DE GRUYTER OPEN

Eur. Pharm. J. 2017, 64 (1): 1-3.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.1515/afpuc-2017-0018

EUROPEAN PHARMACEUTICAL JOURNAL

Pharmacotherapy of adolescent depression - fluoxetine monotherapy or combined treatment?
Farmakoterapia adolescentnej depresie - monoterapia fluoxetínom alebo kombinovaná liečba?

Original Paper

Cesneková D.[1#], Ondrejka I.[1✉#], Oppa M.[1], Tonhajzerová I.[1], Nosáľová, G.[2]

[1]Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin, Biomedical Center Martin, Martin, Slovak Republic

[1]Univerzita Komenského v Bratislave, Jesséniova lekárska fakulta v Martine, Biomedicínske centrum Martin, Martin, Slovenská Republika

[2]Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin, Department of Pharmacology, Martin, Slovak Republic

[2]Univerzita Komenského v Bratislave, Jesséniova lekárska fakulta v Martine, Ústav farmakológie, Martin, Slovenská Republika

Received 10 July, 2017, accepted 26 July, 2017

Abstract Depressive disorder is one of the most common and serious psychiatric diagnosis in paediatric population, often connected with suicidal risk. In recent years, fluoxetine monotherapy is the gold standard in acute phase of depression treatment in children and adolescents, but is not effective enough after an acute phase of treatment. More helpful researches concerning more effective therapeutic strategies of depression in this age are insufficient. The aim of our study is to evaluate the effectiveness and safety of fluoxetine monotherapy in comparison with combined olanzapine/fluoxetine therapy in acute 6-week treatment of depression in adolescence. We found that combined therapeutic strategy, using olanzapine augmentation is predicted to be more useful in the treatment of adolescent depression.

Slovak abstract Depresívna porucha patrí medzi závažné a často sa vyskytujúce ochorenie v pedopsychiatrii, často spojené so suicidálnym rizikom. V súčasnosti je zlatým štandardom akútnej fázy liečby ochorenia fluoxetín, avšak jeho efektivita po akútnej fáze liečby ochorenia nie je dostatočná. Výsledky doterajšieho výskumu týkajúce sa efektívnejšej farmakoterapie depresívnej poruchy u adolescentov sú nedostatočné. Cieľom nášho výskumu bolo posúdiť účinnosť a bezpečnosť monoterapie fluoxetínom a kombinovanej terapie fluoxetín/olanzapín v 6-týždňovej akútnej fáze liečby depresívnej poruchy u adolescentov. Zistili sme, že kombinovaná liečba za použitia augmentácie olanzapínom predstavuje efektívnejšiu terapeutickú stratégiu v liečbe adolescentnej depresie.

Keywords *Depression – fluoxetine – olanzapine – adolescence*

Kľúčové slová: *Depresia – fluoxetín – olanzapín – adolescencia*

INTRODUCTION

Major depressive disorder (MDD) in children and adolescents is a common and recurrent disorder, occurring approximately in 2% of children, 4-8% of adolescents and is twice as prevalent in adolescent girls than in boys (Emslie et al., 2002), typically increasing in prevalence (APA, 2013). Adolescent depression is associated with negative academic, social and health outcomes, including depression in adulthood, increased suicidal risk, substance abuse, significant morbidity and suicidality. Depression presents a great burden to individuals with reduction of overall functioning and affects various psychological variables such as the meaningfulness of life and hope (Farsky et al., 2012). Selective serotonin reuptake inhibitors (SSRIs) are the first-line treatment for depression in children and adolescents (Ondrejka, 2016). Fluoxetine in dosage of 20 mg/day is the only SSRI antidepressant approved by the U.S. Food and Drug Administration (FDA) in paediatric population from 8 to 18 years of age (FDA, 2015). Fluoxetine monotherapy is safe and well-tolerated and has shown efficacy in a number of open-label and double-blind, placebo-controlled trials, but only during acute several weeks' treatment (Heiligenstein et al., 2006). The usefulness of fluoxetine monotherapy is limited by not having enough effectiveness after the acute phase of treatment (Kennard et al., 2006). For this reason, recent interest has focused on the new

* E-mail: Igor.Ondrejka@jfmed.uniba.sk
# Dana Cesnekova and Igor Ondrejka contributed equally to the study (ana partes).

therapeutic strategies consisting of combined or augmented therapeutic options, using, for example, antipsychotic drugs. Olanzapine as a multi-receptor antagonist shows antidepressant effect, especially in psychotic and bipolar depression (Detke et al., 2014). Our results showed that fluoxetine/olanzapine combined therapy is effective in the treatment of unipolar and non-psychotic depression.

MATERIAL AND METHODS

The study sample was composed of 40 adolescent inpatients in Clinic of Psychiatry, University Hospital, Martin (aged 15-17 years, 16.2 ± 1). Inclusion criteria were patients with MDD, who met DSM-5 criteria for non-psychotic MDD and had a CDRS-R total score of > 40 and CGI-S (Clinical Global Impression – Severity) rating of ≥ 4 at study entry and were able to participate in the research. The exclusion criterion was serious symptomatic disease. Study participants were randomized into two lines (20 patients in each line), fluoxetine monotherapy (FXT) and therapy of olanzapine/fluoxetine combination (OFC) for acute 6 weeks phase of treatment of MDD. Fluoxetine was supplied in dosage 20 mg/day and olanzapine in dosage 2.5-5 mg/day according to the patient´s weight. Participants were assessed at baseline, and every week of the 6-weeks treatment (6th week was the end of the acute treatment) using the scales typical for children during the acute phase of treatment. Depressive symptoms were assessed using the CDRS-R (Children´s Depression Rating Scale-Revised); it is a 17-item clinician-rated measure of depression severity and was completed by a clinician at baseline, and every week of 6-weeks' treatment. Clinician´s impression of improvement was evaluated by the CGI-I (Clinical Global Impression Scale Improvement) and was completed every week of 6-weeks' treatment. Adverse effects of the treatment were observed. Data were evaluated by statistical analysis (Pairwise Two-Sided Multiple Comparison Analysis, Dwass, Steel and Critchlow-Fligner Method). The study corresponds with the ethical standards of current scientific research. All collected data are used only for scientific purposes and personal data are not published.

RESULTS

Both fluoxetine monotherapy (FXT) and olanzapine/fluoxetine combined therapy (OFC) were associated with significant mean improvement in CDRS-R total score after 6-weeks' acute phase of treatment, but the mean improvement was greater in the combined therapy (OFC 59.1 ± 7.9, $p < 0.0001$ vs. FXT 58.1 ± 11.5, $p = 0.0002$). Combined therapy has shown significantly greater reduction of depressive symptoms after the 2nd week of treatment in OFC line ($p < 0.0001$) (Figure 1.a, Table 1.). Combined therapy led to a greater clinical improvement in CGI-I total score as compared with fluoxetine monotherapy after 6-weeks' acute phase of treatment (FXT 3.3 ± 1 vs. OFC 3.1 ± 1.6, $p = 0.03$), clinical improvement was significantly greater after the 3rd week of treatment in combined therapy ($p < 0.0001$) (Figure 1.b). The most common side effects of fluoxetine monotherapy were tremor and headache, and in the combined therapy, there were side effects such as headache, increased appetite, weight gain and fatigue.

Table 1. Changes in CGI-I and CDRS-R total score: at baseline and after 6-week of treatment of MDD (FXT - fluoxetine, OLA - olanzapine)

		FXT group	FXT/OLA group
CDRS-R scale	baseline	58.1±11.5	59.1±7.9
	6-th week	35.8±12.7	33.8±11.3
CGI-I scale	1-st week	3.9±0.2	4.0
	6-th week	3.2±1	3.1±1.6

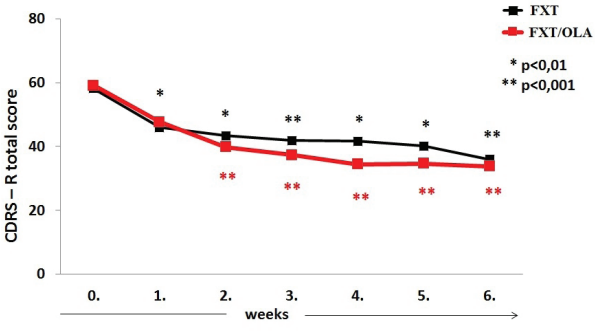


Figure 1. a) Dynamics of depressive symptoms during 6-weeks' treatment of MDD according to CDRS-R objective scale (FXT - fluoxetine monotherapy group, FXT/OLA - fluoxetine/olanzapine combined therapy).

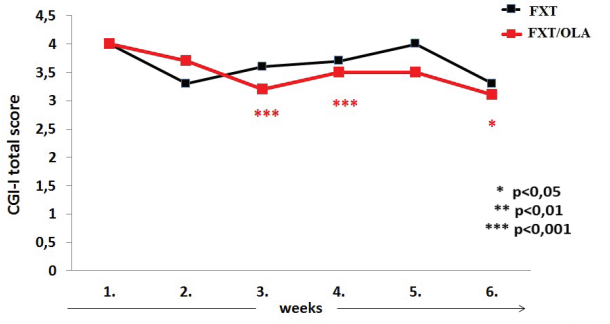


Figure 1. b) Dynamics of clinical improvement during 6-weeks' treatment of MDD according to CGI-I scale (FXT - fluoxetine monotherapy group, FXT/OLA - fluoxetine/olanzapine combined therapy).

DISCUSSION AND CONCLUSION

Our view of the literature using CDRS-R as a measure of treatment effectiveness in the paediatric population indicates

that fluoxetine monotherapy is safe and effective in the acute phase of treatment of MDD in three placebo controlled-trials (Emslie et al., 1997, 2002; March et al., 2004). On the other side, residual symptoms after the acute treatment are common, and relapse/recurrence of depression is typical for more than 30% of adolescents (DeFilippis, Wagner, 2014). According to the study of Mayes et al. (2007), there is a remission rate 41.5% in children and 32.6% in adolescents after the acute phase of fluoxetine monotherapy treatment. Fluoxetine monotherapy is probably not sufficient in treating MDD in children psychiatry. More effective therapeutic strategies are insufficient. In recent years, the combined or augmented therapeutic strategies are preferred. Olanzapine is an effective mood stabilizer and a multi-receptors antagonist with antidepressant effect that binds with high affinity to the receptors of serotonin 5HT2A/2C, 5HT6, dopamine D1-4, histamine H1 and adrenergic α1 receptors. Olanzapine is an antagonist with moderate affinity binding for serotonin 5HT3 and muscarinic M1-5 receptors. Olanzapine binds weakly to GABAA (GABAergic), benzodiazepine, and β-adrenergic receptors and can enhance antidepressant action of fluoxetine - highly specific serotonin reuptake inhibitor. This fact was demonstrated in adult studies of treatment resistant depression, which showed that the olanzapine/fluoxetine therapy is superior to fluoxetine alone (Corya et al., 2006). There are no published studies on MDD in adolescent age comparing fluoxetine monotherapy and combined therapeutic options. There is only one study in bipolar depression showing that olanzapine/fluoxetine combination was superior to placebo in the acute treatment of bipolar I depression in patients who were 10 to 17 years old (Detke et al., 2015), but no more studies are available. According to our study, both therapeutic strategies showed statistically significant effectiveness in the acute 6-weeks' treatment of MDD, but olanzapine/fluoxetine combination is more statistically significant as compared to fluoxetine monotherapy. Except this, we found that for a combined therapy, it is typical to have an earlier onset of action (after 2nd week of treatment). It is possible that the combined therapeutic strategies suggest a higher level of therapeutic effectiveness after the acute treatment, better response to the treatment, shorter time to remission and relapse prevention in patients with MDD. Similar to adult patients, clinically, the most common adverse effects of olanzapine/fluoxetine combination in our patients were increased appetite, weight gain and fatigue. Despite the known adverse effects of olanzapine medication, benefits outweigh the risks (Detke et al., 2015). Combined/augmented treatment in patients with depression is needed, and more research is necessary.

ACKNOWLEDGEMENT

This work is supported by grants VEGA 1/0087/14 and UK/75/2016, UK/90/2016.

References

[1] American psychiatric association. Diagnostic and statistical manual of mental disorders. DSM-5. 5th ed. Arlington, VA: American Psychiatric Association; 2013. ISBN 978-0-89042-554-1.

[2] Corya SA, Williamson D, Sanger TM, Briggs SD, et al. (2006). A randomized, double-blind comparison of olanzapine/fluoxetine combination, olanzapine, fluoxetine, and venlafaxine in treatment-resistant depression. Depress Anxiety 23:364–372.

[3] DeFilippis M, Wagner KD. Management of treatment-resistant depression in children and adolescents. Paediatr Drugs. 2014 Oct;16(5):353-61.

[4] Detke HC, Delbello MP, Landry J, Usher RW. Olanzapine/Fluoxetine combination in children and adolescents with bipolar I depression: a randomized, double-blind, placebo-controlled trial. J Am Acad Child Adolesc Psychiatry 2015;54(3):217–224.

[5] Emslie GJ, Heiligenstein JH, Wagner KD, et al. Fluoxetine for acute treatment of depression in children and adolescents: a placebo-controlled, randomized clinical trial. J Am Acad Child Adolesc Psychiatry 2002; 41 (10): 1205-1215.

[6] Emslie GJ, Rush AJ, Weinberg WA, et al. A double-blind, randomized, placebo-controlled trial of fluoxetine in children and adolescents with depression. Arch Gen Psychiatry 1997; 54 (11): 1031-1037.

[7] Farsky I, Smetanka A, Dubinska S. Spiritualita pacientov s vybranymi psychiatrickymi diagnozami. Osetrovatelstvi a porodni asistence. 2012;3(3):433–441.

[8] Food and drug administration (FDA). Antidepressant Medications: Use in Pediatric Patients. 2015. In https://www.cms.gov/Medicare-Medicaid-Coordination/Fraud-Prevention/Medicaid-Integrity-Education/Pharmacy-Education-Materials/Downloads/ad-pediatric-factsheet11-14.pdf

[9] Heiligenstein JH, Hoog SL, Wagner KD, Findling RL, et al. Fluoxetine 40-60 mg versus fluoxetine 20 mg in the treatment of children and adolescents with a less-than-complete response to nine-week treatment with fluoxetine 10-20 mg: a pilot study. J Child Adolesc Psychopharmacol. 2006 Feb-Apr;16(1-2):207-17.

