetes mellitus

To determine the glycemic effect and safety of dapagliflozin, fourteen double-blind, randomized, controlled clinical trials were conducted with 7056 adult subjects with type 2 diabetes; 4737 subjects in those trials were treated with dapagliflozin. In twelve trials, treatment lasted 24 weeks, 8 had long-term follow-ups ranging from 24 to 80 weeks (up to a total trial duration of 104 weeks), one trial lasted 28 weeks, while one trial lasted 52 weeks with a long-term follow-up of 52 and 104 weeks (total trial duration 208). The average duration of diabetes was between 1.4 and 16.9 years. 50% of subjects had mild impairment of renal function, and 11% had moderate impairment of renal function. Respondents were 51% male, 84% Caucasian, 8% Asian, 4% Black, and 4% from other racial groups. 81% of subjects had a body mass index (BMI) ≥ 27. Furthermore, two 12-week, placebo-controlled trials were conducted in patients with insufficiently well-controlled type 2 diabetes and hypertension.

Improvement of glycemic control and reduction of morbidity and mortality from cardiovascular and renal diseases are integral parts of the treatment of type 2 diabetes.

To evaluate the drug's effect on cardiovascular and renal events, the Cardiovascular Outcomes Trial (DECLARE) was conducted comparing dapagliflozin 10 mg with placebo in 17,190 patients with type 2 diabetes with or without established cardiovascular disease (4, 18).

In a patient population similar to that included in the DECLARE-TIMI 58 study, dapagliflozin was safe for CV outcomes and resulted in lower rates of HHF and CV mortality compared to other GLDs (19).

There is no experience with the use of dapagliflozin for the treatment of chronic kidney disease in non-diabetic patients who do not have albuminuria. Patients with albuminuria may benefit more from treatment with dapagliflozin. The results of a study by Ofri Mosenzon et al indicated an important role for SGLT2 in the primary prevention of diabetes-related kidney disease (20).

The favorable cardiac and renal effects of dapagliflozin are not exclusively dependent on the effect on lowering blood glucose levels and are not limited to patients with diabetes, as demonstrated in the DAPA-HF and DAPA-CKD trials.

Investigation DAPA-HF (*Dapagliflozin And Prevention of Adverse outcomes in Heart Failure*) was an international, multicenter, randomized, double-blind, placebo-controlled trial conducted in patients with heart failure. Of the 4744 patients, 2373 were randomized to treatment with dapagliflozin at a dose of 10 mg, and 2371 received a placebo.

The benefit of dapagliflozin treatment in patients with heart failure was observed both in patients with type 2 diabetes and in those without diabetes. Dapagliflozin reduced the incidence of death from a cardiovascular cause and worsening heart failure, which was the primary pooled outcome measure, with an HR of 0.75 (95% CI: 0.63, 0.90) in patients with diabetes and 0.73 (95% CI: 0.60, 0.88) in those without diabetes (21, 22, 23, 24).

In the cardiovascular outcomes study of dapagliflozin in patients with heart failure and reduced ejection fraction (DAPA-HF trial), 2368 patients have treated with dapagliflozin 10 mg, while 2368 received placebo during a median exposure of 18 months. The patient population included patients with type 2 diabetes and those without diabetes and patients whose eGFR was ≥ 30 ml/min/1.73 m2 (33). Investigation DAPA-CKD (*The Study to Evaluate the Effect of Dapagliflozin on Renal Outcomes and Cardiovascular Mortality in Patients with Chronic Kidney Disease*) was an international, multicenter, randomized, double-blind, placebo-controlled trial conducted in patients with chronic kidney disease (CKD) who had eGFR ≥ 25 and ≤ 75 ml/min/1.73 m 2 and albuminuria (the ratio of albumin to creatinine in urine ≥ 200 and ≤ 5000 mg/g) to determine the effect of dapagliflozin as an add-on to standard therapy on the incidence of the composite outcome measure, which included a sustained decrease in eGFR ≥ 50%, end-stage renal disease (defined as sustained eGFR < 15 ml/min/1.73 m 2, chronic dialysis or receiving a kidney transplant) and death from cardiovascular or renal causes, compared to placebo. Dapagliflozin was superior to placebo in preventing the primary composite outcome measure of ≥ 50% sustained decline in eGFR, end-stage renal disease, and death from a cardiovascular or renal cause (25). Treatment benefit with dapagliflozin was consistently observed in patients with chronic kidney disease who had and did not have type 2 diabetes (26).

2.2.3 Canagliflozin

A total of 10,501 patients with type 2 diabetes participated in ten double-blind, controlled clinical efficacy and safety trials conducted to evaluate the effect of canagliflozin on glycemic control, including 5,151 patients treated with canagliflozin in combination with metformin. In the clinical development program, 1085 patients with an initial eGFR value of 30 ml/min/1.73 m2 to < 60 ml/min/1.73 m2 were evaluated.

Canagliflozin was studied as dual therapy with metformin, dual therapy with a sulfonylurea, triple therapy with metformin and a sulfonylurea, triple therapy with metformin, and pioglitazone, and as an additional therapy with insulin and as monotherapy. Overall, canagliflozin had clinically and statistically significant ($p < 0.001$) results compared to placebo in glycemic control, including HbA1c, percentage of patients achieving glycosylated hemoglobin (HbA1c), < 7%, change from baseline in plasma glucose fasting (FPG) and 2-hour postprandial glucose (PPG). Additionally, reductions in body weight and systolic blood pressure were observed compared to placebo.

In the CANVAS trial, subjects were randomized 1: 1: 1 to receive canagliflozin 100 mg, canagliflozin 300 mg, or a matching placebo

Patients in the pooled canagliflozin-treated groups (pooled data analysis for canagliflozin 100 mg, canagliflozin 300 mg, and canagliflozin titrated from 100 mg to 300 mg) had a lower risk rate of a major adverse cardiovascular event (MACE) compared to placebo (27, 28, 29).

3. Registration status of SGLT2 in B&H and EU

The data are presented in tables 1 and 2.

4. Discussion

In August 2014, the drug from the SGLT2 group empagliflozin (Jardiance, manufactured by Eli Lilly-Boehringer Ingelheim Alliance) received marketing authorization for the treatment of adults with poorly controlled type 2 diabetes. At the end of 2016, Jardiance received marketing authorization again for the treatment of symptomatic chronic heart failure with reduced ejection fraction (HFrEF). This marketing authorization makes Jardiance the first antidiabetic drug approved globally, with the outcome of reducing the risk of cardiovascular death in patients with type 2 diabetes. The approval of the new indication is based on the results of the phase III EMPEROR clinical trial.

The study was conducted on 3,730 adult patients with HfrEF (with or without diabetes). An analysis of the endpoints of the trial found that Jardiance 10 mg tablets reduced cardiovascular death or re-hospitalization due to heart failure by 25%, compared to placebo. The results of this study were presented

Table 1. List of approved SGLT2 inhibitors in B&H

The name of the medicine / INN	Manufacturer	Marketing authorization holder	Date of first authorization	The permit is valid until:
JARDIANCE® *empagliflozin*	Boehringer Ingelheim RCV GmbH & Co.KG, Austrija	Boehringer Ingelheim BH d.o.o.	19.02.2020.	01.03.2025.

Table 2. List of approved SGLT2 inhibitors in the Republic of Croatia

The name of the medicine / INN	Manufacturer	Marketing authorization holder	Date of first authorization	The permit is valid until:
JARDIANCE® *empagliflozin*	Boehringer Ingelheim RCV GmbH & Co.KG, Austrija	Boehringer Ingelheim BH d.o.o.	19.02.2020.	01.03.2025.
INVOKANA® *canagliflozin hemihidrat*	Janssen-Cilag S.p.A. Via C. Janssen	Janssen-Cilag International NV	26.09.2018.	
SYNJARDY® *empagliflozin, metforminklorid*	Boehringer Ingelheim Pharma GmbH & Co. KG	Boehringer Ingelheim International GmbH	1.04.2020.	

at the 2020 European Society of Cardiology Annual Meeting and were published simultaneously in the New England Journal of Medicine.

The final secondary analysis of the study's endpoints shows that Jardiance reduces the relative risk of first hospitalization and recurrent heart failure by 30% compared to placebo. In addition, as a measure of decline in kidney function - the estimated decline in glomerular filtration rate (eGFR) - was slower in the study drug group compared to placebo. In this trial, the safety profile of the drug was within the range of previously known safety information on the drug (30).

Analysis of data from clinical trials conducted for empagliflozin shows the following:

- The risk of a major cardiovascular event (a combination of cardiovascular death and non-fatal heart attack and stroke, MACE) is 14% lower with empagliflozin.
- There were 38% fewer cardiovascular deaths and 35% fewer hospitalizations for heart failure, as well as 39% fewer occurrences or worsening of kidney disease and 32% fewer deaths from any cause. A loss of body weight of 2-3 kg during 12 weeks was recorded. Due to osmotic diuresis, SGLT2-IH significantly reduces both systolic and diastolic arterial pressure, without increasing heart rate.

Given that almost every third patient with diabetes also has an associated cardiovascular disease (marked as having recovered from a heart attack or stroke, unstable angina pectoris, positive ergometry, i.e. revascularization of the coronary, carotid, or peripheral arteries, heart failure), and 2 out of 5 also have chronic kidney disease, these results, and subsequently other available results for other SGLT2s, as well as available data for GLP1-RAs, have seriously changed diabetes treatment recommendations, as reflected in the joint guidelines of the American Diabetes Association and the European Association for the Study of Diabetes, published in 2018.

After metformin therapy, which is still the first choice, it was proposed to introduce GLP1-RA if cardiovascular disease is present, or SGLT2 in case of the presence of kidney disease or heart failure. If there is a contraindication for the use of GLP1-RA, it is recommended to use SGLT2 and vice versa. According to the available evidence, certain drugs from both groups received the order of recommendation, for example, if heart failure is present, the order of preference for SGLT2-IH is empagliflozin, canagliflozin, dapagliflozin. The increasing number of published results of cardiovascular outcome studies creates a need for their timely inclusion in treatment guidelines. The decision to treat patients with type 2 diabetes with SGLT2 to reduce major adverse cardiovascular events, hospitalization for heart failure, cardiovascular death, or exacerbation of chronic kidney disease should be made independently of baseline or target HbA1c values. In other words, in a patient who is well regulated on "older" antidiabetic drugs, and has established cardiovascular or renal disease, or is classified as high risk, correction of therapy is recommended (31, 32).