[10] Kennard B, Silva S, Vitiello B, Curry J, et al. TADS Team. Remission and residual symptoms after short-term treatment in the Treatment of Adolescents with Depression Study (TADS). J Am Acad Child Adolesc Psychiatry. 2006 Dec;45(12):1404-11.

[11] March J, Silva S, Petrycki S, et al. Fluoxetine, cognitive-behavioral therapy, and their combination for adolescents with depression: Treatment for Adolescents With Depression Study (TADS) randomized controlled trial. JAMA 2004; 292 (7): 807-820.

[12] Mayes TL, Tao R, Rintelmann JW, Carmody T, et al. Do children and adolescents have differential response rates in placebo-controlled trials of fluoxetine? CNS Spectr. 2007 Feb;12(2):147-54.

[13] Ondrejka I. Liečba psychofarmakami vdetskom aadolescentnom veku. In: Psychofarmakológia. Vyd. Wolters Kluwer, 2016. S. 467-502. ISBN: 978-80-8168-543-9.

DE GRUYTER OPEN

Eur. Pharm. J. 2017, 64 (1): 4-6.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.1515/afpuc-2017-0014

EUROPEAN PHARMACEUTICAL JOURNAL

Endothelial dysfunction in experimental models of metabolic syndrome – effect of fructose
Endotelová dysfunkcia v experimentálnom modely metabolického syndrómu - význam fruktózy

Original Paper

Kaprinay B.[1,3]✉, Gáspárová Z.[1], Lipták B.[1,3], Frimmel K.[2], Sotníková R.[1]

[1]Slovak Academy of Sciences, Institute of Experimental Pharmacology and Toxicology, Bratislava, Slovak Republic

[2]Slovak Academy of Sciences, Institute of Heart Research, Bratislava, Slovak Republic

[3]Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin, Department of Pharmacology, Martin, Slovak Republic

[1]Slovenská akadémia vied, Ústav experimentálnej farmakológie a toxikológie, Bratislava, Slovenská Republika

[2]Slovenská akadémia vied, Ústav pre výskum srdca, Bratislava, Slovenská Republika

[3]Univerzita Komenského v Bratislave, Jesséniova lekárska fakulta v Martine, Ústav farmakológie, Martin, Slovenská Republika

Received 22 June, 2016, accepted 19 July, 2016

Abstract The aim of the work was to find an experimental model suitable for the study of endothelial dysfunction induced by MS. We used hypertriglyceridemic rats (HTG) that were fed a hypercholesterolemic diet of different composition and duration: a 6-week administration of standard diet with an addition of cholesterol and fat (HTGChol) and a three-month administration of the same diet with an addition of fructose (HTGCholF). We investigated the effect of different diets on aortic endothelial function. The standard diet fed Wistar (W) and HTG rats served as controls. Decision for addition of fructose to HTGChol was done based on in vitro experiments evaluating the effect of high concentration of saccharide in the incubation solution on aortic endothelial function. This intervention caused significant deterioration of relaxation induced by acetylcholine (ACh). While in HTGChol, we did not find significant differences in the function of the aorta compared to W or HTG rats, adding of fructose to high fat diet and prolonging its administration resulted in significantly impaired endothelium-dependent relaxation. It seems that such a model is suitable for the study of endothelial dysfunction in MS and the effect of substances that may protect the endothelium.

Slovak abstract Cieľom práce bolo nájsť experimentálny model, vhodný na štúdium endotelovej dysfunkcie počas MS. Použili sme hypertriglyceridemické potkany (HTG), ktorým sa podávala hypercholesterolemická diéta rôzneho zloženia a trvania. Jednalo sa o 6-týždňové podávanie štandardnej stravy s prídavkom cholesterolu a tuku (HTGChol) a 3-mesačné podávanie rovnakej diéty s prídavkom fruktózy (HTGCholF). Zisťoval sa vplyv jednotlivých diét na funkciu endotelu aorty. Wistar and HTG potkany kŕmené štandardnou diétou slúžili ako kontrola. Pre pridanie fruktózy k vysokotukovej diéte sme sa rozhodli na základe in vitro pokusov, kde sa hodnotila funkcia endotelu po inkubácii aorty v roztoku obsahujúcom vysokú koncentráciu cukru. Po takomto zásahu došlo k významnému poškodeniu relaxácie vyvolanej acetylcholínom v porovnaní s kontrolnou skupinou. Kým u zvierat kŕmených HTGChol sme nenašli významné rozdiely vo funkcii aorty v porovnaní s Wistar a HTG potkanmi, pridanie fruktózy k vysokotukovej diéte a predĺženie jej podávania malo za následok signifikantne poškodenú relaxáciu závislú od endotelu. Zdá sa, že tento model je vhodný na štúdium endotelovej dysfunkcie počas MS a látok, ktoré by mohli endotel ochrániť.

Keywords *Endothelium – Metabolic Syndrome – HTG*

Kľúčové slová: *Endotel – Metabolický syndróm – HTG*

INTRODUCTION

Metabolic syndrome (MS) is associated with serious metabolic abnormalities, which present a high risk for developing cardiovascular diseases. Vascular endothelial dysfunction, which can lead to atherosclerosis, belongs to the first signs of cardiovascular disorders (Oostrom et al., 2002). In our previous work (Kaprinay et al. 2016), we found that hypertriglyceridemic rats fed high fat, high cholesterol diet (HTGChol) are suitable non-obese models of MS, however, without significant changes in the vascular endothelium. The most important components of MS are hyperglycemia and glucose intolerance that are accompanied with oxidative stress – reduction of antioxidant activity and increased production of reactive oxygen species (Anderson et al. 2001, Bae et al. 2001). Oxidative stress has an important role in

* *E-mail: exfabaka@savba.sk*

endothelial dysfunction (Sotníková & Bauer, 2010). Bartuš et al. (2008) found that a rich fructose diet induced serious abnormalities in the cardiovascular system. Therefore, the aim of the work was to evaluate the effect of fructose addition to high fat, high cholesterol diet on the aortic endothelial function during MS.

METHODS

The investigation conformed to the Guide for the Care and Use of Laboratory Animals. Male hypertriglyceridemic rats were divided into three groups: HTG control group fed standard diet, HTGChol group fed high fat, high cholesterol diet for 6 weeks and HTGCholF fed high fat, high cholesterol, fructose rich diet for 3 months. Wistar (W) rats fed standard diet served as control group. The composition of the modified diets was: standard pellets with 1% cholesterol, 7.5% lard, 10% fructose. After the decapitation of animals, function of the isolated thoracic aorta was tested under isometric conditions. Responses of the phenylephrine-precontracted (1µmol/l) arteries to cumulative acetylcholine (Ach) before and after NO-synthase (NOS) inhibition with N-nitro-L-arginine methyl ester hydrochloride (30 µmol/l; L-NAME) were investigated. Data are presented in percentage of phenylephrine-induced contraction and the results are statistically compared using the ANOVA t-test with Bonferroni post test.

RESULTS

Incubation of aorta with a medium containing 44 mmol/l glucose for 24 hours resulted in significantly smaller relaxation to Ach as compared to controls. Responses of aortas from HTG and HTGChol animals to Ach were not statistically different from those of W, while Ach-evoked relaxation of HTGCholF was damaged (Fig. 1). The responses to Ach after NOS-blockade were similar in W and HTG, and the biggest L-NAME-resistant component was found in HTGCholF. The L-NAME-resistant part of the endothelium-dependent relaxation was the smallest in HTGChol (Fig. 2).

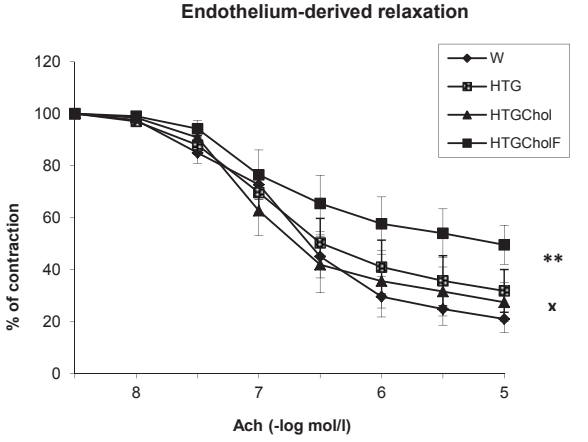


*Figure 1. Responses of the aortas of W, HTG, HTGChol and HTGCholF rats precontracted by phenylephrine (1 µmol/l) to acetylcholine (Ach). Data are means ± SEM obtained from 9-10 experiments. ** p <0.01 HTGCholF vs. W, x p <0.05 HTGCholF vs. HTGChol*

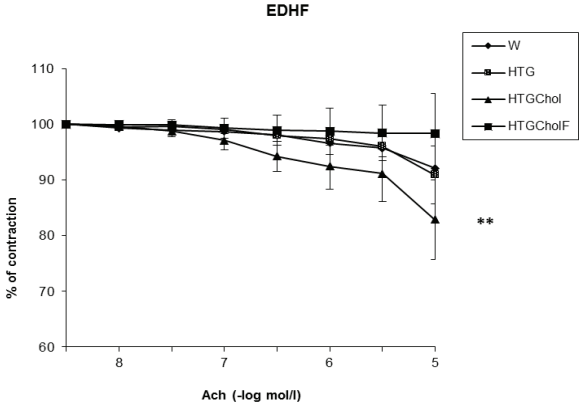


*Figure 2. Responses of the aortas of W, HTG, HTGChol and HTGCholF rats precontracted by phenylephrine (1 µmol/l) to acetylcholine (Ach) after the blockade of NOS by L-NAME. Data are means ± SEM obtained from 9-10 experiments. ** p <0.01 HTGCholF vs. W*

DISCUSSION

Our results showed endothelium dysfunction of the aorta of rats that were fed high cholesterol, high fat diet enriched with fructose. On difference, in HTGChol without fructose, the function of endothelium was preserved, although rats had signs of MS – impaired glucose and lipid metabolism, increased blood pressure and developing liver steatosis (Kaprinay et al., 2016). These results correspond with other authors (Bartuš et al., 2008; Sasaki et al., 1994; Kitagawa et al., 1995; Pisulewski et al., 2005), who did not find any modification of the endothelial function in HTGChol rats. Hyperglycemia and glucose intolerance are important components of human MS. Oxidative stress is a main reason of their pathological influence on the cardiovascular system, including the endothelium. The burst of ROS in the endothelium results in decrease of bioavailability of nitric oxide and subsequently to endothelial dysfunction. These facts supported our decision to add fructose to a high cholesterol, high fat diet. Indeed, the aortas of HTGCholF rats showed significantly lower response to Ach. In the aorta, the part of Ach-induced relaxation is assumed to be mediated by endothelium-derived hyperpolarizing factor (EDHF), which can be identified after the NOS-inhibition (McCulloh et al., 1997). From the group tested, HTGChol aortas showed the biggest part of L-NAME-resistant relaxation to Ach. It seems that HTGChol rats were able to maintain an unchanged endothelium-dependent relaxation by releasing EDHF. As the Ach-induced relaxation of HTGCholF aortas was completely inhibited by L-NAME, EDHF-participation was probably damaged. These results are in accordance with Miller et al.

(1998) and Young et al. (2008) who also found impaired EDHF response in rats administered fructose rich diet.

CONCLUSION

It seems that HTGCholF rats are closer models of MS than HTGChol, because they showed significant signs of endothelial dysfunction, which can lead to atherosclerosis and other cardiovascular diseases.

ACKNOWLEDGEMENT

This study was supported by the VEGA grant No. 2/0054/15.

References

[1] Anderson RA, Evans ML, Ellis GR, et al. The relationships between post-prandial lipaemia, endothelial function and oxidative stress in healthy individuals and patients with type 2 diabetes. Atherosclerosis 2001;154:475–483.

[2] Bae JH, Bassenge E, Kim KB, et al. Postprandial hypertriglyceridemia impairs endothelial function by enhanced oxidant stress. Atherosclerosis 2001;155:517–523.

[3] Bartus M, Kostogrys RB, Kaÿmierczak P, et al. 1-Methylnicotinamide (MNA) prevents endothelial dysfunction in hypertriglyceridemic and diabetic rats. Pharmacol Rep. 2008;60:127–138.

[4] Kaprinay B, Lipták B, Slovák L, et al. Hypertriglyceridemic rats fed high fat diet as a model of metabolic syndrome. Physiol Res. 2016; 65:S515-S518.

[5] Kitagawa S, Sameshima E, Yamaguchi Y, Kwon Y, Shinozuka K, Kunitomo M. Comparison of the effects of hypercholesterolaemia on relaxation responses in aortas of spontaneously hypertensive rats and Dahl salt-sensitive rats. Clin Exp Pharmacol Physiol. 1995;22(suppl):S251-S253.

[6] McCulloch AI, Bottrill FE, Randall MD, Hiley CR. Characterization and modulation of EDHF-mediated relaxations in the rat isolated superior mesenteric arterial bed. Br J Pharmacol.1997;120:1431-1438.

[7] Miller AW, Hoenig ME, Ujhelyi MR. Mechanisms of Impaired Endothelial Function Associated with Insulin Resistance. J Cardiovasc Pharmacol Therapeut. 1998;3:125-133.

[8] Pisulewski PM, Kostogrys RB, Lorkowska B, Bartuś M, Chłopicki S. Critical evaluation of normotensive rats as models for hypercholesterolaemia-induced atherosclerosis. J Anim Feed Sci. 2005;14:339-351.

[9] Sasaki S, Yoneda Y, Fujita H, et al. Association of blood pressure variability with induction of atherosclerosis in cholesterol-fed rats. Am J Hypertens. 1994;7:453-459.

[10] Sotnikova R, Bauer V. Nitric oxide production and its changes in diabetic endothelial dysfunction : chapter 7. Advances in molecular mechanisms and pharmacology of diabetic complications. In: Trivandrum : Transworld Research Networ. India, Kerala; 2010.

[11] Van Oostrom AJ, Cabezas MC, Rabelink TJ. Insulin resistance and vessel endothelial function. J R Soc Med. 2002;95:54-61.

[12] Young EJ, Hill MA, Wiehler WB, Triggle CR, Reid JJ. Reduced EDHF responses and connexin activity in mesenteric arteries from the insulin-resistant obese Zucker rat. Diabetologia2008;51:872-881.

DE GRUYTER OPEN

Eur. Pharm. J. 2017, 64 (1): 7-9.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.1515/afpuc-2017-0005

EUROPEAN PHARMACEUTICAL JOURNAL

The effect of short-term and long-term application of fisetin on experimentally induced airway hyperreactivity

Original Paper

Kazimierová I.,[1,2]✉ Pappová L.,[2] Šútovská M.,[2] Fraňová S.,[1,2]

[1]Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin, Biomedical Center Martin, Martin, Slovak Republic

[2]Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin, Department of Pharmacology, Martin, Slovak Republic

Received 27 February, 2017, accepted 24 March, 2017

Abstract **Background:** Fisetin, a derivate from the flavonol group may possess a variety of pharmacological effects. The aim of the presented study was to evaluate the bronchodilatory effect of fisetin after the acute or the chronic administration to guinea pigs with allergic airway inflammation.
Methods: Experimental animals were sensitized and challenged by ovalbumin. Fisetin was administered in dose 5mg/kg/ p.o., either once after the end of 21-days sensitization or daily during the 21-days sensitization. By using the whole-body plethysmograph, we monitored the specific airway resistance, a parameter of airway hyperreactivity *in vivo*. The changes of the specific airway resistance were evaluated after the short-term inhalation of the bronchoconstriction mediator -histamine (10^{-6} mol.1^{-1}).
Results: Our results showed that the short-term as well as the long-term administration of fisetin caused decrease of the specific airway resistance values. The bronchodilatory effect of fisetin was comparable to the long-acting $beta_2$ sympathomimetic – salmeterol after the long-term administration. The measurements of the bronchodilatory activity after single administration have revealed more prolonged effect of fisetin comparing to the short-acting $beta_2$ sympathomimetic – salbutamol, as this remained even after the 5 hours, when salbutamol was already ineffective.
Conclusion: In conclusion, flavonol – fisetin has shown bronchodilatory potential. In the light of this fact, fisetin may represent potential substance that can be effective in both prevention as well as control of airway inflammation symptoms.

Keywords *Airway hyperreactivity – fisetin – airway inflammation*

INTRODUCTION

The airway hyperreactivity is one of the main attribute of allergic asthma. It is defined as an exaggerated contraction response of the airways to different kind of endogenous and exogenous stimuli (Bossé, 2012). It is believed, that airway inflammation plays a central role in the development of airway hyperreactivity. Airway infiltration by inflammatory cells can impair the integrity of the bronchial epithelium. Due to this change, the release of bronchodilating substances is decreased and the formation of bronchoconstriction active kinins is augmented. Moreover, inflammatory cells such as eosinophils and mast cell release the mediators with the capacity to cause bronchial smooth muscle contraction and exudation of plasma, resulting in the thickening of airway wall and the modification of mechanical properties of airway smooth muscle (O´Byrne & Inman, 2003; Fredberg, 2004).
There are evidences, that flavonoids possess bronchodilatory effect and inhibit the synthesis of pro-inflammatory cytokines and the release of chemical mediators in allergic asthma (Tanaka & Takahashi, 2013). In the light of this fact, the aim of the presented study was to estimate the effect of short-term and long-term application of flavonol derivate fisetin on airway hyperreactivity. The bronchodilatory activity of fisetin was evaluated on experimental model of allergic airway inflammation in guinea pigs. This model simulates the development of airway hyperreactivity (Franova et al., 2013). The ability of fisetin to relax airway smooth muscle was

* *E-mail: Ivana.Kazimierova@jfmed.uniba.sk*

compared with the effect of clinically used bronchodilator drugs. The short-acting beta$_2$-symphatomimetic salbutamol was used to compare the acute bronchodilatory effect of fisetin and the long-acting beta$_2$-symphatomimetic salmeterol as a reference drug for the chronic bronchodilatory effect.

MATERIAL AND METHODS

All processes were approved by the Institutional Ethic Committee of the Jessenius Faculty of Medicine in Martin, Slovakia (permission IRB 00005636) and the experiment procedures were carried out in accordance with the Slovakian and European legislation (decision No. EK 1178/2012). Male guinea pigs (TRIK, 200-350g) were used in the experiment. The animals were obtained from the Department of Toxicology and the Laboratory of Animal Breeding, Dobra Voda, Bratislava, Slovakia (SK CH 24016), and located in animal house of Biomedical Center Martin JFM CU for one week of quarantine; food and water were available ad libitum with a standard air conditioning system.

Material and chemicals: Fisetin, salbutamol, salmeterol, histamine and ovalbumin (OVA, egg albumin grade III) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA).

Experimental design: In both experimental procedures (acute and chronic therapy), the animals were divided into the following groups: i) the healthy control group, ii) the negative control groups – animals sensitized with ovalbumin, iii) the positive control groups – animals sensitized with ovalbumin and treated by the reference drugs – the acute therapy - salbutamol (4 mM once by inhalation for 5 min), the chronic therapy - salmeterol (0.17 mM daily by inhalation for 5 min), iv) the therapeutic groups- animals sensitized with ovalbumin and treated by fisetin (5 mg/kg/ p.o.). Each group consisted of 10 guinea pigs. A model of allergic asthma on guinea pigs by chronic ovalbumin challenge was used in the experiment (Franova et al., 2013). During the acute therapy, the effect of single administration of fisetin was evaluated after the end of 21-day sensitization and the changes in the specific airway resistance were recorded before, 1 and 5 h after application of tested substances. During the chronic therapy, the animals received fisetin daily during the 21-days sensitization. The measurements were performed 24 h after both the last allergen exposure and treatment.

The evaluation of airway reactivity *in vivo*: *In vivo* airway reactivity was evaluated using a double chamber whole-body plethysmograph (Rodents Double-Chamber Plethysmograph, Hugo Sachs Electronik-Harvard Apparatus, type 855 with HSE Pulmodyn Pennock W software). The changes in the specific airway resistance after a short-term (2 min) inhalation of bronchoconstriction mediator – histamine (10^{-6} mol.l^{-1}) were considered as an indicator of airway reactivity *in vivo*. There was an interval of 1 min between histamine exposure and the airway resistance measurement. During this interval, fresh air was insufflated into the breathing chamber.

Statistics: The statistical analysis was performed using ANOVA test followed by Bonferroni *post hoc* test. Differences were considered statistically significant when P-values were below 0.05. All results are expressed as the means ± SEM.

RESULTS

Repetitive exposure of guinea pigs to ovalbumin caused significant increase of the specific airway resistance compared with the healthy control group. The single dose of fisetin induced the highly significant decrease of the specific airway resistance values. In contrast to the effect of salbutamol, fisetin proved its bronchodilatory activity 1 h as well as 5 h after the acute therapy (Fig. 1).

During chronic therapy, the 21-day sensitization of animals by ovalbumin resulted in non-significant increase of the specific airway resistance. Although the long-term administration of fisetin caused a decrease in the specific airway resistance after histamine nebulization compared to negative control group, this effect was not statistically relevant. The similar changes without statistical significance were monitored in animal group treated by reference drug salmeterol (Fig. 2).

DISCUSSION

Fisetin (3, 3´, 4´, 7- tetrahydroxyflavone), a flavonoid is mostly discussed in literature sources for its various spectra of pharmacological properties. Several experimental studies have showed its anti-inflammatory, anti-oxidant activities, cardioprotective, neuroprotective and anticancer properties (Khan et al., 2013). Moreover, fisetin belongs to the group of flavonols, that exhibit the well-known bronchodilatory activity (Ko, 2003). Flavonol derivates such as quercetin, kaempferol and morin proved the ability to suppress allergen-induced airway hyperreactivity and airway infiltration by inflammatory cells. Gong et al. (2012) showed that kaempferol administration suppressed eosinophil infiltration of the airways, which are the source of bronchoconstriction active substances. Recent studies in allergy confirmed that quercetin is more effective

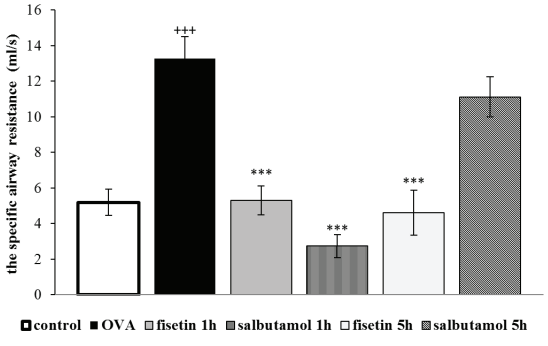


*Figure 1. The specific airway resistance values after the acute administration of fisetin.Data are expressed as the means ± SEM; statistical significance +++P<0.001 control vs. OVA, ***P<0.001 tested substances vs. OVA.*

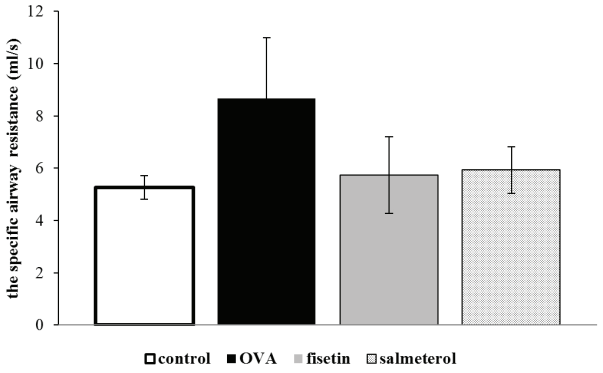


Figure 2. The specific airway resistance values after the chronic administration of fisetin. Data are expressed as the means ± SEM.

in the stabilization of mast cells than Cromolyn sodium (Weng et al., 2012). Franova et al. (2016) found out, that the bronchodilatory activity of morin was higher than the effect of salmeterol.

In current study, we studied the bronchodilatory effect of other flavonol derivate fisetin under in vivo conditions. Our results confirmed the ability of fisetin to relax airway smooth muscle, which correlate with the findings of previously mentioned studies. The 21-day application of fisetin decreased the airway reactivity comparing to treatment naïve animals with allergen induced airway inflammation. However these results were not statistically significant, observed decrease in specific airway resistance was similar to that of reference drug salmeterol. We assume that the mentioned tendency of fisetin to reduce airway smooth muscle contraction after long-term administration of fisetin is a result of its suppressive effect on airway inflammation, which was demonstrated in the study of Goh et.al. (2012).

Regardless the anti-inflammatory activity, fisetin was able to attenuate the bronchoconstriction response even after single administration, when the anti-inflammatory impact of fisetin should be minimal. Therefore it is highly probable, that fisetin can act directly on the mechanisms regulating the bronchoconstriction. Indeed recent study has demonstrated that fisetin inhibits phospholipase Cβ and phosphodiesterase-4D3, the enzymes which play the important role in maintenance of airway smooth muscle contraction (Brown et al., 2016).

CONCLUSIONS

In conclusion, according to the above-mentioned results, we can say, that the present study demonstrated the beneficial effect of fisetin on the airway hyperreactivity related to allergic inflammation. We believe, that our findings may contribute to the development of drugs useful in prevention or in the treatment of airway inflammatory diseases such as asthma.

ACKNOWLEDGEMENTS

This work was supported by the project „Biomedical Center Martin" ITMS code: 26220220187, the project is co-financed by EC sources; the Slovak Research and Development Agency under the contract No. APVV-0305-12; CEKR I; CEKR II; Grant VEGA No. 1/0165/14 and Grant MZ 201/35-UKMA-12.

References

[1] Bossé Y. Asthmatic airway hyperresponsiveness: the ants in the tree. Trends in Molecular Medicine. 2012; 18: 627-633.

[2] Brown A, Danielsson J, Townsend EA, et al. Attenuation of airway smooth muscle contractility via flavonol-mediated inhibition of phospholipase-Cβ. Am J Physiol Lung Cell Mol Physiol. 2016; 310:L747-L758.

[3] Franova S, Joskova M, Sadlonova V, Pavelcikova D, Mesarosova L, Novakova E, Sutovska M. Experimentalmodel of allergic asthma. Advances in Experimental Medicine and Biology. 2013; 756: 49-55.

[4] Franova S, Kazimierova I, Pappova L, Joskova M, Plank L, Sutovska M. Bronchodilatory, antitussive and anti-inflammatory effect of morin in the setting of experimentally induced allergic asthma. Journal of Pharmacy and Pharmacology. 2016; 68(8):1064-1072.

[5] Fredberg JJ. Bronchospasms and its biophysical basis in airway smooth muscle. In Respiratory Research. 2004; 5: 2.

[6] Goh FY, Upton N, Guan S, et al. Fisetin, a bioactive flavonol, attenuates allergic airway inflammation through negative regulation of NF-κB. European Journal of Pharmacology. 2012; 679: 109-116.

[7] Gong JH, Shin D, Han SY, Kim JL, Kang YH. Kaempferol suppresses eosinophil infiltration and airway inflammation in airway epithelial cells and in mice with allergic asthma. J Nutr. 2012; 142: 47-56.

[8] Khan N, Syed DN, Ahmad N, Mukhtar H. Fisetin: A dietary antioxidant for health promotion. Antioxidant Redox Signaling. 2013; 19(2): 151-162.

[9] Ko WCH, Liu PY, Chen JL, Leu IJ, Shin CM. Relaxant effects of flavonoids in isolated in guinea pig trachea and their structure – activity relationship. In Planta Medica. 2003; 12: 1086-1090.

[10] O´Byrne PM, Inman MD. Airway Hyperresponsiveness. Chest Journal. 2003; 123: 411-416.

[11] Tanaka T, Takahashi R. Flavonoids and asthma. Nutrients. 2013; 5: 2128-2143.

[12] Weng Z, Zhang B, Asadi S, et al. Quercetin is more effective than cromolyn in blocking human mast cellcytokine release and inhibits contact dermatitis and photosensitivity in humans. PLoSOne. 2012; 7: 33805.

DE GRUYTER OPEN

Eur. Pharm. J. 2017, 64 (1): 10-12.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.1515/afpuc-2017-0001

EUROPEAN PHARMACEUTICAL JOURNAL

Influence of smooth muscle contractility by inhibition of crac channels activity

Original Paper

Kocmálová M.,[1,2]✉ Kazimierová I.[1,2]

[1]Comenius University in Bratislava,
Jessenius Faculty of Medicine in Martin,
Biomedical Center Martin, Martin, Slovak Republic

[2]Comenius University in Bratislava,
Jessenius Faculty of Medicine in Martin,
Department of Pharmacology,
Martin, Slovak Republic

Received 14 December, 2016, accepted 20 January, 2017

Abstract The present in vitro study was focused on the differences in expression and activity of calcium release-activated calcium (CRAC) channels of human term-pregnant and non-pregnant myometrium. The expression of Orai1 protein, as a functional subunit of CRAC channel, was significantly higher than in non-pregnant myometrium. Lower Orai1 protein expression did not influence the amplitude of contractile response of term-pregnant myometrium, but higher Orai1 expression observed in non-pregnant myometrium was related to the different influence of CRAC blocker on contraction frequency.