5. Conclusion

Sodium-glucose co-transporter 2 (SGLT2) inhibitors are a newer class of drugs for the treatment of type 2 diabetes. SGLT2 is used together with diet and exercise in patients with type 2 diabetes, alone or in combination with other drugs for the treatment of diabetes.

In addition to type 2 diabetes, they are approved for the treatment of symptomatic chronic heart failure with reduced ejection fraction, as well as for the treatment of chronic kidney disease. Dozens of multicenter randomized clinical trials have been conducted for each substance, which confirmed the safety and effectiveness of SGLT2 within the specified indication area. The main representatives of SGLT2 inhibitors are empagliflozin, dapagliflozin, canagliflozin, and ertugliflozin. The mentioned substances are approved in the EU as a single-substance agent and as a fixed dose combination with metformin. Some drugs of this group are in the phase of clinical trials or the phase of the approval process for a place on the market.

The Agency for Medicines and Medical Devices of Bosnia and Herzegovina has registered the drug Jardiance 10 and 25 mg film-coated tablets. In 2016, empagliflozin, the active substance of Jardiance, received marketing authorization for the treatment of symptomatic chronic heart failure with reduced ejection fraction (HFrEF) (with or without type 2 diabetes), making it the first anti-

diabetic drug approved globally, with the outcome of reducing the risk of cardiovascular death in patients with type 2 diabetes. The approval of the new indication is based on the results of phase III clinical trial EMPEROR, the analysis of which established that empagliflozin reduces the risk of a major cardiovascular event (combination of cardiovascular death and non-fatal heart attack and stroke, MACE) by 14%. In addition to the above, there were 38% fewer cardiovascular deaths, 35% fewer hospitalizations due to heart failure, 39% fewer occurrences or worsening of kidney disease, and 32% fewer deaths from any cause.

In the last published joint guidelines of the American Diabetes Association and the European Association for Diabetes Research in December 2018, the recommendations for the pharmacological treatment of type 2 diabetes following the needs of the patient, with an emphasis on the secondary prevention of cardiovascular incidents in high-risk patients, are broken down in detail.

According to the available evidence, the increasing number of published results of cardiovascular outcome trials creates a need for the timely inclusion of SGLT2 in treatment guidelines. The decision to treat patients with type 2 diabetes with SGLT2 to reduce major adverse cardiovascular events, hospitalization for heart failure, cardiovascular death, or exacerbation of chronic kidney disease should be made independently of baseline or target HbA1c values. If heart failure is present, the order of SGLT2 preference is empagliflozin, then canagliflozin, and dapagliflozin.

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Baicalin suppresses proliferation and migration of the VSMCs via regulating the CDH5 /miR-125b-5p pathway

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Abstract

The effect of baicalin on the proliferation and differentiation of hcy-induced VSMCs was observed and the mechanism of action was investigated. Hcy-induced VSMCs were divided into five groups: control, Hcy (200 μmol/L); Hcy + miR-125b-5p mimic, and Hcy+baicalin(10μM, 20μM, 50μM). Targeting gene CDH5 of miR-125b-5p were screened by gene prediction software TargetScan, miR and TarBase. MTT assay was used to detect cell viability of VSMCs. Western blot was used to detect the expression of PCNA, Cyclin D1 and CDH5. In addition, the targeting relationship between miR-125b-5p and CDH5 was verified by dual luciferase reporter gene detection technology. It was observed that treatment of 20 and 50 μM baicalin has significant inhibition in the proliferation and migration of the VSMCs. Furthermore, the miR-125b-5p expression was down-regulated and its targeted gene CDH5 was up-regulated in Hcytreating VSMCs, while the baicalin reversed the effect of Hcy, suggesting the miR-125b-5p/CDH5 may be involved in the protective effect of baicalin. Conversely, the miR-125b-5p can decrease the luciferase activity of the wild-type CDH5 3'UTR reporter vector, and had no effect on the mutant-type CDH5. Collectively, present study demonstrates that baicalin inhibits Hcy-induced proliferation of VSMCs via miR-125b-5p/CDH5 signaling.

Key words: *Baicalin, VSMCs, CDH5, miR-125b-5p*

Introduction

Cardiovascular disease, a leading cause of mortality worldwide, is caused mainly by atherosclerosis, a chronic inflammatory disease of blood vessels[1]. The pathogenesis of AS is very complicated. Lipid infiltration, vascular flatness, excessive proliferation and migration of vascular smooth muscle cells (VSMC) and chronic inflammatory reaction were regarded as a doctrinal mechanism[2]. Therefore, regulation of the proliferation of VSMC is important in the early stage of AS development.

Baicalin was flavonoids derived from the root of Scutellaria baicalensis Georgi. It has been reported that baicalin shows a stronger treatment for the of inflammation, hyperlipemia, favus, jaundice and scald. After the application of baicalin for intervention in the atherosclerosis model, the VE-cadherin levels were significantly reduced[3]. The proliferation of VSMCs were inhibited, which may be related to the mechanisms baicalin can reduce the intracellular calcium load and suppresses ET-stimulated endothelial respiratory outbreaks and excessive release of oxygen radicals[4]. VE-cadherin is a component of endothelial cell-to-cell adherens junctions, and it has a key role in the maintenance of vascular integrity[5]. Study previously demonstrated that downregulation of VE-cadherin expression within intimal neo vessels in human atherosclerotic lesions is accompanied by increased entry of immunocompetent cells into the intimal areas of Neovascularization. These studies allowed us to investigate the effect of baicalin on Hcy-induced VSMCs proliferation[6].

Recent studies have demonstrated that microRNAs (miRNAs) expressed in the vascular system are involved in the control of VSMC proliferation[7]. Additionally, the expression of miR-125b-5p was decreased in the arteries with arteriosclerosis obliterans and PDGF-BB-stimulated VSMCs. miR-125b-5p suppressed VSMC proliferation and migration but promoted VSMC apoptosis by directly targeting SRF. Furthermore, exogenous miR-125b-5p expression inhibited vascular neointimal formation in balloon-injured rat carotid arte-

ries[8]. Biological analysis indicated that CDH5(as an VE-cadherin) may become an important target for miR-125b-5p to regulate the growth of vascular endothelium. However, whether miR-125b-5p is involved ininhibiting AS of baicalin has not been reported yet. Hence, we evaluated whether baicalin exerted anti-proliferative effect in Hcy treating VSMCs, and further investigated whether miR-125b-5p was involved in the anti-proliferation of baicalin in VSMCs by targeting CDH5.

Materials and methods

Procurement of materials

Baicalin were procured from Solarbio (Beijing, China). miR-125b-5p and miR-NC were obtained from Ribobio (Guangzhou, China). MTT (Sigma Aldrich, USA). CDH5, PCNA, CyclinD1 were was obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The instruments used in the study were microscope (Olympus Crop, Tokyo, Japan, Carbon dioxide (CO_2) incubator (BPN 50CH (UV)), BeyoECL Plus reagent (Beyotime, Shanghai, China), Trizol reagent (Invitrogen, Thermo Fisher Scientific, Inc.), All-in-OneTM First-Strand cDNA Synthesis kit (GeneCopoeia, Rockville, MD, USA).

Cell culture

Human aortic vascular smooth muscle cells were purchased from Shandong Academy of Medical Sciences (Shandong, China). were maintained in DMEM (Gibco/Thermo Fisher Scientific, San Jose, USA) supplied with 10 % fetal bovine serum in a humidified atmosphere of 5% CO_2.

Drug treatment

All cultures were incubated with serum-free media for 24h prior to treatment. Cultures of VSMCs were divided into the following groups: control (phosphate-buffered saline [PBS]); Hcy (200 μmol/L); Hcy+baicalin(CSA 21967-41-9,synthesized by Jinan Yuedi, concentred with 10μM 20μM 50μM); miR-125b-5p inhibitor control +Hcy +baicalin; miR-125b-5p inhibitor (Biomics Biotech)+ baicalin + Hcy: VSMCs were separately transfected with the miR-125b-5p inhibitor control and the miR-125b-5p inhibitor and pretreated with baicalin for 24 h, and then treated with Hcy. Total cellular RNA and total protein were collected for corresponding indicators after 48h. The negative control of miR-125b-5p(NC group 5'-GUCAGAGATCCAGGGACUCT-3) was added in the luciferase assay.

Cell growth inhibition by MTT assay

Cell proliferation was measured by the MTT assay. cells were seeded into 96-well plates (10^4cells/well), serum-starved and treated as described above for 24h. The samples were applied carefully on the monolayers of cells for 48 h. Then 10 μL of MTT stock solution (5 mg/mL in PBS) was added in each well and the plate was incubated for 4 h. The blue-coloured formazan that formed was dissolved in 100 μL of DMSO per well. The plates were incubated on a shaker at room temperature for 10 min, and the optical density (OD) was assayed at 570 nm on a microplate reader.

Transwell assay

For migration assay, 2×10^5transfected VSMCs cells were seeded in 12-well plates and incubation overnight. Then a wound was made by 10 μL sterile pipette tip and the cell debris were washed by PBS twice. The cells were continuously cultured for 24 h. The photographs were taken at 0 and 24 h under an inverted microscope. The percent of the wound was quantified.

Western blot

After VSMCs dealed according to different groups, total protein were collected, measured by BCA protein concentration determination, and then were loaded onto SDS-PAGE gels and transferred electrophoretically to PVDF membranes. Next, the membranes were blocked with 5 % nonfat milk for 2 h and incubated with the primary antibodies at 4 °C overnight. The membranes were washed and further incubated with a secondary antibody marked by horseradish peroxidase at room temperature for 2h. The blots were visualized with a chemiluminescent detection system according to the manufacturer's instructions.

Real-time quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Reverse transcription was performed, and cDNA was synthesized from 2 μg of total RNA using TaqMan miRNA Reverse TranscriptionKit (Applied Biosystems, Foster City, CA, USA). The PCR amplification for the quantification of the CDH5 and miR-125b mRNAs was performed using an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City,CA, USA). The primers were as follows: miR-125b-5p 5'-CAGTCTCTAGGTCCCT GAGA-3' and reverse primer: 5'-TTTGGCACTAGCACATT-3'. CDH5, forward primer: 5'-GCGACTACCAGGACGCTTTCA-3' and reverse primer: 5'-CATGTATCGGAGGT CGATGGTG -3'. β-actin: forward primer, 5'-GGCACCCAGCACAATGAA-3' and reverse primer: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'.

Luciferase assay

The base sequence of miR-125b-5p was cloned around the target site of CDH5 3'-UTR and added to the double luciferase reporter vector as the wild-type vector CDH5 UTRwt. The mutational-type vector CDH5 UTRmut can be obtained through site-directed mutagenesis at the target site. Then the dual-luciferase assay tool cells of 293T was inoculated in a 12-well plate. The vector of CDH5 3'-UTR and miR-125b-5p minic were co-transfected to 293T cells. Divided into four groups: CDH5-UTRwt+NC, CDH5-UTRwt+miR-125b-5p minic, CDH5-UTRmut+NC, CDH5-UTRmut+miR-125b-5p minic. After 48h of transfection, cells were lysed with reporter lysis buffer, and the luciferase activities were measured using the Dual-Luciferase Reporter Kit (Promega) according to the manufacturer's instructions.

Targeted gene prediction

Online gene prediction software such as miRanda, TarBase, and TargetScan were used to predict the target genes of miR-125b-5p. A luciferase assay assay further validated the fact.

Statistical analysis

All experiments were conducted in triplicate. The data were presented as the mean + SEM. Comparisons between two groups were analysed by the unpaired t-test, and we used One-way ANOVA followed by Dunnett's t-test for comparisons among three or more groups. Statistical analyses were performed with SPSS 13.0 software, and a value of $P < 0.05$ was considered to be statistically significant.

Results and discussion

Atherosclerosis was thought of as a chronic inflammatory disease, involving in multiple inflammatory factors such as IL-6,hs-CRP,ve-cadherin[9]. VE-cadherin-mediated adherencing junctions have an essential role in maintaining VSMCs barrier functions[10]. Baicalin was flavonoids derived from the root of Scutellaria baicalensis Georgi and has been shown to harbor a broad spectrum of biological activities, including antiinflammatory, antioxidant and antiproliferation activities[11]. Protective mechanism of baicalin on endothelial cell injury was related to down-regulation the level of CDH5(as a VE-cadherin), decreasing intracellular calcium load and increasing calcium-calmodulin complex[12]. However, the molecular mechanisms of how baicalin inhibit the express of CDH5 during development of atherosclerosis remain largely obscure. In this study, we explored the roles of baicalin in the proliferation and migration of VSMCs and the related mechanism. It was observed that baicalin may affect the proliferation and migration of VSMCs via regulating the CDH5 /miR-125b-5p signaling pathway.

The effect of baicalin at various concentrations on VSMCs proliferation in response to Hcy was determined by MTT assay. The significant cell proliferation was observed in VSMCs cell treated by Hcy. When the Hcy-induced VSMCs were pretreated with baicalin, we found that the proliferation of VSMCs were reversed in a concentration-dependent manner (Fig.ure 1A, $P < 0.05$ n=3). The inhibiton of proliferation in miR-125b-5p inhibitor group indicated that the miR-125b-5p involved in the growth of Hcy-induced VSMCs. Moreover, proliferation related protein were detected as shown in Figure 1B. In the baicalin+Hcy group, the protein levels of PCNA

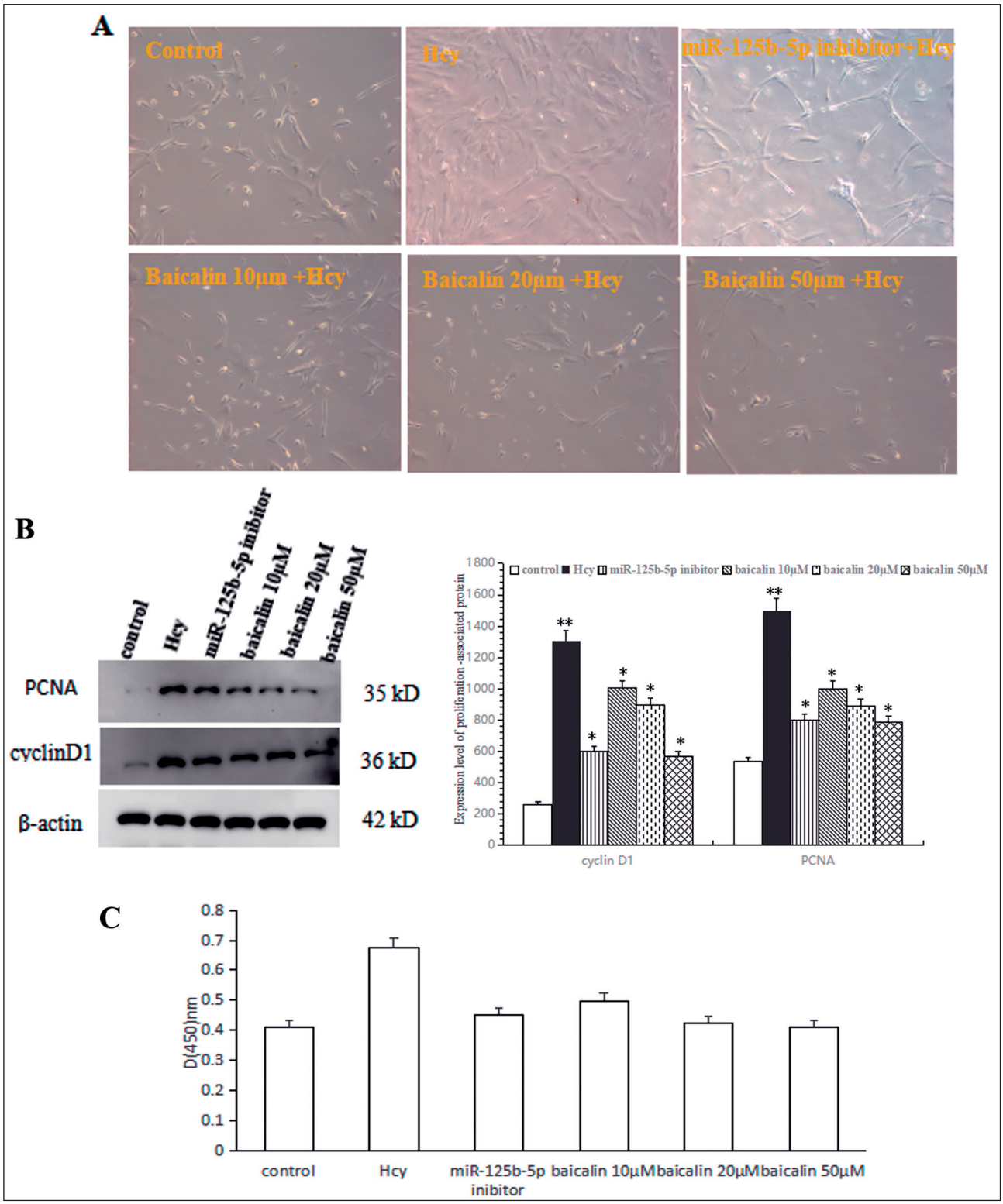

*Figure 1. Cell viability and expression levels of proliferation related indicators were detected. The cells were treated with Hcy and baicalin (10μM, 20μM, 50μM) for 24 h. (A) and (B) Cell viability was determined by MTT assay. (C) and (D)The relative protein expression of Cyclin D1 and PCNA was analyzed using Western Blot, compared with β-actin as an internal control. The data are presented as the mean ± standard deviation (SD) of at least three independent experiments (n=3). ** p < 0.01 the versus control group, *p< 0.05 the versus*

and cyclinD1 were significantly downregulated compared with Hcy group. These results demonstrated that baicalin inhibited VSMCs proliferation induced by Hcy (Figure 1C, $P < 0.05$ n=3).

Hcy group

Next, we investigated the effect of baicalin and miR-125b-5p on the migration of VSMCs cells in vitro. The scratch wound assay indicated that incubation with Hcy resulted in significantly greater wound healing than in the control group.The Migration area and number of penetrating cells were increased in the cell pretreatment with baicalin compared to Hcy group. The cell mobility of control group, Hcy group and baicalin were (38.32±1.06)%, (76.85±1.38)%, (45.48±2.68)%, (46.89±2.75)%, (45.39±2.75)%, (40.22±2.30)% separately (Figure 2, $P < 0.05$ n=3). Consequently, we can conclude that baicalin inhibit significantly the proliferation and migration of VSMCs induced by Hcy.