Keywords *Oral N-acetylcysteine – Inhaled N-acetylcysteine – mucus clearance – airway reactivity*

INTRODUCTION

Calcium ions (Ca2+) play a substantial mediatory and regulatory role in nearly all cell activities. 3 types of ion channels are responsible for Ca2+ metabolism: receptor-operated calcium channels, voltage-gated calcium channels (VGCC) and store-operated calcium channels (Wray et al., 2001)).

VGCC is the principal mechanism of Ca2+ influx in spontaneously active myometrial tissue. The VGCC family L-type plays a vital role in the generation of phasic contractility of the uterus. Myometrial contractility depends on increase in intracellular Ca2+ resulting from the transmembrane influx of Ca2+ and the release of Ca2+ from endoplasmic reticulum (ER) (Noble et al., 2009). The main representative of store-operated channels is CRAC channel, which exhibits low conductance, strong inward rectification and remarkable Ca2+ selectivity (Sutovska et al., 2013; Parekh 2010). The CRAC channel is composed of two main elements: Orai1 channel protein of the plasma membrane and stromal interaction molecule 1 (STIM1) protein of ER (Hoth and Penner, 1992; Potier and Trebak, 2008). A decrease of Ca2+ concentration in the ER triggers changes in STIM1 conformation, the sensor of Ca2+ concentration in the ER. Opening the Orai1 channels causes an influx of Ca2+ into the cytoplasm of the cell. The rising concentration of Ca2+ in the cytoplasm induces closing of the channel by Ca2+-dependent inactivation (Derler et al., 2012).

Recently, the expression of CRAC channels in smooth muscle (SM), including the myometrium, has been confirmed (7, 8). Therefore, this in vitro experimental study aimed to specify the role of CRAC channels in the contractility of human term-pregnant and non-pregnant myometrium.

MATERIAL AND METHODS

All processes were approved by the Institutional Ethics Committee of the Jessenius Faculty of Medicine, (decision No. EK 1124/2012). Patient recruitment was conducted by the provision of information sheets, and written informed

* E-mail: Michaela.Kocmalova@jfmed.uniba

Table 1. CRAC blocker influence on frequency (per hour) and amplitude of contraction (mN) of pregnant and non-pregnant myometrium. All data are expressed as average ± SEM. Results with P < 0.05 were considered statistically significant. P vs. Oxy.

	Non-pregnant n=7		Pregnant n=6	
	Frequency	**Amplitude of contraction**	**Frequency**	**Amplitude of contraction**
Oxy (1μM)	41.2±2.9	1.5±0.2	48.3±4.0	2.3±0.2
Sal (100μM)	28.3±3.2 *	0.9±0.1 *	28±2.3 **	1.3±0.2 **
CRAC (1μM)	20.3±3.3 **	1.1±0.1	34.7±3.1 *	2.1±0.1
CRAC (10μM)	26.3±2.6 *	1±0.1 *	33.7±4.4 **	2±0.1 *
CRAC (100μM)	29.3±2.5	0.9±0.1 *	27.5±5.8 **	1.9±0.1 *

consent was obtained. Samples of myometrium were collected in cooperation with the Clinic of Gynaecology and Obstetrics. Human myometrium originated from woman without any underlying conditions and undergoing elective caesarean section at term (week of pregnancy 38.7 ± 0.7) and non-pregnant myometrium of women undergoing total hysterectomy (aged 47 ± 2.24) due to uterine myoma from macroscopic healthy tissue.

Uterus SM reactivity was verified using the organ tissue bath methodology (Franova et al., 2009). Myometrium samples were incubated for one hour in Krebs-Henzeleit solution. Oxytocin was applied directly into the chamber to induce SM contractions. Cumulative doses (1μM, 10μM, 100μM) of tested substance CRAC channel blocker 3-fluoropiridine-4carboxylic acid (FPCA) and salbutamol (100μM) were applied into the chamber after 5 minutes from oxytocin application. The amplitude and frequency of SM contraction was recorded.

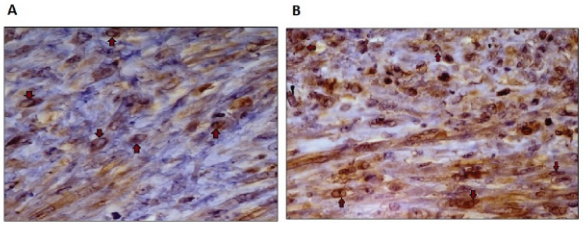
The modulator of CRAC channels activity FPCA was purchased from TOCRIS (USA). Oxytocin and salbutamol were obtained from Sigma Aldrich SR. All substances were dissolved in aqua pro injection.

Orai1 protein expression, a pore-forming subunit of the CRAC channel, was determined by immunohistochemistry. The reaction was visualized using the LSAB visualization kit and diaminobenzidine chromogen. The Orai1 expression was evaluated using a rating scale, where 1 stood for no degree of positivity, 2 mild or sporadic positivity and level 3 distinct positivity.

Fisher's exact test was selected to evaluate the IHC features and amplitude of contraction was evaluated using one-way ANOVA with Bonferroni post-hoc test.

RESULTS

Uterus SM contractions, induced by oxytocin, were reduced after FPCA application. The amplitude of contractions was significantly reduced after the addition FPCA at 10 and 100 μM concentration in both groups (Tab. 1). The FPCA ability to reduce oxytocin-induced contraction were compared to



Tissue	1	2	3
Non-pregnant	1	3	2
Pregnant	6	1	0

Figure 1. The immunohistochemical analysis of A) pregnant and B) non-pregnant myometrium. Cells with distinct plasma membrane Orai1 positivity are marked by red arrows. 1 - no degree of positivity, 2 - mild positivity, 3 - distinct positivity.

clinically used drug salbutamol. FPCA effect in samples from non-pregnant women was comparable with salbutamol, but in samples from pregnant group, salbutamol was more effective than FPCA (Tab. 1). The frequency of uterine contractions from pregnant group reduced significantly after the application of CRAC channel blocker. The higher concentration of the tested substance led to an increase in the contraction frequency (Tab. 1). FPCA reduced the contraction frequency of myometrium from patients with myoma, which was almost comparable to the reference drug (Tab. 1).

Higher expression of Orai1 protein was in samples from non-pregnant group than in samples of pregnant myometrium (Fig. 1).

DISCUSSION

Recently, the expression of CRAC channels in smooth muscle, including the myometrium, has been confirmed in several studies. These studies confirmed the presence of CRAC channels not only in smooth muscle of myometrium but also in smooth muscle of respiratory tract and others (Sutovska et al. 2013; Potier and Trebak, 2008; Derler et al. 2012). The present study not only confirmed the expression of Orai1

protein, the main pore-forming subunit of CRAC channels in myometrium, but for the first time also distinguished distinct changes between Orai1 protein expression in non-pregnant myometrium and in term-pregnant myometrium.

Functional part of this experimental study aimed to specify the role of CRAC channels in the contraction/relaxation of human term-pregnant and non-pregnant myometrium. Data presented in this paper provide strong evidence of CRAC channel's involvement in the contracting activity of myometrium. Moreover, lower expression of Orai1 protein did not influence the amplitude of the contractile response of term-pregnant myometrium on cumulative doses of CRAC blocker, but higher expression of Orai1 in non-pregnant myometrium was very probably related to different influence of this agent on frequency of contraction.

ACKNOWLEDGEMENT

The authors acknowledge Katarina Jesenska for her outstanding technical assistance, Marian Adamkov for immunohistochemical analysis and Karol Dokus for obtaining specimens. This study was supported by the Centre of Experimental and Clinical Respirology I, Centre of Experimental and Clinical Respirology II, BioMed and 'The increasing of opportunities for career growth in research and development in the medical sciences' projects co-financed from EC sources, grant MZ 2012/35-UKMA-12, APVV-0305-12 and grant VEGA no. 1/0127/13.

References

[1] Derler I, Madl J, Schütz G, Romanin CH. Structure, regulation and biophysics of I(CRAC), STIM/Orai1. Adv Exp Med Biol 2012; 740: 383–410.

[2] Franova S, Janicek F, Visnovsky J, Dokus K, Zubor P, Sutovska M, Nosalova G. Utero-relaxant effect of PDE4-selective inhibitor alone and in simultaneous administration with β2-mimetic on oxytocin-induced contractions in pregnant myometrium. Int J Gynaecol Obstet. 2009; 35: 20-25.

[3] Hoth M, Penner R. Depletion of intracellular calcium stores activates a calcium current in mast cells. Nature 1992; 355: 353–356.

[4] Noble K, Matthew A, Burdyga T, Wray S. A review of recent insights into the role of the sarcoplasmic reticulum and Ca entry in uterine smooth muscle. Eur J Obstet Gynecol Reprod Biol 2009; 144 (1 suppl): S11–S19.

[5] Parekh AB. Store-operated CRAC channels: Function in health and disease. Nat Rev Drug Discov 2010; 9: 399–410.

[6] Potier M, Trebak M. New developments in the signalling mechanisms of the store-operated calcium entry pathway. Pflugers Arch 2008; 457: 405–415.

[7] Sutovska M, Kocmalova M, Adamkov M, Vybohova D, Mikolka P, Mokra D, Hatok J, Antosova M, Franova S. The long-term administration of Orai 1 antagonist possesses antitussive, bronchodilatory and anti-inflammatory affects in experimental asthma model. Gen. Physiol. Biophys.2013; 32:251–259

[8] Wray S, Kupittayanant S, Shmygol A, Smith RD, Burdyga T. The physiological basis of uterine contractility: A short review. Exp Physiol 2001; 86: 239–246.

DE GRUYTER OPEN

Eur. Pharm. J. 2017, 64 (1): 13-16.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.1515/afpuc-2017-0013

EUROPEAN PHARMACEUTICAL JOURNAL

Efficacy of vortioxetine monotherapy compared with combined therapy vortioxetine and olanzapine in the treatment of major depression – first results

Účinnosť monoterapie vortioxetínom v porovnaní s kombinovanou liečbou vortioxetínom a olanzapínom v liečbe depresie – prvé výsledky

Original Paper

Oppa M., Ondrejka I.[1]✉, Cesnekova D.[1], Tonhajzerova I.[1], Nosalova G.[2]

[1]*Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin, Biomedical Center Martin, Martin Slovak Republic*

[1]*Univerzita Komenského v Bratislave, Jesénova lekárska fakulta v Martine, Biomedicínske centrum Martin, Martin, Slovenská Republika*

[2]*Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin, Department of Pharmacology, Martin, Slovak Republic*

[2]*Univerzita Komenského v Bratislave, Jesénova lekárska fakulta v Martine, Ústav farmakológie, Martin, Slovenská Republika*

Received 22 June, 2016, accepted 19 July, 2016

Abstract Vortioxetine is a novel antidepressant with two mechanisms of action – direct effect on several serotonin receptors and serotonin reuptake inhibition. Atypical antipsychotics, such as olanzapine, used in the augmentation of antidepressants causes not only a better response to treatment, but also increased number of remissions. The aim of our work was to evaluate the efficacy of vortioxetine monotherapy compared to the combined treatment vortioxetine and olanzapine in adult patients with depression during the acute phase of treatment lasting 6 weeks. Depressive symptomatology was assessed by the MADRS scale, anxiety symptoms were assessed by the HAM-A scale and global clinical impression were evaluated by the CGI-S scale. The number of patients in full-analysis set was 28. The results showed statistically significant improvement in CGI-S for both groups. Patients with vortioxetine monotherapy showed significant improvement in MADRS total score from the third week of treatment ($p = 0.009$) compared to patients with combined therapy that showed significant improvement since the end of first week of treatment ($p = 0.036$). Both groups showed significant improvement in HAM-A total score from the second week of treatment. Our results show the possibility of olanzapine in the augmentation strategy in treatment of major depressive disorder in adult patients.

Slovak abstract Vortioxetín je nové multimodálne antidepresívum s dvomi mechanizmami účinku – priamym efektom na niektoré sérotonínové receptory a inhibíciou spätného vychytávania sérotonínu. Atypické antipsychotiká, ako je olanzapín, používané ako augmentácia antidepresívnej liečby, spôsobujú nielen lepšiu odpoveď na liečbu, ale aj vyšší počet remisií. Cieľom našej štúdie bolo zhodnotiť účinnosť monoterapie vortioxetínom v porovnaní s kombinovanou liečbou vortioxetín a olanzapín v rámci liečby akútnej fázy depresie počas 6 týždňov. Depresívnu symptomatiku sme hodnotili podľa škály MADRS, úzkostnú symptomatiku podľa škály HAM-A a celkový klinický dojem podľa škály CGI-S. Analyzovaný počet pacientov bol 28. Výsledky ukázali významné zlepšenie v škále CGI-S u oboch terapeutických vetiev. U pacientov liečených vortioxetínom v monoterapií došlo k významnému zlepšeniu v škále MADRS na konci tretieho týždňa liečby ($p = 0.009$), v porovnaní s pacientami liečenými kombinovanou liečbou, kde došlo k zlepšeniu v škále MADRS už na konci prvého týždňa liečby ($p = 0,036$). Obe skupiny preukázali významné zlepšenie v škále HAM-A počas druhého týždňa liečby. Naše výsledky poukazujú na možnosť použitia olanzapínu v rámci augmentačnej stratégie liečby depresívnej poruchy u dospelých pacientov.

Keywords *Vortioxetine – Olanzapine – Depression*

Kľúčové slová: *Vortioxetín – Olanzapín – Depresia*

* *E-mail: igor.ondrejka@jfmed.uniba.sk*

This work is supported by grants VEGA 1/0087/14 and UK/90/2016, UK/75/2016.