MiRNAs are endogenous non-coding small RNA of approximately 21-25 nucleotides that negatively regulated target genes at the post-transcriptional level[13,14]. A large amount of literature shows that the miR-125 family participates in the occurrence and development of a variety of cardiovascular and cerebrovascular diseases, including myocardial ischemia, atherosclerosis, ischemia-reperfusion injury, ischemic stroke, and heart failure directly or indirectly[15]. CDH5, a VE-cadherin distributed over vascular epithelium, was regarded as a unique molecular target for controlling atherosclerosis[16-18]. The influence of CDH5 and miR-125b-5p signaling pathway in Hcy-induced activation of VSMCs was analyzed by transfecting miR-125b-5p and CDH5. The miR-125b-5p level was remarkably decreased in the Hcy group compared to the control group, which indicated the key role of miR-125b-5p in the anti-proliferation of hcy-induced VSMCs. Hcy treatment in VSMCs prominently declined the expression of miR-125b-5p and increased the protein level of CDH5. Conversely, when pretreated with baicalin, the mRNA expressions of miR-125b-5p and CDH5 induced by Hcy were reversed (Figure 3 $P < 0.05$ n=3). The lower expressions of miR-125b-5p in Hcy group showed that miR-125b may be-

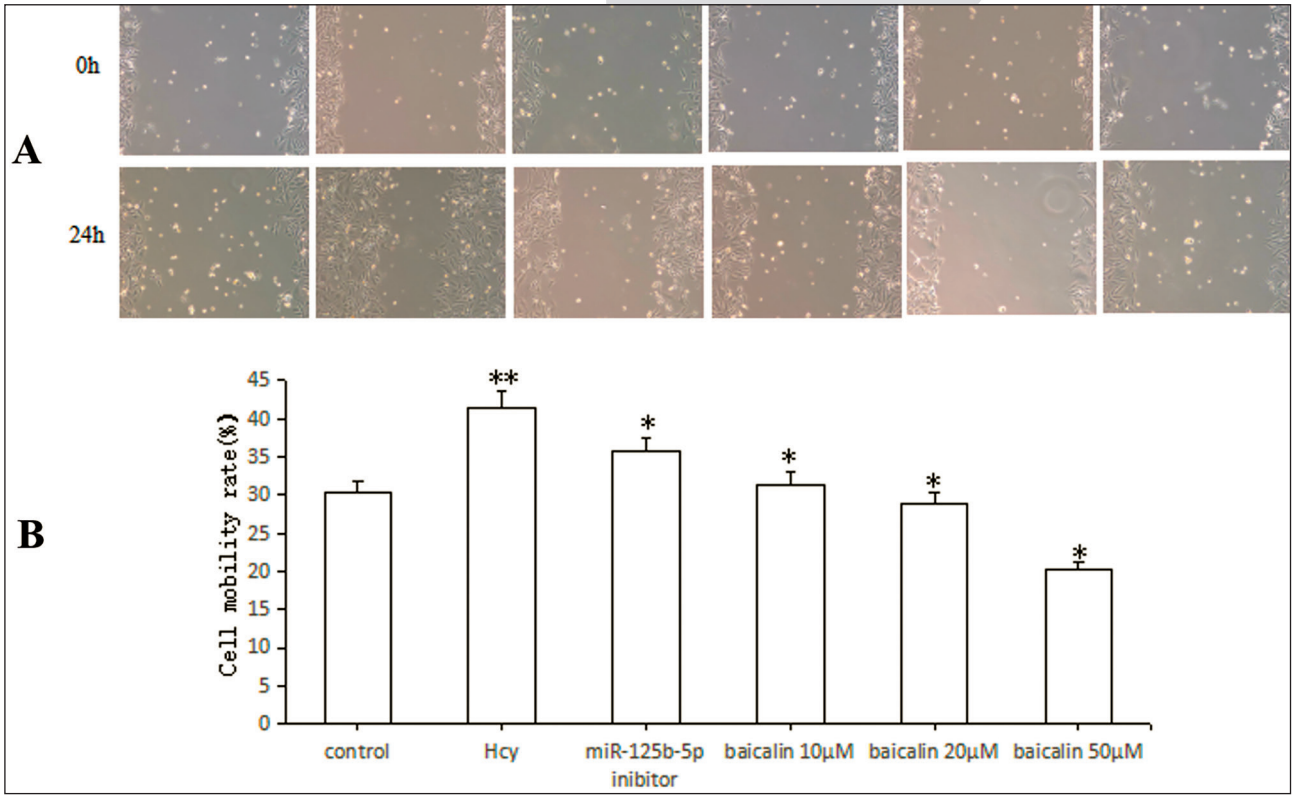

*Figure 2. The effect of migration was determined in Hcy-induced VSMCs induced by scratch wound assay. The data are presented as the mean ± standard deviation (SD) of at least three independent experiments (n=3). ** $p < 0.01$ the versus control group, *$p < 0.05$ the versus Hcy group.*

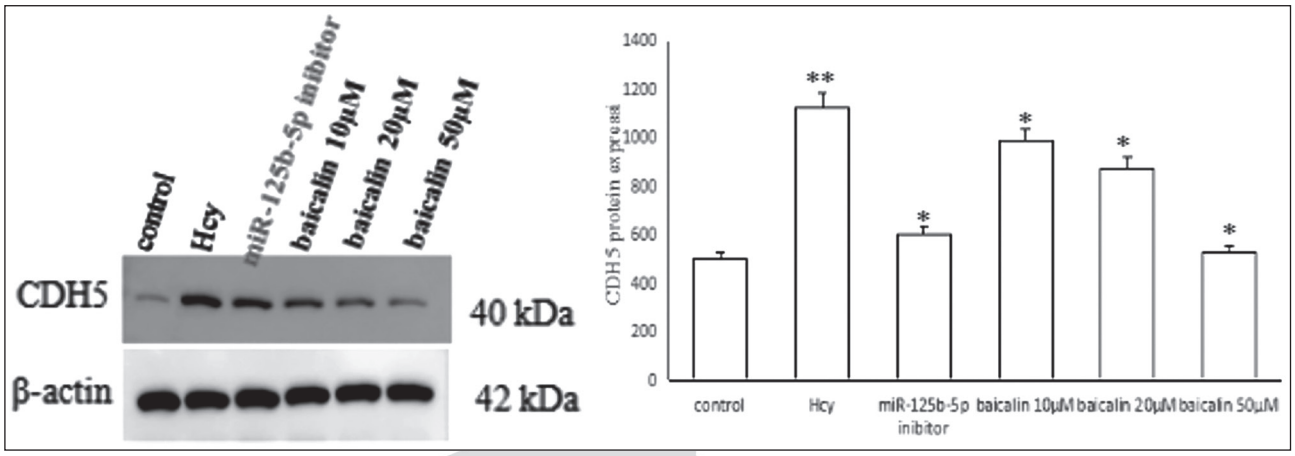

*Figure 3. Effect of baicalin on the expression level of (a) miR-125b-5p and (b) CDH5.The data are presented as the mean ± standard deviation (SD) of at least three independent experiments (n=3). ** p < 0.01 the versus control group, *p< 0.05 the versus Hcy group.*

come an important target inhibiting the proliferation of VSMCs. When pretreated with baicalin, the expressions of miR-125b-5p and CDH5 induced by Hcy were reversed. The fact indiceted that baicalin supressed VSMCs proliferation by promoting the miR-125b-5p expression.

To further verify the underlying mechanism of miR-125b-5p in baicalin inhibiting Hcy-induced VSMCs proliferation, we determined the expression of CDH5. Targeting gene CDH5 of miR-125b-5p were screened by gene prediction software TargetScan, miRanda and TarBase. miR-125b-5p and CDH5 have a binding site in 3'UTR region. CDH5-WT and CDH5-MUT carrier were constructed. The luciferase activity of wild-type CDH5 3'UTR vector co-transfected with miR-125b mimic group was significantly lower than the control group (Figure 4A, P < 0.05 n=3). The

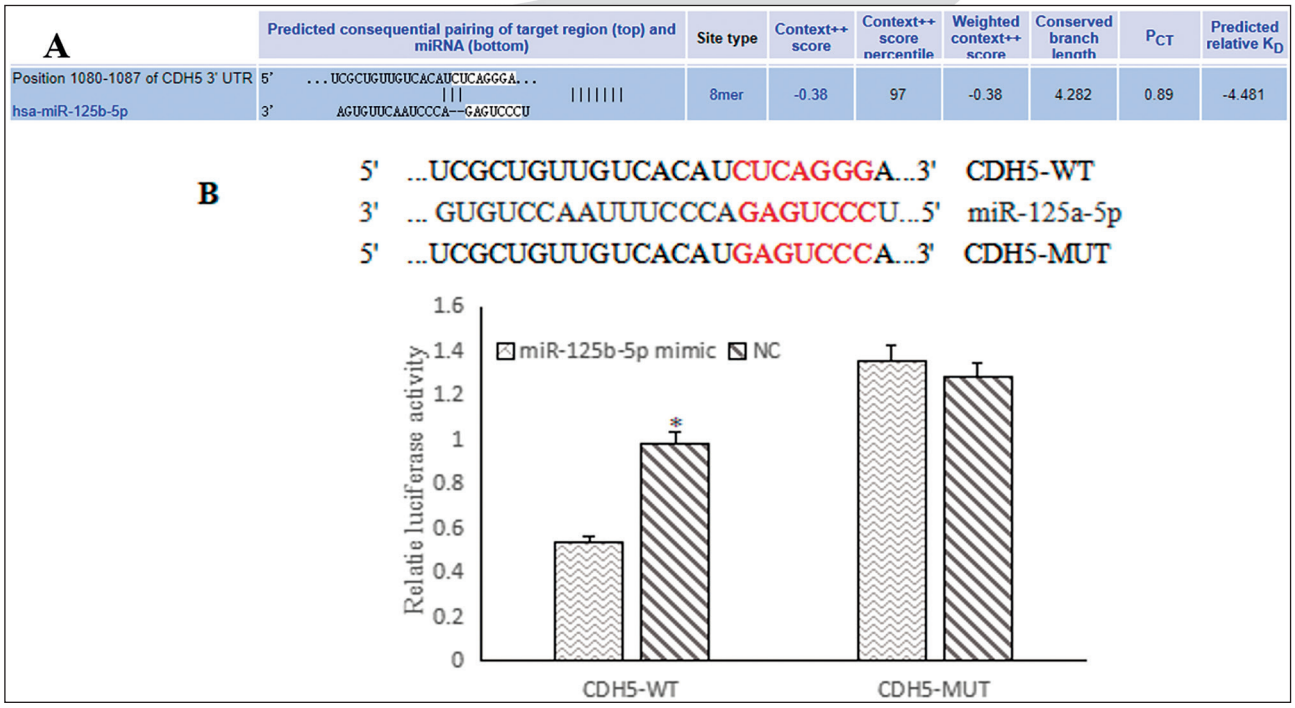

Figure 4. Prediction and validation of miR-125b-5p target gene.
(A) Targeting gene CDH5 of miR-125b-5p were screened by gene prediction software TargetScan.
*(B) CDH5 was verfied as the target of miR-125b-5p by the luciferase assay. The data are presented as the mean ± standard deviation (SD) of at least three independent experiments (n=3). * p < 0.05 the versus NC group.*

miR-125b-5p can decrease the luciferase activity of the wild-type CDH5 3'UTR reporter vector, and had no effect on the mutant-type CDH5 3'UTR reporter vector (Figure 4B). The results illustrated that miR-125b-5p directly targets CDH5.

In conclusion, this study clearly demonstrated that baicalin can inhibit the Hcy-induced VSMCs proliferation. Moreover, miR-125b-5p reversed the Hcy-induced proliferation of VSMCs. Baicalin upregulates the expression of miR-125b-5p and downregulates its target gene CDH5. These findings reveal that baicalin inhibits Hcy-induced proliferation of VSMCs via miR-125b-5p/CDH5 pathway. Our present study provides a theoretical basis by which baicalin can serve as a promising agent to protect against Hcy-induced cardiovascular disease.

Acknowledgements

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Ispitivanje antimikrobne rezistencije izolata gram-negativnih bakterija sa molekularno dokazanim ESBLi karbapenemazama

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Abstract

Problem: The development of resistance of bacteria to antibiotics is one of the leading problems of today's medicine. While previous multi-resistant bacteria were mostly nosocomial pathogens that did not spread particularly well in the outpatient setting, enterobacteria are a group of microorganisms widespread in the human body with the potential to cause both nosocomial and nosocomial infections. drug. Bacteria have evolved to perfection to develop mechanisms for the accumulation of antibiotic resistance genes. Bacteria that produce *extended-spectrum beta-lactamases* (ESBLs), in addition to hospitalized patients, are increasingly being isolated in the outpatient population. *Extended-spectrum beta-lactamases* (ESBLs), which are widespread among enterobacteria, predominantly in *Klebsiella pneumoniae* and *Escherichia coli* strains, have been a major problem in recent years. Respondents and methods: The study was realized as a prospective one, in the time period 2017/2019. in the Microbiological Laboratory of the Institute of Public Health of Sarajevo Canton and in the Service for Monitoring the Susceptibility of Bacteria and Fungi, Laboratory for Molecular Bacteriology and Mycology, Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana.

Key words: *resistance, bacteria, antibiotics, extended-spectrum beta-lactamases, Klebsiella pneumoniae, Escherichia coli.*

Sažetak

Problem: Razvoj otpornosti (rezistencije) bakterija na antibiotike predstavlja jedan od vodećih problema današnje medicine. Dok su dosadašnje multiplorezistentne bakterije uglavnom predstavljale bolničke patogene koji se nisu posebno uspješno širili u vanbolničkoj sredini, enterobakterije čine grupu mikroorganizama široko rasprostranjenih u ljudskom tijelu s potencijalom izazivanja kako bolničkih, tako i vanbolničkih infekcija.Većina bakterija posjeduje različite mehanizme kojima se postiže rezistencija na neki lijek. Bakterije su kroz evoluciju do savršenstva razvile mehanizme za akumulaciju gena rezistencije na antibiotike. Bakterije koje produciraju beta-laktamaze proširenog spektra (ESBL), osim u hospitaliziranih pacijenata, sve se češće izoliraju i u vanbolničkoj populaciji. Posljednjih godina veliki problem predstavljaju beta-laktamaze proširenog spektra (engl. *Extended-spectrum beta-lactamases*, ESBL) koje su široko rasprostranjene među enterobakterijama, predominantno u sojevima *Klebsiella pneumoniae* i *Escherichia coli.*

Ispitanici i metode: Studija je realizovana kao prospektivna, u vremenskom periodu 2017/2019. godina u Mikrobiološkom laboratoriju Zavoda za javno zdravstvo Kantona Sarajevo i u Službi za spremljanje občutljivosti bakterij in gliv, Laboratorij za molekularno bakteriologijo in mikologijo, Inštitut za mikrobiologijo in imunologijo, Medicinska fakulteta Univerza v Ljubljani. Istraživanjem su obuhvaćeni multirezistentni izolati gram-negativnih bakterija, uzročnika infekcija: *Escherichia coli* i *Klebsiella pneumoniae* izolovanih iz urina i drugih bioloških uzoraka vanbolničkih pacijenata u Kantonu Sarajevo. Istraživanjem se sprovelo fenotipsko i genotipsko dokazivanje beta-laktamaza proširenog spektra (ESBL) i karbapenemaza na uzorku od 114 multirezistentnih izolata.

Ključne riječi: *otpornost, bakterija, antibiotik, beta-laktamaze proširenog spektra, Escherichia coli, Klebsiella pneumoniae.*

Uvod

Razvoj otpornosti (rezistencije) bakterija na antibiotike predstavlja jedan od vodećih problema današnje medicine. U protekloj deceniji, u mnogim

dijelovima svijeta, problem rezistencije je postao naročito izražen među gram-negativnim bakterijama (1). Rezistencija bakterija na antibiotike predstavlja problem koji se javio istovremeno sa uvođenjem antibiotika u kliničku praksu, ali na značaju dobija zbog sve većeg broja i raznovrsnosti rezistentnih mikroorganizama. Proučavanjem porijekla različitih mehanizama rezistencije došlo se do zaključka da je rezistencija bakterija na antibiotike široko rasprostranjena u prirodi i da se javlja i u sredinama u kojima nema humanog uticaja. Produkcija prirodnih antibiotika predstavlja jedan od kompetitivnih mehanizama kojim su se bakterije borile za nutrijente. Da bi opstale, osjetljive vrste su razvile odbrambene mehanizme (2). Dok su dosadašnje multiplorezistentne bakterije uglavnom predstavljale bolničke patogene koji se nisu posebno uspješno širili u vanbolničkoj sredini, enterobakterije čine grupu mikroorganizama široko rasprostranjenih u ljudskom tijelu s potencijalom izazivanja kako bolničkih, tako i vanbolničkih infekcija (3,4,5,6). Bakterije koje produciraju beta-laktamaze proširenog spektra (ESBL), osim u hospitaliziranih pacijenata, sve se češće izoliraju i u vanbolničkoj populaciji. Posljednjih godina veliki problem predstavljaju beta-laktamaze proširenog spektra (engl. *Extended-spectrum beta-lactamases*, ESBL) koje su široko rasprostranjene među enterobakterijama, predominantno u sojevima *Klebsiella pneumoniae* i *Escherichia coli* (7,8).

Smjernice za kontrolu i prevenciju antimikrobne rezistencije preporučuju uvođenje sistema nadzora i brzog izvještavanja u trendovima i značajnim promjenama u rezistenciji bakterija. Osnovni mehanizam djelovanja beta-laktamskih antibiotika (penicilina, cefalosporina, karbapenema i monobaktama) je inaktivacija penicilin vežućih proteina (PBP), koji su neophodni u sintezi peptidoglikana bakterijskog ćelijskog zida. Najčešći mehanizam rezistencije *Escherichiae coli* i *Klebsiella pneumoniae* na beta-laktamske antibiotike je sinteza beta-laktamaza, a prisutnost beta-laktamaza proširenog spektra i karbapenemaza u pripadnika porodice *Enterobacteriaceae*, posebno *Klebsiella pneumoniae* i *Escherichia coli*, od velike je mikrobiološke važnosti. Zbog kliničke i epidemiološke važnosti mnogo je istraživanja posvećeno ESBL. Stvaranje beta-laktamaza je najvažniji faktor koji doprinosi rezistenciji gram-negativnih bakterija na beta-laktamske antibiotike. Sve je više poznato da produkcija ESBL nije važna samo za nozokomijalne infekcije, već postaje važno javnozdravstveno pitanje s obzirom na povećanu prevalenciju infekcija ovim sojevima u vanbolničkoj populaciji. Porast učestalosti ESBL-producirajućih sojeva i njihovo značenje za odabir prave antimikrobne terapije, ukazao je na potrebu razvoja adekvatnih laboratorijskih testova za detekciju ESBL i njihovu identifikaciju (9,10,11).

Nastanak rezistencije bakterija na više antimikrobnih lijekova istovremeno, postaje sve veći javnozdravstveni problem s obzirom na često sužen izbor efikasnih antibiotika ili čak odsustvo efikasnog lijeka za liječenje bakterijskih infekcija.

Beta-laktamski antibiotici se uobičajeno koriste za liječenje bakterijskih infekcija. U ovu grupu antibiotika spadaju penicilini, cefalosporini, karbapenemi i monobaktami (12). Beta-laktamaze proširenog spektra djelovanja (engl. *extended spectrum beta-lactamases*, ESBL) su enzimi koji hidroliziraju beta-laktamski prsten antibiotika, pri čemu poništavaju dejstvo antibiotika u terapiji. Postoje mnogi izvještaji o ovim enzimima izolovanim u bakterijskim rodovima porodice *Enterobacteriaceae,* kao i roda *Pseudomonas* (13).

Međutim, ESBL su najučestalije izolirane u bakterija *Klebsiella pneumoniae* i *Escherichia coli* (14). ESBL su prvo izolirane u izolatima iz bolničkih infekcija, a danas i u vanbolničkim infekcijama (15). Antimikrobna rezistencija je prepoznata na globalnom nivou kao jedna od najvećih prijetnji za javno zdravlje ljudi. Naročito su značajne infekcije izazvane rezistentnim gram-negativnim bacilima, koji se sve češće bilježe širom svijeta (1).

Antimikrobna rezistencija gram-negativnih bakterija se zasniva na ekspresiji enzima koji inaktivišu antibiotik ili na neenzimskim mehanizmima. Enzimski kao i neenzimski mehanizmi mogu biti urođeno prisutni kod date vrste na hromozomskim genima ili se mogu steći kao posljedica dva genetička događaja: 1) mutacije hromozomskih gena koji dovode do: a) povećanja ekspresije urođenih mehanizama rezistencije (antibiotik- inaktivišućih enzima ili efluks pumpe), b) promjene u propustljivosti ovojnica gubitkom porina spoljašnje membrane ili c) modifikacije ciljnog mjesta i 2) horizontalnog genskog transfera mobilnih genetičkih elemenata na kojima se nalaze geni rezistencije (najčešće beta-laktamaze, aminoglikozid-modifikujući enzimi i neenzimski mehanizmi (1).