INTRODUCTION

Major depressive disorder (MDD) is a disease with lifetime prevalence of around 13% and incidence rate of 4%.[1] It presents a great burden to individuals with reduction of overall functioning.[2] Despite a large number of antidepressants, complete remission of depressive symptomatology is achieved only in 30 to 40% of patients with MDD treated with antidepressants from SSRI and SNRI group.[3] Also, the sexual dysfunction is a problem when using conventional antidepressants. From the findings of meta-analysis focused on the incidence of serious sexual dysfunction, only antidepressant without serotonergic activity were able to maintain low levels of sexual dysfunction similar to placebo.[4]
Vortioxetine is a novel antidepressant with multimodal mechanism of action: it acts on several serotonin receptors (5-HT1A agonist, 5-HT1B partial agonist and 5-HT1D, 5-HT3 and 5-HT7 antagonist), but it also causes serotonin reuptake inhibition through inhibition of serotonin transporter (SERT). In experimental studies, vortioxetine showed the normalization of serotoninergic, noradrenergic, and dopaminergic transmission. It is proved that vortioxetine is efficacious in various types of depression; in addition, it is also efficacious in patients who did not respond sufficiently to SSRIs and SNRIs treatment. Furthermore, vortioxetine is well tolerated with low rates of sexual dysfunction despite the patient's serotoninergic activity. Vortioxetine also showed statistically significant improvement in cognitive functions in depressed patients.[5]
Olanzapine is an atypical antipsychotic from the MARTA group (multi acting receptor targeted agents) structurally related to clozapine with no risk of agranulocytosis. It has an affinity for several serotonin (5-HT2A, 5-HT2C, 5-HT3 and 5-HT6), dopamine (D1-5), acetylcholine (M1-5), α1-adrenergic and histamine H1 receptors.[6] Olanzapine agonism at 5-HT1A receptors result in an increased release of noradrenaline in the prefrontal cortex and ncl. accumbens, which is associated with improvement in cognitive function. Its antagonism on the 5-HT2A receptors enhances the release of noradrenaline and serotonin, resulting in a reduction of suppression of firing in the locus coeruleus induced by SSRIs. Moreover, the increased release of dopamine in the prefrontal cortex leads to an improved regulation of mood and cognitive function.[7]
The aim of our work was to evaluate the efficacy of vortioxetine monotherapy compared to a combined treatment of vortioxetine and olanzapine in adult patients with MDD.

MATERIAL AND METHODS

The design of the study was open-label clinical randomized trial without blind testing or using placebo control group. The duration of the study was 6-weeks.

The including criteria were diagnosis of depressive episode (F32) or recurrent depressive disorder (F33) according to ICD-10 classification, age 18–65 years and the ability to participate in the study. The excluding criteria were serious symptomatic disease, which could distort the results and cognitive deficit that did not allow adequate cooperation in the study. Eligible patients were randomized (1:1) into two equal therapeutic groups (vortioxetine monotherapy, vortioxetine and olanzapine combined therapy). Vortioxetine was dosed from 10 to 20 mg per day, olanzapine was dosed from 5 to 10 mg a day.
The depressive and anxious symptomatology was evaluated by standard scales used in psychiatric research every week during the acute phase of treatment lasting 6 weeks. Depressive symptomatology was assessed by the 10-item MADRS scale (Montgomery and Asberg Depression Rating Scale). The anxiety symptoms were assessed by the 15-item HAM-A scale (Hamilton Anxiety Rating Scale). Also, the global clinical impressions were evaluated by the CGI-S scale (Clinical Global Impression - Severity). After that, the mean MADRS, HAM-A and CGI-S total scores were calculated and statistically assessed by using non-parametric t-test and ANOVA for comparison of efficacy of treatment in each therapeutic group and Kruskal-Wallis test for inter-group comparison.

RESULTS

From the 29 patients included in the study, 28 went for the full-analysis set (FAS), 14 in each therapeutic group, with one drop-out from the study.
The results of t-test from the CGI-S scale showed statistically significant improvement in both groups. The mean CGI-S score in patients on vortioxetine monotherapy was 5.1 ± 0.7 in the first week compared to 2.7±0.9 in the sixth week of treatment. The CGI-S mean score in patients on combined therapy was 5.4±0.8 in the first week compared to 2.4±1.5 in the sixth week of treatment. There were no significant differences between groups.

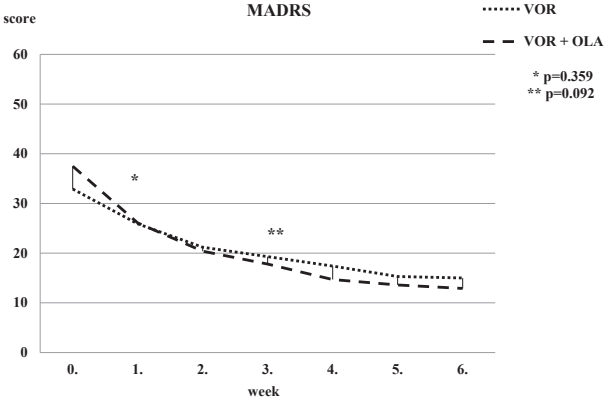


Figure 1. MADRS total score dynamics during the treatment. VOR – vortioxetine, OLA – olanzapine

Both groups showed a statistically significant decrease in MADRS total score after 6 weeks of treatment. Patients treated with vortioxetine monotherapy had mean MADRS total score 32.9±7.1 in the first week and 15±7.5 after six weeks of treatment with p = 0.0004. Patients treated with combined therapy had mean MADRS total score 37.5±6.6 in the first week and 12.9±12.9 after six weeks of treatment with p = 0.0031. The dynamics of decreasing MADRS total score during the treatment is shown in Figure 1. Patients with vortioxetine monotherapy showed significant improvement from the third week of treatment (p = 0.009) compared to the patients with a combined therapy that showed significant improvement since the end of first week of treatment (p = 0.036). There was no statistical significance between the groups in MADRS total score.

Both groups showed a statistically significant decrease in HAM-A total score after 6 weeks of treatment. Patients with vortioxetine monotherapy had mean HAM-A total score 27.9±4.3 in the first week and 13.5±5.8 after six weeks with p = 0.0001. Patients treated with combined therapy had mean HAM-A total score 30.3±10.1 in the first week and 10.9±10.1 after six weeks with p = 0.0052. Each group showed significant improvement from the second week of treatment with no significant difference between the groups. The dynamics of decreasing HAM-A total score is shown in Figure 2.

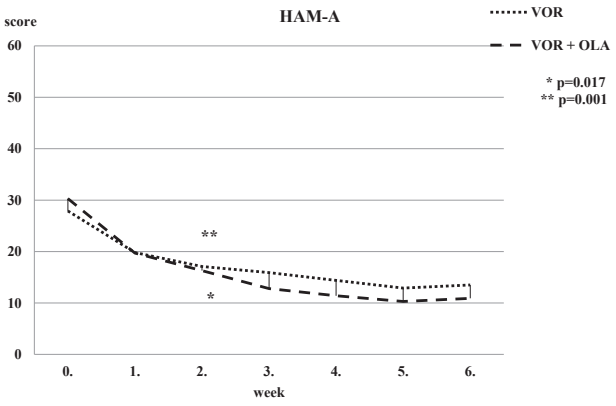


Figure 2. HAM-A total score dynamics during the treatment. VOR – vortioxetine, OLA – olanzapine

DISCUSSION

This study evaluated the efficacy of vortioxetine monotherapy compared to the combined therapy vortioxetine and olanzapine after 6 weeks of treatment in adult patients with depression. Clinical relevance was shown by an improvement in the CGI-S score in both groups.

Vortioxetine efficacy in reducing the depressive symptoms has been proved in 8 out of 12 short-term (6, 8 or 12 weeks) clinical trials[1] and it is consistent with the results of our study. The significant improvement in the MADRS total score was observed from the third week of treatment. However, the combined therapy with olanzapine showed significant improvement in the MADRS total score since the end of first week. These findings suggest the augmentation effect of olanzapine on reducing depressive symptomatology. The results from studies showed that typical antipsychotics are not sufficiently effective in reducing at least two symptoms of depression, which are loss of interest and psychomotor inhibition,[8] but atypical antipsychotics used in augmentation of antidepressants causes not only a better response to treatment, but also an increased number of remissions.[9]

In both groups, we showed significant effect in reducing anxiety symptoms demonstrated by a decrease of HAM-A total score from the second week of treatment. It could be beneficial in the treatment of depression, because in approximately half of all individuals with MDD, high levels of anxiety symptoms are observed.[10] The anxiolytic efficacy of vortioxetine is also proved in the treatment of generalized anxiety disorder based on the results of recent meta-analysis of data from 4 short-term, randomized controlled trials.[11]

Our results show the possibility of olanzapine in the augmentation strategy in treatment of major depressive disorder in adult patients with an effect in reducing depressive symptomatology.

References

[1] Alvarez E, Perez V, Artigas F. Pharmacology and clinical potential of vortioxetine in the treatment of major depressive disorder. Neuropsychiatric Disease and Treatment. 2014;10:1297–1307.

[2] Farsky I, Smetanka A, Dubinska S. Spiritualita pacientov s vybranymi psychiatrickymi diagnozami. Osetrovatelstvi a porodni asistence. 2012;3(3):433–441.

[3] Nemeroff CB. Prevalence and management of treatment resistant depression. J Clin Psychiatry. 2007;68(Suppl 8):17–25.

[4] Serreti A, Chiesa A. Treatment-emergent sexual dysfunction related to antidepressants: a meta-analysis. J Clin Psychopharmacol. 2009;29(3):259–266.

[5] Jarema M, Dudek D, Czernikiewicz A. Cognitive dysfunctions in depression – underestimated symptom or a new dimension? Psychiatr. Pol. 2014;48(6):1105–1116.

[6] Pecenak J, Korinkova V et al. Psychofarmakologia. 2016. Bratislava: Wolters Kluwer. ISBN 978-80-8168-542-2.

[7] Bobo V, Shelton RC. Olanzapine and fluoxetine combination therapy for treatment-resistant depression: review of efficacy, safety, and study design issues. Neuropsychiatric Disease and Treatment. 2009;5:369–383.

[8] Raskin A, Schulterbrandt JG, Reatig N, McKeon JJ. Differential response to chlorpromazine, imipramine, and placebo. A study of subgroups of hospitalized depressed patients. Arch Gen Psychiatry. 1970;23:164–173.

[9] Papakostas GI, Shelton RC, Shmith J, Fava M. Augmentation of antidepressants with atypical antipsychotic medications for treatment-resistant major depressive disorder: a meta-analysis. J Clin Psychiatry. 2007;68:826–831.

[10] Kessler RC, Sampson NA, Berglund P et al. Anxious and non-anxious major depressive disorder in the World Health Organization World Mental Health Surveys. Epidemiol. Psychiatr. Sci. 2015;24:210–226.
[11] Pae CU, Wang SM, Han C, Lee SJ, Patkar AA, Masand PS, Serretti A. Vortioxetine, a multimodal antidepressant for generalized anxiety disorder: a systematic review and meta-analysis. J. Psychiatr. Res. 2015;64: 88–98.

DE GRUYTER OPEN

Eur. Pharm. J. LXIII, 2017 (1): 17-21.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.1515/afpuc-2017-0002

EUROPEAN PHARMACEUTICAL JOURNAL

Effect of inhaled and oral n-acetylcysteine on airway defense mechanism

Original Paper

Pappová L.,[1,2]✉ Kazimierová I.,[1,2] and Kocmálová M.[1,2]

[1]*Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin, Biomedical Centre Martin, Martin, Slovak Republic*

[2]*Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin, Department of Pharmacology, Martin, Slovak Republic*

Received 13 December, 2016, accepted 14 December, 2016

Abstract **Aim:** N-acetylcysteine is the prototype of mucolytic agents. The aim of this study was to evaluate the acute and chronic effect of inhaled and oral N-acetylcysteine on airway reactivity, cough reflex and ciliary beat frequency and parameters of mentioned defense mechanisms were assessed in physiological conditions.
Methods: An experiment was performed using healthy guinea pigs treated with inhaled (0.6 M; 5min) and oral N-acetylcysteine (20 mg/kg), administrated either acutely as a single dose or chronically during 7 days. The cough reflex and specific airway resistance were assessed by *in vivo* method, using a double chamber plethysmograph box. The ciliary beat frequency was evaluated in *in vitro* conditions on tracheal brushed samples using light microscope coupled to high speed video camera.
Results: Inhaled and oral N-acetylcysteine, either administrated as a single dose or during 7 days, have shown a tendency to decrease sensitivity of the cough reflex and increase the airway reactivity. Acute administration of inhaled and oral N-acetylcysteine had no statistically relevant effect on the ciliary beat frequency, whereas chronic administration of both inhaled and oral N-acetylcysteine led to a marked reduction in the ciliary beat frequency.
Conclusion: Chronic administration of oral and inhaled N-acetylcysteine had a negative impact on the ciliary beat frequency, which represents one of the key factors determining the rate of mucociliary clearance. Thus, administration of N-acetylcysteine is less likely to increase the expulsion of mucus by ciliary movement. In addition, the observed tendency of inhaled and oral N-acetylcysteine to increase the airway reactivity may limit its use in conditions with severe airflow obstruction.

Keywords *N-acetylcysteine – mucus clearance – ciliary beat frequency – airway reactivity*

INTRODUCTION

Airway mucus hypersecretion is one of the prominent features of severe respiratory diseases. Pharmacological approach for relieving mucus accumulation in airways currently involves several classes of agents, including mucolytics. Mucolytics have gained importance as the drugs that can degrade the mucin polymers of mucus gel, and by reducing the viscosity and elasticity of mucus are thought to increase the mucus expulsion, either by ciliary movement or cough reflex (Rogers, 2007). Best known of these agents is N-acetylcysteine (NAC). Although, it was introduced as, and it is still considered to be a mucolytic agent, this activity up to date has not been well established. The doubt about its effectiveness in chronic respiratory diseases arises from the uncertainties about the mechanism of action and controversial results of long-term clinical trials (Seagrave et al., 2012). Actually, none of the current recommendations for the treatment of chronic obstructive disease (GOLD, 2016) support the use of mucolytic agents as a part of the standard therapy due to lack of evidence. Moreover, several studies have demonstrated a dose dependent effect of NAC on the viscoelastic properties of secretion in vitro as well as after inhaled administration, but this effect is not seen when given orally, which may be associated with poor excretion of NAC into the airway lumen after oral administration (Fuloria & Rubin, 2000). Nevertheless, some published evidence suggests, that oral NAC may improve pulmonary function in selected patients with chronic

* *E-mail: Lenka.Pappova@jfmed.uniba.sk*

lung disease, but observed clinical benefit is more likely due to the antioxidant properties. On the contrary, there is no data that supports the clinical use of inhaled NAC, as it may cause bronchospasm (Rubin, 2014).