U liječenju infekcija izazvanih enterobakterijama koriste se najčešće beta-laktamski antibiotici, fluorokinoloni i aminoglikozidi. Beta-laktamski antibiotici inhibiraju sintezu ćelijskog zida. Najčešće korišteni predstavnici efikasni protiv enterobakterija su ampicilin, amoksicilin, piperacilin, tikarcilin bez ili sa inhibitorima beta-laktamaza (klavulanskom kiselinom, sulbaktamom i tazobaktamom), cefalosporini proširenog spektra (ceftazidim, cefotaksim, cefepim) i karbapenemi (imipenem, meropenem i ertapenem) (16). Beta-laktamski antibiotici su izuzetno dobri lijekovi jer djeluju na strukture koje ne postoje u humanim ćelijama. Njihova efikasnost je nažalost sve manja uslijed globalne pojave i širenja rezistencije naročito posljednjih decenija.

Ispitanici i metode istraživanja

Studija je realizovana kao prospektivna, u vremenskom periodu 2017/2019. godina u Mikrobiološkom laboratoriju Zavoda za javno zdravstvo Kantona Sarajevo i u Službi za spremljanje občutljivosti bakterij in gliv, Laboratorij za molekularno bakteriologijo in mikologijo, Inštitut za mikrobiologijo in imunologijo, Medicinska fakulteta Univerza v Ljubljani. Istraživanjem su obuhvaćeni multirezistentni izolati gram-negativnih bakterija, uzročnika infekcija: *Escherichia coli* i *Klebsiella pneumoniae* izolovanih iz urina i drugih bioloških uzoraka vanbolničkih pacijenata u Kantonu Sarajevo. U ispitivanje rezistencije uključivao se samo prvi izolat određene bakterijske vrste izoliran u istog pacijenta.

Istraživanjem su obuhvaćeni svi podaci o vanbolničkim pacijentima i rezultati antibiograma o osjetljivosti/rezistenciji uzročnika na pojedine antibiotike.

Svim pacijentima su uzimani biološki uzorci u okviru rutinske, medicinski indicirane bakteriološke analize. Biološki materijali su inokulirani na odgovarajuće hranljive podloge i propisno inkubirani. Gram- negativni izolati su se identificirali standardnim mikrobiološkim metodama.

Uzorci biološkog materijala su se inokulirali na krvni (5%) Columbia agar base i McConcey agar, inkubirani su preko noći na 37 C. Nakon utvrđivanja bakterijskog porasta, gram-negativni bacili su identificirani standardnim mikrobiološkim metodama, a biohemijskim testovima se određivala njihova pripadnost određenoj vrsti (17).

Određivanje osjetljivosti/otpornosti na antimikrobne lijekove

Antimikrobna osjetljivost/otpornost je testirana disk-difuzijskom metodom (Kirby-Bauer) na Mueller-Hinton agaru u skladu sa Clinical and Laboratory Standards Institute (CLSI) standardima. Sojevi *E.coli* i *K.pneumoniae* se testiraju na: amoksicilin, amoksicilin+klavulanska kiselina, piperacilin/tazobaktam, cefaleksin, cefazolin, cefuroksim, cefotaksim, ceftriakson, ceftazidim, cefepim, cefiksim, meropenem, ertapenem, gentamicin, amikacin, tetraciklin, ciprofloksacin, norfoksacin, sulfametoksazol-trimetoprim, nitrofurantoin. Sojevi *Pseudomonas aeruginosa* se testiraju na: piperacilin/tazobaktam, ceftazidim, cefepim, imipenem, meropenem, ciprofloksacin, gentamicin, amikacin. Utvrđeni MDR sojevi se dodatno testiraju na kolistin.

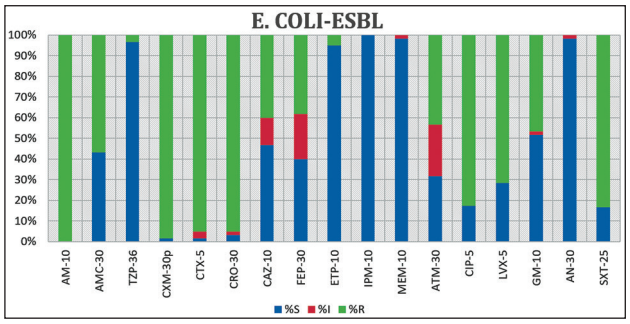
Disk-difuzijski test

Suspenzija testiranog soja se nanosi na površinu Mueller-Hinton agara na koji se zatim nanose diskovi s antibioticima pomoću dispenzera ili pincete. Suspenzija soja se priprema od 3 do 5 kolonija koje se pokupe bakteriološkom ezom sa površine čvrste hranljive podloge i pravi se suspenzija u 5 ml fiziološke otopine. Antibiotik iz diska difundira po površini agara tako da se stvara gradijent koncentracija i na određenoj udaljenosti od ruba diska ovisno o osjetljivosti soja on prestaje rasti. Promjer inhibicijske zone se mjeri i izražava u mm. Na osnovu širine inhibicijske zone određuje se da li je ispitivani soj osjetljiv, umjereno osjetljiv ili rezistentan. Debljina agara treba iznositi 4 mm.

Rezultati istraživanja

U Laboratoriju za molekularnu bakteriologiju i mikologiju Instituta za mikrobiologiju i imunologiju Medicinskog fakulteta Univerziteta u Ljubljani, ispitana je antimikrobna osjetljivost/rezistencija 114 izolata sa molekularno dokazanim ESBL i karbapenemazama. Od toga 60 izolata je bilo *Escherichia coli* i 54 izolata *Klebsiella pneumoniae*.

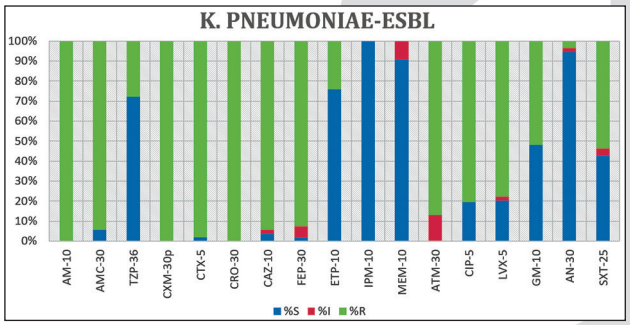
Izolati su testirani na AM-10, AMC-30, TZP-36, CXM-30p, CTX-5, CRO-30, CAZ-10, FEP-30, ETP-10, IPM-10, MEM-10, ATM-30, CIP-5, LVX-5, GM-10, AN-30 i SXT-25.



Grafikon 1. Prikaz rezistentnosti ESBL- Escherichiae coli

Na grafikonu 1. prikazana je distribucija i odnos utvrđene antimikrobne rezistencije na testirane antibiotike kod ESBL-producirajućih sojeva *Escherichiae coli*.

Rezultati pokazuju utvrđenu antimikrobnu rezistenciju na AM-10 (ampicilin) 100,00%, AMC-30 (amoksicilin) 48,30%, TZP-36 (piperacilin/tazobaktam) 3,30%, CXM- 30p (cefuroksim) 98,30%, CTX-5 (cefotaksim) 95,00%, CRO-30 (ceftriakson) 95,00%, CAZ-10 (ceftazidim) 40,00%, FEP-30 (cefepime) 38,30%, ETP-10 (ertapenem) 5,00%, IPM (imipenem) 0,00%, MEM (meropenem) 0,00%, ATM-30 (aztreonam) 43,30%, CIP-5 (ciprofloksacin) 80,00%, LVX-5 (levofloksacin) 71,70%, GM-10 (gentamicin) 46,70%, AN-30 (amikacin) 30,00% i SXT-25 (sulfamethoxasol/trimethoprim) 83,30%.



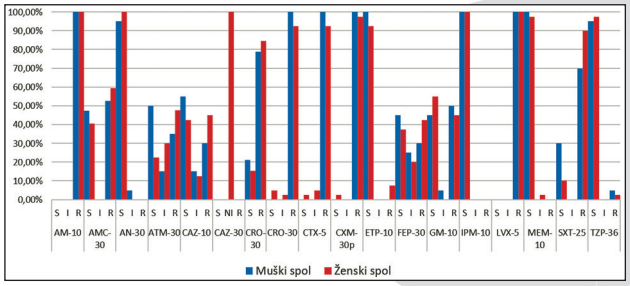
Grafikon 2. Prikaz rezistentnosti ESBL- Klebsiella pneumoniae

Na grafikonu 2. prikazana je distribucija i odnos utvrđene antimikrobne rezistencije na testirane antibiotike kod ESBL-producirajućih sojeva *Klebsiella pneumoniae*.

Rezultati pokazuju utvrđenu antimikrobnu rezistenciju na AM-10 (ampicilin) 100,00%, AMC-30 (amoksicilin) 92,60%, TZP-36 (piperacilin/tazobaktam) 25,90%, CXM- 30p (cefuroksim) 100,00%, CTX-5 (cefotaksim) 98,10%, CRO-30 (ceftriakson) 100,00%, CAZ-10 (ceftazidim) 94,40%, FEP-30 (cefepime) 5,60%, ETP-10 (ertapenem) 24,10%, IPM (imipenem) 0,00%, MEM (meropenem) 0,00%, ATM-30 (aztreonam) 87,00%, CIP-5 (ciprofloksacin) 75,90%, LVX-5 (levofloksacin) 77,80%, GM-10 (gentamicin) 51,90%, AN-30 (amikacin) 3,70% i SXT-25 (sulfamethoxasol/trimethoprim) 53,70%.

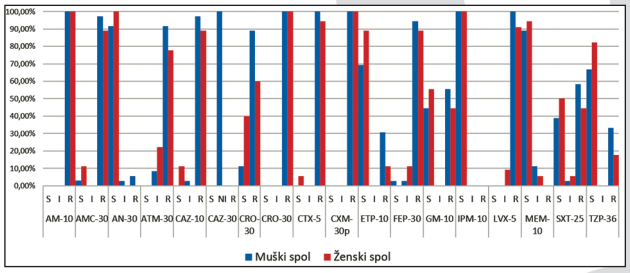
Analizom rezultata antibiograma kod izolata sa molekularno dokazanim ESBL i karbapenemazom, utvrđena je rezistencija na AM-10 (ampicilin) kod svih 60/60 (100,00%) izolata *E. coli* i kod svih 54/54 (100,00%) izolata *K. pneumoniae*. Rezistencija na AMC-30 (amoksicilin) utvrđena je kod 29/60 (48,30%) izolata *E. coli* i kod 50/54 (92,60%) izolata *K. pneumoniae*. Rezistencija na TZP-36 (piperacilin/tazobaktam) utvrđena je kod 2/60 (3,30%) izolata *E. coli* i 14/54 (25,90%) izolata *K. pneumoniae*. Rezistencija na CXM-30p (cefuroksim) utvrđena je kod 59/60 (98,30%) izolata *E.coli* i 54/54 (100,00%) izolata *K. pneumoniae*. Rezistencija na CTX-5 (cefotaxime) utvrđena je kod 57/60 (95,00%) izolata *E. coli* i 53/54 (98,10%) izolata *K. pneumoniae*. Rezistencija na CRO-30 (ceftriaxone) utvrđena je kod 57/60 (95,00%) izolata *E. coli* i 54/54 (100,00%) izolata *K. pneumoniae*. Rezistencija na CAZ-10 (ceftazidime) utvrđena je kod 24/60 (40,00%) izolata *E. coli* i 51/54 (94,40%) izolata *K. pneumoniae*. Rezistencija na FEP-30 (cefepime) utvrđena je kod 23/60 (38,30%) izolata *E. coli* i 50/54 (92,60%) izolata *K. pneumoniae*. Rezistencija na ETP-10 (ertapenem) utvrđena je kod 3/60 (5,00%) izolata *E. coli* i 13/54 (24,10%) izolata *K. pneumoniae*. Rezistencija na IPM-10 (imipenem) nije utvrđena ni kod jednog izolata *E. coli* i *K. pneumoniae*. Kod svih izolata utvrđena je visoka senzitivnost (100,00%) na imipenem. Također nije utvrđena rezistencija ni kod antimikrobnog testiranja na MEM-10 (meropenem). Kod sojeva *E. coli* utvrđena je visoka senzitivnost 59/60 (98,30%) uz postojanje rezultata I (intermedijaran) u 1/60 (1,7%) izolata *E. coli*. Kod izolata *K.pneumoniae* utvrđena je senzitivnost u 49/54 (90,70%) i rezultat (I) kod 5/54 (9,30%) izolata *K. pneumoniae*. Rezistencija na ATM-30 (aztreonam) utvrđena je kod 26/60 (43,30%) izolata *E. coli* i 47/54 (87,00%) izolata *K. pneumoniae*. Rezistencija na CIP-5 (ciprofloksacin) utvrđena je kod 48/60 (80,00%) izolata *E. coli* i 41/54 (75,90%) izolata *K. pneumoniae*. Rezistencija na LVX-5 (levofloxacin) utvrđena je kod 43/60 (71,70%) izolata *E. coli*

i 42/54 (77,80%) izolata *K. pneumoniae*. Rezistencija na GM-10 (gentamicin) utvrđena je kod 28/60 (46,70%) izolata *E. coli* i 28/54 (51,90%) izolata *K. pneumoniae*. Rezistencija na AN-30 (amikacin) nije utvrđena kod izolata *E. coli*, a kod izolata *K.* pneumoniae utvrđena je u 2/54 (3,70%). Na amikacin je utvrđena visoka senzitivnost izolata *E. coli* 59/60 (98,30%) i kod *K. pneumoniae* 51/54 (94,40%). Rezistencija na SXT-25 (sulphamethoxasol-trimethroprim) utvrđena je kod 50/60 (83,30%) izolata *E. coli* i kod 29/54 (53,70%) izolata *K. pneumoniae*.



Grafikon 3. Rezistencija utvrđena na osnovu PCR analize (spolna distribucija) – Escherichia coli

Antimikrobna rezistencija sa rezultatom (100,00%) utvrđena je kod testiranih izolata *Escherichiae coli* od vanbolničkih pacijenata muškog spola na AM-10, CRO-30, CTX-5, CXM-30p i LVX-5. Kod testiranih izolata od vanbolničkih pacijenata ženskog spola, antimikrobna rezistencija 100% utvrđena je na AM-10 i LVX-5 (grafikon 3).



Grafikon 4. Rezistencija utvrđena na osnovu PCR analize (spolna distribucija) - Klebsiella pneumoniae

Antimikrobna rezistencija sa rezultatom (100,00%) utvrđena je kod testiranih izolata *Klebsiellae pneumoniae* od vanbolničkih pacijenata muškog spola na AM-10, CRO-30, CTX-5, CXM-30p i LVX-5. Kod testiranih izolata od vanbolničkih pacijenata ženskog spola, antimikrobna rezistencija 100% utvrđena je na AM-10, CRO-30 i CXM-30p. Analizom rezistencije u odnosu na spol, utvrđena je rezistencija (100,00%) kod muškog spola sa izolatima *E. coli* na AM-10, CRO-30, CTX-5, CXM-30p i LVX-5 i ženskog spola 100,00% na AM-10 i LVX-5; kod muškog spola sa izolatima *K. pneumoniae* utvrđena je 100,00% rezistencija na AM-10, CRO-30, CTX-5, CXM-30p, LVX-5 i kod ženskog spola na AM-10, CRO-30 i CXM-30p.

Analizom antimikrobne rezistencije *Escherichiae coli* u odnosu na dobnu distribuciju, utvrđeno je postojanje najveće rezistencije (100,00%) kod vanbolničkih pacijenata u dobnoj grupi od 0-18 godina na AM-10, CRO-30, CTX-5, CXM-30p, LVX-5, SXT-25. U dobnoj grupi od 19-35 godina na AM-10, CXM-30p, LVX-5, SXT-25. U dobnoj grupi od 36-65 godina na AM-10, CRO-30, CTX-5, CXM-30p, LVX-5. U dobnoj grupi više od 65 godina na AM-10, CRO-30, CTX-5, CXM-30p i LVX-5. Analizom antimikrobne rezistencije *Klebsiella pneumoniae* u odnosu na dobnu distribuciju, utvrđeno je postojanje najveće rezistencije (100,00%) kod vanbolničkih pacijenata u dobnoj grupi od 0-18 godina na AM-10, AMC-30, ATM-30, CAZ-10, CRO-30, CTX-5, CXM-30p, GM-10, CVX-5. U dobnoj grupi od 19-35 godina na AM-10, AMC-30, ATM-30, CAZ-10, CRO-30, CXM-30p, FEP-30, GM-10, LVX-5, SXT-25, TZP-36. U dobnoj grupi od 36-65 godina na AM-10, CRO-30, CXM-30p. U dobnoj grupi više od 65 godina na AM-10, CIP-5, CRO-30, CTX-5 i CXM-30p.

Analizom antimikrobne rezistencije *Escherichiae coli* u odnosu na vrstu pozitivnog uzorka, utvrđena je rezistencija (100,00%) kod brisa rane na AM-10, CIP-5, CRO-30, CTX-5, CXM-30p, LVX-5. Kod izolata brisa (vagine) vulve na AM-10, ATM-30, CIP-5, CRO-30, CTX-5, CXM-30p, FEP-30, GM-10, LVX-5, SXT-25. Kod izolata iz urina na AM-10 i LVX-5. Analizom antimikrobne rezistencije *Klebsiella pneumoniae* u odnosu na vrstu pozitivnog uzorka, utvrđena je rezistencija 100,00% sa najvećom zastupljenošću kod brisa rane na AM-10, AMC-30, ATM-30, CAZ-10, CIP-5, CRO-30, CTX-5, CXM-30p, FEP-30, GM-10, LVX-5, SXT-5. Kod izolata iz brisa uha na AM-10, AMC-30, ATM-30, CAZ-10, CRO-30, CTX-5, CXM-30p, LVX-5. Kod izolata brisa (vagine) vulve na AM-10, AMC-30, ATM-30, CRO-30, CXM-30p, FEP-30, LVX-5, SXT-5. Kod izolata iz urina na AM-10, CRO-30, CTX-5 i CXM-30p.

Diskusija

Multirezistentni izolati bakterija predstavljaju rezervoar multiplih genetičkih elemenata koji su odgovorni za nastanak rezistentnih fenotipova. Racionalna upotreba antibiotika, naročito beta-laktama, kao što su treća generacija cefalosporina i karbapenema, ima za cilj smanjenje selektivnog pritiska kojem su ove bakterije izložene. Gram-negativne crijevne bakterije spadaju u najčešće uzročnike bolničkih i vanbolničkih infekcija. Rezistencija enterobakterija predstavlja rastući problem u svijetu (18).

Multirezistentni sojevi mogu vertikalnim transferom da prenose determinante rezistencije i omoguće širenje soja i njegovu povećanu zastupljenost. Također, ovi sojevi mogu da postanu donori i da horizontalno prenesu determinante rezistencije na druge sojeve, vrste i rodove (18). Dodatni problem predstavljaju multirezistentni regioni koji imaju pluripotentni potencijal rezistencije, tako da upotreba jednog antibiotika dovodi do rezistencije na mnoge druge. Proučavanjem različitih mehanizama rezistencije naročito među bakterijama nađenim u prirodi, stičemo znanja o novim mogućim mehanizmima.

Metodama disk-difuzijskog sinergističkog testa identificirano je 114 ESBL-producirajućih izolata. Analizom rezultata antibiograma kod izolata sa molekularno dokazanim ESBL i karbapenemazom, utvrđena je rezistencija na AM-10 (ampicilin) kod svih 60/60 (100,00%) izolata *E. coli* i kod svih 54/54 (100,00%) izolata *K. pneumoniae*.