In this study, we centred our attention on the role of NAC in modulation of mucus clearance. Besides the well- established mucolytic activity, we were interested if oral and inhaled NAC also have the ability to affect the mucus clearance, directly acting on ciliary beat frequency (CBF) as one of the key regulator of the mucociliary clearance rate (Braiman & Priel, 2008), and cough sensitivity. These parameters were evaluated in physiological conditions to avoid the indirect impact of other mentioned N-acetylcysteine activities (antioxidant) on CBF or cough sensitivity. Moreover, we also addressed the possible effect of N-acetylcysteine on airway reactivity, as it has been shown that inhaled N-acetylcysteine may cause a bronchospasm, and thus, may worsen the airway obstruction (Reinero et al., 2011).

MATERIAL AND METHODS

The experiment was approved by the Institutional Ethics Committee of Jessenius Faculty of Medicine (permission IRB 00005636). All experimental procedures were performed according Slovakian and European Community regulations for the use of laboratory animals and guidelines on animal welfare (decision No. 1249/2013). Healthy adult male TRIK-strain guinea pigs were purchased from the accredited breeding facility, from the Department of Experimental Pharmacology, Slovak Academy of Sciences, Dobrá Voda, Slovakia (SKCH24011). Animals were housed under the control conditions with free access to food and water.

In the experiment, the commercially available product of N-acetylcysteine (ACC inject; sol. inj.) was used. Other chemicals such as citric acid and bronchoconstrictor mediator (histamine), were obtained from Sigma Aldrich Chemicals (St. Louis, MO, US).

Experimental groups

The experiment was carried out using healthy male guinea pigs weighting 300-400 g. The animals were randomly divided into experimental groups comprising 10 animals per group (n=10). Animals assigned to the therapeutic groups were treated with inhaled (0.6 M; 5 min) and oral NAC (20 mg/kg), administrated either acutely as a single dose or chronically during 7 days. The guinea pigs in control group received only water vehiculum (1 mL. kg^{-1}) in the case of oral NAC administration, or the animals were exposed to saline aerosol instead of NAC aerosol. NAC solution was aerosolized by jet nebulizer (PARI jet nebuliser, Paul Ritzau, Pati-Werk GmbH, Germany, output 5 $1.s^{-1}$, particles mass median diameter 1,2 µm) delivering the aerosol of drug to head chamber of double body plethysmograph (HSE type 855, Hugo Sachs Electronic, Germany), where the animals were placed.

The evaluation of cough reflex in vivo

Healthy conscious guinea pigs were individually placed in a double chamber plethysmograph box (HSE type 855, Hugo Sachs Electronic, Germany) and cough evaluation was performed according to the method described by Franova et al. (Franova et al., 2013). Briefly, the cough reflex was provoked chemically, by the exposure of animals to citric acid aerosol in a time interval of three minutes, during which the number of cough efforts were counted. Citric acid solution at a concentration of 0.3 mol/L was aerosolized by jet nebulizer (PARI jet nebuliser, Paul Ritzau, Pati-Werk GmbH, Germany, output 5 $1.s^{-1}$, particles mass median diameter 1,2 µm). Sudden enhancement of expiratory flow during coughing was detected by pneumotachograph connected to the nasal chamber of the double body plethysmograph. In accordance with ERS guidelines, cough effort was defined as sudden PC-recorded enhancement in expiratory airflow, which was simultaneously accompanied by a characteristic cough sound and movement. Sound and movement typical for cough reflex were evaluated and recognised by two trained observers and compared using video recordings (Morice et al., 2007).

The evaluation of airway smooth muscle reactivity in vivo

Airway smooth muscle reactivity was evaluated on conscious animals using a double chamber plethysmograph box (HSE type 855, Hugo Sachs Electronic, Germany). For the assessment of airway reactivity, the values of specific airway resistance (sRAW) were used. As per Pennoc, sRaw is a parameter calculated from the phase shift between nasal and thoracic respiratory flows, using the HSE respiratory software PULMODYN PENNOCK. Changes in the thoracic and nasal airflow were provoked according to a method described by Kazimierová et al. (Kazimierová et al., 2015). Briefly, the animals were exposed for thirty seconds to histamine aerosol at concentration 10^{-6} mol/L. An interval of 1 min was provided between the exposure to histamine and sRaw measurement, during which fresh air was insufflated into the nasal chamber.

The evaluation of ciliary beat frequency in vitro

After sacrificing the animals, the small window was dissected in precisely cleaned upper part of the trachea, in order to expose the tracheal epithelium for brushing the collection of ciliated cells by cytological brush. Acquired material was resuspended on a heated microscope slide into a drop of warm saline solution (36,5°C ±0,5) and obscured by a cover slide. The undisrupted strips of ciliated epithelium with the presence of beating cilia were selected using an inverted phase contrast microscope (Zeiss Aixo vert. A1; carl Zeiss AG, Göttingen, Germany) and recorded by high speed video camera (Basler A504kc; Adept Turnkey Pty Ltd, Brookvale, Australia) with the

frame rate 256–512 frames per second. The recorded short video sequences of beating regions, approximately 10 video sequences per sample, were analysed by the LabwiewTM software generating ciliary regions of interest (ROI). For every ROI, the median of ciliary beat frequency was calculated and used as an evaluation parameter. The final value of the ciliary beat frequency (CBF) expressed in Hz, was an average of ten median values obtained from each specimen.

Statistical analysis

All results are represented as means ± SEM. Statistical analysis was performed using one-way analysis of variance ANOVA. A P value of less than 0.05 was taken as a threshold for statistical significance.

RESULTS

Cough reflex

The changes in cough reflex, evaluated in '*in vivo*' were assessed before drug administration to obtain a control group. The effect of oral and inhaled NAC on cough reflex was recorded 2 hours after the single dose and 2 hours after the last dose of a 7-day treatment. As the evaluation parameter, the number of cough efforts induced by citric acid inhalation were used. Inhaled and oral NAC, either administrated as a single dose or during 7 days, showed a tendency to decrease the number of chemically induced cough efforts, however these results were not statistically significant (Table 1).

Airway reactivity

The airway contraction under '*in vivo*' conditions was provoked by histamine inhalation, and as an evaluation parameter, the values of specific airway resistance were used. The measurement of changes in specific airway resistance was realized before and 2 hours after the oral and inhaled NAC administration. Both, single and 7-day treatments with oral and inhaled NAC caused a small increase in the values of specific airway resistance. However, none of the NAC administrations (neither oral nor acute therapy) showed any statistically relevant bronchoconstrictor activity (Table 1).

Ciliary beat frequency

Oral and inhaled NAC effect on ciliary movement was evaluated '*in vitro*' by recording the changes in CBF. Acute administration of oral and inhaled NAC had no significant effect on ciliary movement, although inhaled acute NAC administration has shown the tendency to supress CBF slightly in comparison to the acute administration of oral NAC, after which no effect on CBF was observed. On the contrary, chronic administration of both, inhaled and oral NAC, significantly reduced CBF (Fig. 1).

DISCUSSION

Airway surface fluid, composed of mucus gel layer and periciliary sol fluid, forms an important part of the defense mechanisms of the airways. The upper mucus layer acts as a medium in which the inhaled foreign particles are trapped, whereas the less viscous periciliary fluid maintains the gel layer at the optimal distance from the airway epithelium and provides an ideal environment for ciliary beating. To protect the airway epithelium, the mucus is constantly removed by means of beating cilia and replenished by new one in a process called mucociliary transport (MCT). MCT represents one of the most important mucus clearance mechanisms; its rate depends on the viscoelastic properties of mucus layer, depth of periciliary fluid and on the kinetic parameters of beating cilia. The depth and composition of airway surface fluid are regulated by mucin secretion and by the hydration, mediated through the active ion transport across the airway epithelium (Fahy & Dickey, 2010). Some studies have demonstrated that NAC, in addition to exert the mucolytic activity, may improve the hydration of airways through the downregulation of the

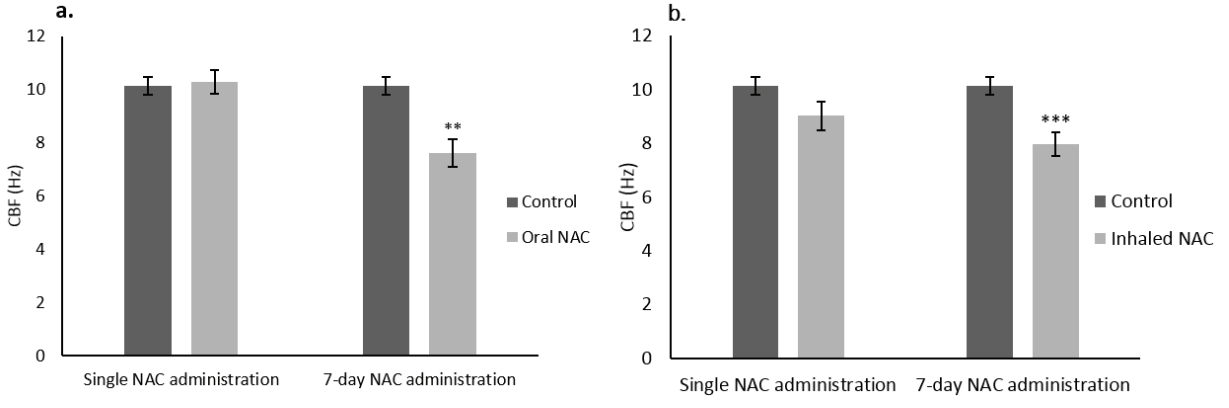


*Figure 1. Changes in ciliary beat frequency. Changes in ciliary beat frequency (CBF) induced by oral (a.) and inhaled (b.) N-acetylcysteine (NAC) administrated as a single dose or as a 7-day treatment. Statistical significance: ** p<0,01 vs. control; *** p<0,001 vs. control (one-way ANOVA and Bonferroni post hoc test).*

Table 1. Changes in cough efforts and specific airway resistance. Changes in number of cough efforts and specific airway resistance (sRaw) after chronic (7 days) and acute administration of inhaled (inh.) and oral (p.o.) N-acetylcysteine (NAC). Data are expressed as the means ± SEM. Statistical analysis: Student's t-test.

	NAC inh. acutely		NAC inh. 7 days		NAC p.o. acutely		NAC p.o. 7days	
	Before administration	After administration	Before administration	After administration	Before administration	After administration	Before administration	After administration
Number of cough efforts	7,875 ± 1,008	6,000 ±1,402	8,500 ± 1,880	6,250 ± 1,333	8,000 ± 1,633	6,571 ± 1,974	8,000 ± 0,724	7,428 ± 0,841
sRaw (ml/s)	6,470 ± 1,119	8,728 ± 1,452	5,483 ± 1,695	6,567 ± 2,335	5,331 ± 2,021	7,528 ± 2,442	8,069 ± 2,061	11,182 ± 2,238

epithelial sodium channels and the enhancement of chloride efflux into the airway lumen (Rochat et al., 2004; Varelogianni et al., 2010). However, both mentioned effects may be beneficial in the regulation of MCT, and NAC does not seem to improve MCT. Seagrave et al. attributed the observed decline in MCT rate to excessive reduction of mucus viscosity induced by NAC, which may result in disruption of effective coupling between the mucus and beating cilia (Seagrave et al., 2012). Although both periciliary fluid and mucus layer are important components of MCT, the reduction in ciliary movement can have a great impact on MCT. Thus, we focused our attention to evaluate the direct effect of NAC on CBF, which represents the driving force for MCT (Braiman & Priel, 2008). According to our study, the decrease in MCT may be associated with the negative effect of NAC on ciliary movement. We recorded significant reduction in CBF after the 7-day treatment with inhaled as well as oral NAC. Acutely, slight decrease in CBF was observed only after NAC inhalation, whereas acute oral NAC therapy had no effect on ciliary movement.

In the case of mucociliary dysfunction, the cough becomes the most important mechanism for mucus clearance (Munkholm & Mortensen, 2014). Regarding the negative effect of NAC on CBF, the suppression of cough reflex sensitivity would lead to considerable retention of mucus in the airways. For mentioned reason, it is important to know the effect of NAC on cough reflex sensitivity. Although, chronic and acute administration of oral NAC has shown a tendency to decrease the number of cough efforts slightly, these results were not statistically relevant. However, NAC, by the mucolytic action, may decrease the mucus adhesivity to airway epithelium, facilitating the mucus expulsion by cough (Rubin, 2010). No data convincingly demonstrates that any classic mucolytic agent including the NAC improves the ability to expectorate mucus (Rubin, 2007).

Furthermore, since the significant airflow limitation due to the mucus accumulation may be worsened by airway narrowing (Rogers, 2007), the determination of the NAC effect on the reactivity of bronchial smooth muscle formed part of this study. Unfortunately, regardless of the duration or route of administration, NAC induced a small increase in the airway reactivity provoked by histamine inhalation *in vivo*. Although this rise in airway reactivity was insignificant, it can be considerable in some hyper-reactive airway diseases, as demonstrated in the study carried out on cats with experimental asthma (Reinero et al., 2011).

In conclusion, we can summarize, that in our study we did not record any significant difference between oral and inhaled NAC administration. Chronic administration by both routes led to a significant decrease in CBF, which may have a great impact on mucus accumulation. Although in our physiological conditions, NAC negatively affected mucus clearance, in pathological conditions, its mild anti-inflammatory and antioxidant activity may be of benefit if combined with bronchodilators, which also possess ciliostimulatory activity (Pappová et al., 2016).

ACKNOWLEDGEMENT

Work was supported: APVV 0305-12, VEGA 1/0165/14 a MZ 2012/35- UK, CERK II, UK/62/2016; by the project 'Biomedical Centre Martin' ITMS: 26220220187 'We support research activities in Slovakia/ The project is co-financed from EU sources.'

References

[1] Braiman A, Priel Z. Efficient mucociliary transport relies on efficient regulation of ciliary beating. Respir. Physiol. Neurobiol. 2008; 163: 202–207.

[2] Fahy JV, Dickey BF. Airway mucus function and dysfunction. N. Engl. J. Med. 2010; 363: 2233–2247.

[3] Franova S, Joskova M, Sadlonova V, Pavelcikova D, Mesarosova L, Novakova E, Sutovska M. Experimental model of allergic asthma. Adv Exp Med Biol. 2013; 756:49-55

[4] Fuloria M, Rubin BK. Evaluating the efficacy of mucoactive aerosol therapy. Respir. Care 2000; 45: 868–873.