Razlika u incidenciji i prevalenciji ESBL-producirajućih bakterija ovisi o više različitih faktora, ali najčešće je povezana sa nekontrolisanom, nepravilnom i neracionalnom upotrebom antibiotika (19). Zloupotreba antibiotika, osobito beta-laktamskih antibiotika širokog spektra djelovanja, olakšava nastajanje otpornosti i pojavu infekcija bakterijama koje produciraju ESBL, kao i prenos ESBL sojeva iz bolničke sredine u vanbolničku. U studiji u Indiji Tada i sur. su izvijestili prevalenciju ESBL kod vanbolničkih pacijenata od 16,7% (20).

U brojnim studijama naglašena je selekcijska uloga antibiotika, naročito cefalosporina treće generacije, u širenju ESBL-producirajućih bakterija (21). Posljednjih godina veliki problem kliničaru i mikrobiologu predstavljaju beta-laktamaze proširenog spektra koje su široko rasprostranjene među enterobakterijama, predominantno u sojeva *K.pneumoniae* i *E.coli* (22). Ove beta-laktamaze prenose se plazmidima uz čestu istodobnu prisutnost gena za rezistenciju na fluorokinolone, aminoglikozide i sulfonamide, što ograničava izbor lijekova u liječenju infekcija ovim bakterijama (22).

Osim otpornosti na sve beta-laktamske antibiotike (osim na karbapeneme), ESBL-producirajući sojevi su multiplo otporni i na niz drugih antibiotika, najčešće istovremeno na aminoglikozide, trimetoprim-sulfamethoxazol i kinolone (23,24,25). Prema smjernicama CLSI-a (*Clinical Laboratory Standard Institute*) (2012) ESBL-producirajući sojevi trebali bi se smatrati otpornim na sve beta-laktamske antibiotike, osim karbapenema, neovisno o rezultatima *in vitro* testiranja (26,27).

Lee DS i sur. u svom istraživanju potvrdili su hipotezu da uslijed selektivnog pritiska koji se javlja kod prekomjerne upotrebe cefalosporina treće generacije dolazi do diseminacije ESBL među pripadnicima porodice *Enterobacteriaceae* (28).

Pojava izolata koji luče ESBL osamdesetih godina prošlog stoljeća, predstavlja odgovor bakterija na široku upotrebu cefalosporina, kao što su ceftazidim i cefotaksim u terapiji (23). Izolatima koji luče ESBL je svojstveno da su osim na cefalosporine, otporni istovremeno i na druge antimikrobne lijekove, aminoglikozide, kinolone i kotrimoksazol zbog toga što su geni koji kodiraju rezistenciju na te antibiotike često locirani na istom plazmidu kao i gen koji kodira ESBL (23,29 ,30). Mnoge studije iznose razloge povećane prevalencije CTX-M beta-laktamaza, a jedna od njih je upotreba cefalosporina treće generacije u terapiji infekcija, osobito ceftriaksona (31). U posljednjih nekoliko godina došlo je do širenja infekcija uzrokovanih ESBL sojevima i u vanbolničkoj sredini (32,33,34). U epidemiologiji ESBL treba uzeti u obzir više različitih nivoa, kao što je individualni pristup svakom pojedinom pacijentu, vrstu medicinske institucije i geografsko područje (35).

Posljednjih 20 godina, više novih beta-laktamskih antibiotika je razvijeno na posebno dizajniran način sa ciljem postizanja otpornosti na hidrolitičku akciju bakterijskih beta-laktamaza (36). Međutim, sa svakom novom klasom lijeka koja je bila korištena u liječenju pacijenata, bakterije su gradile otpornost stvaranjem novih beta-laktamaza (36). Moguće da je prekomjerno korištenje novih antibiotika u liječenju pacijenata stvorilo nove varijante beta-laktamaza (36).

Zloupotreba antibiotika, posebno beta-laktamskih antibiotika širokog spektra djelovanja, olakšava nastajanje otpornosti i pojavu infekcija bakterijama koje produciraju ESBL, kao i prenos ESBL sojeva iz bolničke sredine u vanbolničku (37,38). Prenošenje ESBL sojeva iz bolnice u vanbolničku sredinu je dokazano, naročito kod pacijenata nakon hospitalizacije (39), osobito onih koji su bili hospitalizirani u jedinicama intenzivne njege, a potom otpušteni na kućnu njegu (33). Dokaz prenosa ESBL u vanbolničku sredinu trebale bi pokazati i buduće studije, u cilju identifikacije rezervoara i načina prenosa ESBL (40).

ESBL izolate sa multiplom otpornošću važno je nadzirati (167). Laboratorijske studije u sjevernom dijelu Izraela otkrile su faktore rizika kod nastanka urinarnih infekcija u vanbolničkoj sredini, uzrokovanih ESBL-producirajućim izolatima Klebsiella spp., a to su prethodna hospitalizacija, prethodna antibiotska terapija, muški spol dobne starosti preko 60 godina i dijabetes melitus (41).

Zbog toga je, prije svega, kontinuirano praćenje svih bakterijskih izolata, u bolničkoj kao i u vanbolničkoj sredini, veoma važno kako bi se dobili podaci neophodni za sprovođenje empirijskog liječenja na lokalnom nivou, i kako bi se uvela kontrolirana upotreba antibiotika (42).

Tako je najveća učestalost ESBL u uzorcima bolničkih pacijenata ranije bila najvjerovatnije posljedica istraživanja fokusiranih isključivo na uzorke bolničkih pacijenata, te je njihovo prisustvo u uzorcima vanbolničkih pacijenata bilo potcijenjeno (33). ESBL su otkrivene u gram-negativnih štapićastih bakterija sa različitom prevalencijom, međutim, najveći broj ovih enzima otkriven je u porodici *Enterobacteriaceae* (35).

Multirezistentni sojevi *Klebsiella pneumoniae* predstavljaju značajne uzročnike naročito bolničkih, ali i vanbolničkih infekcija kao što su infekcije urinarnog trakta, pneumonija, septikemija, meningitis i infekcije mekih tkiva. Sojevi *Klebsiella pneumoniae* imaju sposobnost akumuliranja plazmida koji nose gene rezistencije za veliki broj antibiotika kao što su penicilini, cefalosporini, karbapenemi, aminoglikozidi i fluorokinoloni (43). Prenos gena rezistencije sa *Klebsiella pneumoniae* na *Escherichia coli* može doprinijeti širenju multirezistentnih izolata u zajednici uzrokujući prije svega teške urinarne infekcije (44). Sa druge strane, sojevi *Escherichia coli* koji nose gene rezistencije mogu dospjeti u crijevni trakt ljudi sa životinja, ili iz vodene sredine putem hrane, vode ili preko ruku. Multirezistentni izolati *Escherichia coli* kod ljudi mogu da uzrokuju brojna oboljenja, ali i da prenesu determinante rezistencije na druge patogene bakterije, što za posljedicu ima razvoj teže bolesti, produženo trajanje bolesti i zbog toga često terapijski neuspjeh (45).

Zaključci

ESBL – producirajući sojevi su utvrđeni kod 114 izolata, od toga 60 (47,0%) izolata je *Escherichia coli* i 54 (53,0%) izolata *Klebsiella pneumoniae*.

Antimikrobna rezistencija na cefalosporine kod ESBL- producirajućih sojeva *E. coli* utvrđena je na cefotaksim 95,0%, ceftriakson 95,0%, ceftazidim 40,0%, cefepime 38,3% i cefuroksim 98,3%; kod ESBL-producirajućih sojeva *K. pneumoniae* rezistencija na cefotaksim 98,1%, ceftriakson 100,0%, ceftazidim 94,4%, cefepime 92,6% i cefuroksim 100,0%.

Antimikrobna rezistencija na karbapeneme ESBL- producirajućih sojeva *E. coli* utvrđena je na ertapenem u 5,0%, na imipenem je utvrđena visoka senzitivnost 100,0%, na meropenem je utvrđena senzitivnost 98,3% uz postojanje rezultata intermedijaran u 1,7%; kod ESBL-producirajućih sojeva *K. pneumoniae* utvrđena je rezistencija na ertapenem u 24,1%, na imipenem visoka senzitivnost 100,0%, na meropenem senzitivnost u 90,7% i rezultat intermedijaran 9,3%.

Antimikrobna rezistencija na monobaktame (aztreonam) ESBL-producirajućih sojeva *E. coli* utvrđena je kod 43,3% , rezistencija ESBL-producirajućih sojeva *K. pneumoniae* utvrđena je kod 87,0%.

Antimikrobna rezistencija na ostale testirane antibiotike utvrđena je kod ESBL-producirajućih sojeva *E.coli* na ampicilin 100,0%, amoksicilin 48,3%, piperacilin/tazobaktam 3,3%, ciprofloksacin 80,0%, levofloxacin 71,7%, gentamicin 46,7%, sulphamethoxasol-trimethroprim 83,3%; rezistencija ESBL-producirajućih sojeva *K. pneumoniae* na ampicilin je 100,0%, amoksicilin 92,6%, piperacilin/tazobaktam 25,9%, ciprofloksacin 75,9%, levofloksacin 77,8%, gentamicin 51,9%, amikacin 3,7% i sulphametoxasol-trimethroprim 53,7%.

Antimikrobna rezistencija sa rezultatom (100,0%) utvrđena kod izolata sa molekularno dokazanim ESBL i karbapenemazama, bila je najza-

stupljenija kod pacijenata muškog spola, u starosnoj grupi od 19-35 godina, sa izolatima *K. pneumoniae* iz brisa rane.

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Abstract

In this paper the instructions for preparing camera ready paper for the Journal are given. The recommended, but not limited text processor is Microsoft Word. Insert an abstract of 50-100 words, giving a brief account of the most relevant aspects of the paper. It is recommended to use up to 5 key words.

Key words: Camera ready paper, Journal.

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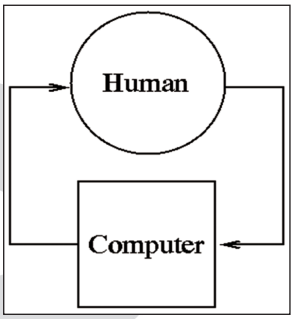

Figure 1. Text here

Conclusion

Be brief and give most important conclusion from your paper. Do not use equations and figures here.

Acknowledgements (If any)

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