[5] GOLD. Global Strategy for the Diagnosis, Management and Prevention of COPD, Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2015.

[6] Kazimierová I, Jošková M, Pecháňová O, Šutovská M, Fraňová S. Effects of provinol and its combinations with clinically used antiasthmatics on airway defense mechanisms in experimental allergic asthma. Adv Exp Med Biol. 2015;838:27-34.

[7] Morice AH, Fontana GA, Belvisi MG, Birring SS, Chung KF, Dicpinigaitis PV, Kastelik JA, McGarvey LP, Smith JA, Tatar M et al. ERS guidelines on the assessment of cough. Eur. Respir. J. 2007; 29: 1256–1276.

[8] Munkholm M, Mortensen J. Mucociliary clearance: pathophysiological aspects. Clin. Physiol. Funct. Imaging 2014; 34: 171–177.

[9] Reinero CR, Lee-Fowler TM, Dodam JR, Cohn LA, DeClue AE, Guntur VP. Endotracheal nebulization of N-acetylcysteine increases airway resistance in cats with experimental asthma. J. Feline Med. Surg. 2011; 13: 69–73.

[10] Rogers D. Mucoactive agents for airway mucus hypersecretory diseases. Respir. Care. 2007; 52: 1176-93.

[11] Rochat T, Lacroix J-S, Jornot L. N-acetylcysteine inhibits Na+ absorption across human nasal epithelial cells. J. Cell. Physiol. 2004; 201: 106–116.

[12] Rubin BK. Secretion properties, clearance, and therapy in airway disease. Transl. Respir. Med. 2014;2: 6.

[13] Rubin BK. The role of mucus in cough research. Lung. 2010; 188 (1 Suppl): S69-72.

[14] Seagrave J, Albrecht HH, Hill DB, Rogers DF, Solomon G. Effects of guaifenesin, N-acetylcysteine, and ambroxol on MUC5AC and mucociliary transport in primary differentiated human tracheal-bronchial cells. Respir. Res. 2012;13: 98.

[15] Varelogianni G, Oliynyk I, Roomans GM, Johannesson M. The effect of N-acetylcysteine on chloride efflux from airway epithelial cells. Cell Biol. Int. 2010; 34: 245–252.

[16] Rubin BK. Mucolytics, expectorants, and mucokinetic medications. Respir. Care. 2007; 52: 859–865.

[17] Pappová L, Jošková M, Kazimierová I, Šutovská M, Fraňová S. Combination Therapy with Budesonide and Salmeterol in Experimental Allergic Inflammation. Adv. Exp. Med. Biol. 2016: 935: 25–34.

DE GRUYTER OPEN

Eur. Pharm. J. 2017, 64 (1): 22-25.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.1515/afpuc-2017-0008

EUROPEAN PHARMACEUTICAL JOURNAL

Combination treatment with valsartan and amlodipine intensifies evening suppression of Bmal1 clock gene in kidneys of spontaneously hypertensive rats

Kombinovaná liečba valsartanom a amlodipínom zvyšuje večernú supresiu Bmal1 génu v obličkách spontánne hypertenzných potkanov

Original Paper

Potucek P.✉, Radik M., Doka G., Kralova E., Krenek P., Klimas J.

Comenius University in Bratislava, Faculty of Pharmacy, Department of Pharmacology and Toxicology, Bratislava, Slovak Republic / *Univerzita Komenského v Bratislave, Farmaceutická fakulta, Katedra farmakológie a toxikológie, Bratislava, Slovenská Republika*

Received 16 March, 2017, accepted 12 May, 2017

Abstract Blood pressure (BP) rhythm is exhibited in a circadian pattern regulated by complex system of endogenous factors. Administration of pharmacological treatment at the right time can influence the efficacy of treatment; but while kidneys play significant role in BP regulation, little is known about their role in chronopharmacotherapy. This study aimed to compare differences between morning and evening dosing with valsartan and amlodipine combination in both short-term and long-term settings and to elucidate the role of kidneys in chronopharmacology. Spontaneously hypertensive rats aged between 8 and 10 weeks were daily treated with 10mg/kg of valsartan and 4 mg/kg of amlodipine, either in the morning or in the evening with treatment duration of 1 and 6 weeks. After short-term treatment, only morning treatment group demonstrated significantly better outcomes in terms of BP control when compared to placebo. After long-term treatment, both treatment groups gained superior results in BP control against placebo; however, no significant difference was seen between morning and evening treatment. Interestingly, clock gene expression in kidney has been significantly modulated only in the evening-treated groups, with treatment intensifying the reduced Bmal1 levels, while Per2 expression was less altered. However, no direct relation with the outcomes of the therapy has been observed, suggesting that pharmacotherapy may serve as an independent modulator of peripheral circadian clock in the kidney.

Slovak abstract Rytmus krvného tlaku má charakteristickú cirkadiánnu štruktúru, ktorá je podmienená komplexným systémom endogénnych faktorov. Podávanie farmakologickej liečby v správnom čase môže mať vplyv na účinnosť liečby, úloha obličiek, ktoré významne regulujú tlak krvi, je však v chronofarmakoterapii hypertenzie málo prebádaná. Cieľom tejto práce bolo porovnať rozdiely v účinnosti medzi ranným a večerným podávaním kombinácie valsartanu s amlodipínom pri krátkodobej aj dlhodobej liečbe a objasniť vplyv obličiek v chronofarmakológii. Spontánne hypertenzným potkanom vo veku 8-10 týždňov bol denne podávaný valsartan v dávke 10mg/kg a amlodipín v dávke 4mg/kg, buď ráno alebo večer s dĺžkou liečby 1 a 6 týždňov. Po krátkodobej liečbe sa iba pri rannom podávaní podarilo preukázať významné rozdiely oproti placebu v znížení hodnôt krvného tlaku. Po dlhodobej liečbe sa pri oboch liečených skupinách dosiahli lepšie výsledky v porovnaní s placebom, medzi večernou a rannou liečbou však už nebol pozorovaný významný rozdiel. Expresia hodinových génov v obličkách bola významné modulovaná iba pri večernej liečbe, pričom sa zvýšila potlačená hladina Bmal1, zatiaľ čo expresia Per2 bola ovplyvnená v menšej miere. Vplyv na expresiu hodinových génov však nemal preukázateľnú koreláciu s výsledkami liečby, čo naznačuje, že farmakoterapia by mohla pôsobiť na expresiu periférnych hodinových génov v obličkách nezávisle od signalizačnej dráhy, ktorou je sprostredkované zníženie tlaku a redukcia hypertrofie ľavej komory.

Keywords *Chronotherapy – valsartan – amlodipine – spontaneously hypertensive rats (SHR) – Bmal1 – clock genes*

Kľúčové slová: *Chronoterapia – valsartan – amlodipín – spontánne hypertenzné potkany (SHR) – Bmal1 – hodinové gény*

INTRODUCTION

Cardiovascular function, including blood pressure, displays daily rhythms, providing the basis for the formation of chronopharmacology, i.e. an implementation of chronobiology into pharmacodynamics and pharmacokinetics. Indeed, dosing time-dependent differences in efficacy of antihypertensive treatment have been reported in humans

* *E-mail: petar.potucek@gmail.com*

(Hermida et al., 2003) and in rat (Liu et al., 2011), a primarily nocturnal animal. Data available from combinational therapy are still very limited (Potucek & Klimas, 2013), but it is generally assumed that chronopharmacological profiles of each drug might contribute to the dosing-time-dependent influences on efficacy.

Oscillations in cardiovascular functions are strictly controlled by an endogenous oscillator, the circadian clock pathway. In mammals, specific clock genes constitute the core of the molecular clock (Takahashi et al., 2008). These genes are involved in interlocking positive and negative transcriptional and translational feedback loops that drive the oscillation of circadian gene expression in individual cells. The rhythmic transcriptional enhancement of various genes is governed by heterodimer of two transcription factors, circadian locomotor output cycles protein kaput (CLOCK) and brain and muscle Arnt-like protein-1 (BMAL1). The CLOCK-BMAL1 heterodimer activates the transcription homologs of Period (Per)1, Per2, Per3 and Cryptochrome (Cry)1, Cry2. PER and CRY proteins then translocate back into the nucleus and inhibit the activity of CLOCK-BMAL1, comprising the negative feedback limb of the circadian clock loop, resulting in the downregulation of its own expression. Outside of the clock loop (i.e. without feedback on CLOCK/BMAL1), the heterodimer controls cellular physiology and biochemical processes through output genes in a time-of-day-dependent manner.

Among core genetic constituents, Bmal1 and Per2 have been identified to play a critical role in the circadian regulation of cardiovascular function, particularly of BP, with a causative role in the development of hypertension (Woon et al., 2007; Richards et al., 2014). Although a number of studies have observed the influence of circadian clock on cardiac function, its impact on kidneys is less explored. In this study, we tested the influence of morning or evening administration of combination of antihypertensives on systolic blood pressure and on renal expression of Bmal1 and Per2.

METHODS

Experimental design

We used eight-week-old male SHRs (Department of Toxicology and Laboratory Animal breeding (SAS, Dobra Voda, Slovak Republic). They were maintained under a constant environment with a controlled light/dark cycle for 2 weeks before the experiment. Valsartan and amlodipine besylate (a kind gift from Novartis, Ireland) were dispersed in a 0.5% solution of methylcellulose. The same vehicle was used for control groups as a placebo treatment. All experimental procedures involving the use of experimental animals were approved by the State Veterinary and Food Administration of the Slovak Republic and by the Ethics Committee of the Faculty of Pharmacy, Comenius University in Bratislava.

We performed two experiments with different duration: 1 week and 6 weeks. Animals were randomized into groups based on the kind of treatment (AV – amlodipine+valsartan-treated; Con – controls receiving placebo). These were divided into two sub-groups based on the time of treatment (M – morning; E – evening). Animals in the AV groups were daily given 10 mg/kg dose of valsartan and 4 mg/kg dose of amlodipine by oral gavage in the morning between 7 and 8 a.m. and in the evening between 7 and 8 p.m. The same schedule was used in placebo-treated controls. Dosage was adjusted to actual body weight twice a week.

Systolic BP measurements and termination

At the end of experimental treatments, arterial systolic blood pressure (sBP), as a measure of hypertension was measured prior to daily treatment by the tail-cuff plethysmographic method in pre-trained conscious animals pre-warmed in thermostatic cages. Measurements were repeated several times and the mean of six consecutive values after stabilization was taken. Twenty-four hours after the final dose (i.e. morning or evening, respectively), animals were sacrificed and the left kidney was removed and cortex was prepared and used for further investigation.

RNA isolation and RT-PCR

We studied the mRNA levels of two crucial circadian genes Bmal1 and Per2 in renal cortex by real-time PCR reaction. Tissue samples were homogenized in liquid nitrogen and total RNA was isolated by phenol/chloroform extraction (TriReagent®, Sigma Aldrich, USA). The quality of isolated RNA was verified by agarose gel electrophoresis and spectrophotometric analysis (NanoDropND-1000, Thermo Scientific, USA). Two micrograms of total RNA were reverse-transcribed using high-capacity cDNA Reverse Transcription Kit with RNAse inhibitor® (Applied Biosystems, USA). Real-time PCR (StepOne Plus System, Applied Biosystems, USA) was performed using SYBR Select Master Mix (Applied Biosystems, USA). The results were normalized to the expression of endogenous reference genes (Beta-2 microglobulin, B2m; Hprt1, hypoxanthine phosphoribosyltransferase 1) and calibrated to the control group. The following primer sequences were used: Bmal1 (forward: 'GCACTCACACATGGTTCCAC', reverse: 'CATTCCGCAAGGTGTCCTAT'), Per2 (forward: 'GCACAAAGTCAGGGTAGGCC', reverse: 'GCATCAGTAGCCGGTGGATT'), B2m (forward: 'ATGGAGCTCTGAATCATCTGG', reverse: 'AGAAGATGGTGTGCTCATTGC') and Hprt1 (forward: 'CAGCTTCCTCCTCAGACCGCTTT', reverse: 'TCACTAATCACGACGCTGGGACTG'). All primers (Sigma-Aldrich, USA) were designed by Primer3 version 0.4.0 into intron/exon boundaries to avoid the amplification of genomic DNA. All primers were verified to yield a single PCR product with the correct molecular weight and the absence of signal was confirmed when reverse transcription was omitted (Radik et al., 2016).

*Table 1. Relative mRNA levels of Bmal1 and Per2 in kidneys of controls (Con) and amlodipine+valsartan (AV)-treated rats, respectively. Values are mean ± SD (n = 10–14 per group; *P < .05 vs. Con)*

Clock gene	Treatment duration	Morning		Evening	
		Con	AV	Con	AV
Bmal1	1 week	1.00±0.12	1.05±0.13	0.29±0.10	0.08±0.10*
	6 weeks	1.00±0.05	1.11±0.03	0.29±0.02	0.16±0.02*
Per2	1 week	1.00±0.12	0.93±0.06	1.03±0.09	1.16±0.24
	6 weeks	1.00±0.11	0.97±0.09	1.03±0.11	1.80±0.20*

Statistical analysis

All variables are reported as mean ± standard deviation (SD) or standard error of the mean (SEM). Means were compared using ANOVA with subsequent Tukey's HSD multiple comparison test for normally distributed data or Kruskal–Wallis test followed by pairwise Wilcoxon test with Holm–Bonferroni correction for non-parametric data, with $p < 0.05$ considered as statistically significant. Mean PCR efficiency estimates (E) per amplicon and quantification cycle (Cq) values per sample were determined with LinRegPCR software (version 2015.0) and efficiency corrected relative expression ratios were calculated (all reactions had E > 1.8 and Cq < 35). Data were handled by GraphPad Prism (GraphPad Software, Inc., version 6).

RESULTS

Following 1-week treatment, we observed reduction of sBP in the morning but not in the evening dosing group, while at the of the 6-week period, treatment was efficient against placebo in both treated groups (Fig. 1).

As expected, mRNA expressions of Bmal1 in renal cortex were suppressed in the evening (Tab. 1). Interestingly, evening treatment intensified the reduced Bmal1 levels after one as well as after 6 weeks of therapy. Per2 expression was less altered, and we found significantly increased mRNA expression only in the evening-treated group (Tab. 1).

DISCUSSION

Chronopharmacological studies comparing morning and evening dosing of amlodipine+valsartan combination conducted so far have given contradictory results (Hermida et al., 2010; Asmar et al., 2011). In our study, comparison against placebo group revealed that BP control was established faster with morning dosing, but no difference was seen between morning and evening dosing after long-time treatment. So while the long-term treatment with the combination of antihypertensives was demonstrated to be equal regardless of administration time, morning dosing (i.e. during inactive period of rat) exhibited accelerated onset of action and the efficacy of the treatment was established earlier than with the evening dosing (i.e. during active period of rat).

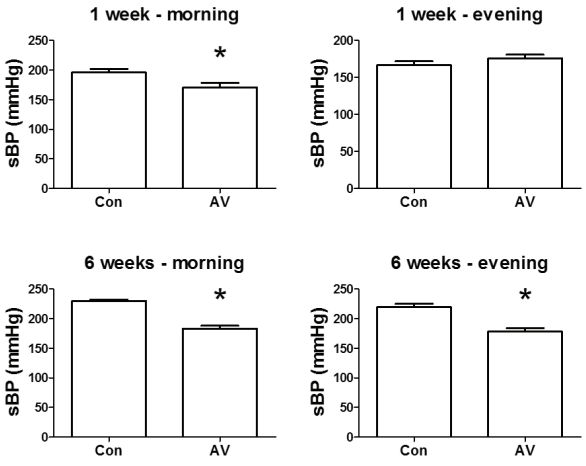


*Figure 1. Systolic blood pressures of SHRs treated for 1 week or 6 weeks with placebo (Con) or valsartan+amlodipine combination (AV). Values are mean ± SEM (n = 10–14 per group; *P <.05 vs. corresponding Con)*

In SHR model, abnormalities in the expressions of clock genes are limited to hypertrophied left ventricles as concurrently to significant differences in the left ventricular levels of Bmal1 and Per2 (Naito et al., 2003) and lack of significant alterations in clock gene expression were reported in aortas and kidneys in SHRs when compared to normotensive rats (Naito et al., 2003; Cui et al., 2011). Consequently, studies pay less attention to peripheral circadian clock pathway in kidneys during the progression of hypertension, though kidneys are crucial modulators of blood pressure (Vavrinec et al., 2011; Ulu et al., 2013; Vavrinec et al., 2013). Indeed, the hypertensive renal injury occurs several months later than the development of hypertension and left ventricular hypertrophy in SHRs (Braun et al., 2013; Klimas et al., 2015). However, we observed antihypertensive treatment-induced alterations already in young SHRs, which suggest that treatment might alter the circadian clock independently of disease development in the kidneys of SHRs. Although the observed effect of antihypertensive drugs on circadian clock pathway seems to be unspecific, it may have significant influence on the both therapeutic outcome as well as safety of particular drugs. Whether this action provides benefits or not is related to therapeutic failure in patient subpopulations, which requires further investigation.

CONCLUSION

In conclusion, we obtained evidence that chronopharmacological effect of amlodipine and valsartan combination observed in short-term treatment was diminished in long-term settings. Our results provided experimental evidence supporting the idea that the combination therapy of valsartan and amlodipine is lacking significant chronopharmacological properties in long-term treatment, while in acute treatment, this combination might be more beneficial when administered during inactive phase. Additionally, our findings showed that the combination of valsartan and amlodipine influences circadian clock pathway in kidneys independently from sBP, i.e. pharmacotherapy may serve as an independent modulator of circadian clock. Still, it remains to be clarified whether modulation of circadian clock contributes someway to the outcome of treatment. If yes, this would further support the concept of the optimization of antihypertensive treatment by timing along the circadian scale.

References

[1] Asmar R, Gosse P, Quere S, Achouba A. Efficacy of morning and evening dosing of amlodipine/valsartan combination in hypertensive patients uncontrolled by 5 mg of amlodipine. Blood Press Monit. 2011;16(2):80-86.

[2] Braun MC, Herring SM, Gokul N et al. Hypertensive renal disease: susceptibility and resistance in inbred hypertensive rat lines. J Hypertens. 2013;31(10):2050-2059.

[3] Cui H, Kohsaka A, Waki H, Bhuiyan ME, Gouraud SS, Maeda M. Metabolic cycles are linked to the cardiovascular diurnal rhythm in rats with essential hypertension. PLoS One. 2011;6(2):e17339.

[4] Hermida RC, Ayala DE, Fontao MJ, Mojon A, Fernandez JR. Chronotherapy with valsartan/amlodipine fixed combination: improved blood pressure control of essential hypertension with bedtime dosing. Chronobiol Int. 2010;27(6):1287-1303.

[5] Hermida RC, Calvo C, Ayala DE et al. Administration time-dependent effects of valsartan on ambulatory blood pressure in hypertensive subjects. Hypertension. 2003;42(3):283-290.

[6] Klimas J, Olvedy M, Ochodnicka-Mackovicova K et al. Perinatally administered losartan augments renal ACE2 expression but not cardiac or renal Mas receptor in spontaneously hypertensive rats. J Cell Mol Med. 2015;19(8):1965-1974.

[7] Liu Y, Ushijima K, Ohmori M et al. Chronopharmacology of angiotensin II-receptor blockers in stroke-prone spontaneously hypertensive rats. J Pharmacol Sci. 2011;115(2):196-204.

[8] Naito Y, Tsujino T, Kawasaki D et al. Circadian gene expression of clock genes and plasminogen activator inhibitor-1 in heart and aorta of spontaneously hypertensive and Wistar-Kyoto rats. J Hypertens. 2003;21(6):1107-1115.

[9] Potucek P, Klimas J. Chronotherapy of hypertension with combination treatment. Pharmazie. 2013;68(12):921-925.

[10] Radik M, Doka G, Malikova E, Krenek P, Klimas J. Voluntary exercise and testosterone therapy caused increase in percentage of Myh6 and expression of oxidative stress marker Cybb in left ventricles of rats. Eur Pharm J. 2016;63(1):12-15.

[11] Richards J, Diaz AN, Gumz ML. Clock genes in hypertension: novel insights from rodent models. Blood Press Monit. 2014;19(5):249-254.

[12] Takahashi JS, Hong HK, Ko CH, McDearmon EL. The genetics of mammalian circadian order and disorder: implications for physiology and disease. Nat Rev Genet. 2008;9(10):764-775.

[13] Ulu N, Mulder GM, Vavrinec P et al. Epidermal growth factor receptor inhibitor PKI-166 governs cardiovascular protection without beneficial effects on the kidney in hypertensive 5/6 nephrectomized rats. J Pharmacol Exp Ther. 2013;345(3):393-403.

[14] Vavrinec P, Henning RH, Goris M, Landheer SW, Buikema H, van Dokkum RP. Renal myogenic constriction protects the kidney from age-related hypertensive renal damage in the Fawn-Hooded rat. J Hypertens. 2013;31(8):1637-1645.

[15] Vavrinec P, van Dokkum RP, Goris M, Buikema H, Henning RH. Losartan protects mesenteric arteries from ROS-associated decrease in myogenic constriction following 5/6 nephrectomy. J Renin Angiotensin Aldosterone Syst. 2011;12(3):184-194.

[16] Woon PY, Kaisaki PJ, Braganca J et al. Aryl hydrocarbon receptor nuclear translocator-like (BMAL1) is associated with susceptibility to hypertension and type 2 diabetes. Proc Natl Acad Sci U S A. 2007;104(36):14412-14417.

DE GRUYTER OPEN

Eur. Pharm. J. 2017, 64 (1): 26-29.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.1515/afpuc-2017-0007

EUROPEAN PHARMACEUTICAL JOURNAL

The role of cytokines in degenerative spine disorders

Original Paper

Sutovsky J.,[1] Kocmalova M.,[2,3]✉ Benco M.,[1] Kazimierova I., Pappova L.,[2,3] Frano A.,[4] Sutovska M.,[2,3]

[1]*Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin, Martin University Hospital, Neurosurgery Clinic, Martin, Slovak Republic*

[2]*Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin, Biomedical Centre Martin, Martin, Slovak Republic*

[3]*Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin, Department of Pharmacology, Martin, Slovak Republic*

[4]*Charles University Prague, 1st Medical Faculty, The Hospital Na Bulovce, The Ortopedic Clinic, Prague, Czech Republic*

Received 21 February, 2017, accepted 11 April, 2017

Abstract Background: Degenerative spine disorders (DSD) are the most frequent reason of morbidity in adults. Commonly DSD includes degenerative disorders of intervertebral discs (IVDs), spinal stenosis and degenerative spondylolisthesis (SL). There is increasing evidence about significant role of cytokines in DSD pathogenesis, symptomathology and progression, but their protective levels remain still unknown.
Material and Methods: The aim of presented study was to provide quantitative and qualitative analysis of cytokine, chemokine and growth factors levels in individual parts of IVDs - annulus fibrosus (AF) and nucleus pulposus (NP) - separately and in facet joints (FJ) subchondral bone of patients with DSD and in controls - healthy subjects during a multiorgan procurement procedure. Bio-Plex® assay was used to measure concentrations of 27 different cytokines in tissue of patients with DSD. Their concentrations in tissues of healthy subjects during a multiorgan procurement procedure represented protective levels.
Results: The Bio-Plex® assay revealed significant differences between the patients suffered from degenerated and herniated IVDs and from lumbar SL and controls in cytokines, chemokines and growth factor profiles suggested that pro-inflammatory changes of both NP and AF were dominated in herniated IVDs, whereas the same tissue of lumbar SL patients exhibited much more complex changes in cytokine levels suggested o only ongoing inflammation (IL-6, IL-8, MCP-1, TNF-α), abut also anti-inflammatory processes (IL-ra, IL-10) or connective tissue remodeling (PDGF-bb, IL-17, VEGF). The different mediators were found elevated in lumbar SL samples of subchondral FJ bone. These also confirmed ongoing inflammation, accelerated bone resorption and formation and increased fibroblasts activity in FJ bone.
Conclusion: The study supported the significant involvement of several cytokines, chemokines and growth factors in the pathogenesis of DSD. These cytokines should represent future potential targets for new biological treatment able to slow DSD progression as well as factor determining prognosis of DSD.

Keywords *Degenerative lumbar spondylolisthesis – herniated intervertebral disc – cytokine levels – annulus fibrosus – nucleus pulposus*

INTRODUCTION

The degenerative spine disorders (DSD) includes disorders of intervertebral discs (IVDs) and degenerative spondylolisthesis. DSD represents the most frequent reason of morbidity in adults and its incidence rises with age. The common clinical signs of DSD are pain and mobility impairment, as the consequence of spinal roots and spinal cord compression by herniated IVDs, spinal canal stenosis, osteoarthrosis and damage of facet joints (FJs) cartilage. The pain can appear without evident neuronal compression due to the local release of cytokines and chemokines. It has been recently appointed a significant role of cytokines, not only in pain mediation, but also in DSD progression (Wuertz and Haglund, 2013).

IVDs consist of annulus fibrosus (AF) and nucleus pulposus (NP). While collagen represents the main compound of AF, NP comprises proteoglycans and water. One of the early symptoms of IVD degeneration is reduced hydration of NP, followed by subsequent biochemical and structural changes that change the mechanic loading of spine (Risbud and Shapiro, 2014).

* *E-mail: Michaela.Kocmalova@jfmed.uniba*

The microtraumatic changes appear in AF that initiate the local release of cytokines and chemokines, which trigger local inflammatory reaction and simultaneously regulate the anabolic and catabolic processes in IVDs. Typical feature of degenerated IVDs is the loss of physiological balance between matrix production and degradation, which can possibly result in protrusion or herniation of NP even in complete failure of IVD structure (Wuertz and Haglund, 2013; Risbud and Shapiro, 2014). Spondylolisthesis (SL) is defined as anterior slippage of cranial vertebra in relation to the adjacent vertebra. Although there are 5 types of SL: dysplastic, isthmic, traumatic, pathologic and degenerative. Degenerative SL, localized in lumbar spine, is by far the most frequent. It is generally accepted that degenerative SL occurs as the result of alterative inflammation and FJs subchondral bone remodelling(Wuertz and Haglund, 2013), or as a result of FJ overload at IVD failure.

The cytokines play an important regulatory and amplifying role under the physiological conditions as well as in IVD degeneration or in SL development (Wuertz and Haglund, 2013; Lee et al., 2013). The catabolic processes in IVD tissues are significantly accelerated by interleukin-1 (IL-1), IL-2, IL-4, IL-6, IL-8 and tumour necrosis factor-α (TNF-α) (Wuertz and Haglund, 2013; Risbud and Shapiro, 2014; Lee et al., 2013). Simultaneously, IL-1ß, IL-6, IL-8 and TNF-α stimulate synthesis of neuronal growth factor (NGF) and vascular endothelial growth factor (VEGF) that regulate the neuronal proliferation and neoangiogenesis in AF, NP and FJs subchondral bone, control bone remodelling and irritate nociceptors (Wuertz and Haglund, 2013; Lee et al., 2013; Raggatt and Partridge, 2010).

Despite several recently published papers describing the changes incytokine levels in DSD, the role of cytokines in disease pathogenesis is not still completely explained. One of the reasons is that we still do not know their levels produced under the physiological conditions in healthy subjects, which have a protective character. Although several studies compared cytokine levels in the IVDs of operated patients with their levels in healthy cadaveric tissue samples, but donors at the time of collection were dead for more than 24 hours (Le Maitre et al., 2007). According to the limited stability of cytokines, it is not possible to exclude that such an increase in the patient's cytokine levels may have been caused due to secondary factors (Friebe and Volk, 2008). The works, for example, comparing the cytokine levels in patients with DSD and healthy subjects, are not presented in scientific literature. This study was designed to exclude the secondary distort of results. The control subjects are living organ donors and sample handling methods allow to maintain stable cytokine levels. The data obtained this way should significantly contribute to the clarification of cytokine and chemokine roles in the pathophysiology and symptomatology of DSD.

MATERIAL AND METHODS

The main aim of our study was to investigate the cytokines, chemokines and the profile of growth factors in the samples of AF, NP and FJs bone from patients with SL, AF and NP from patients with degenerated and herniated IVDs as well as from healthy donors.

All processes were approved by the Institutional Ethic Committee of the Jessenius Faculty of Medicine registered in the Institutional Review Board/Institutional Ethic Board Office (IRB 00005636) in accordance with the Slovakian and European legislation (decision No. EK 1209/2012). The patient's recruitment was performed by providing information sheets describing the study, and the patients provided written informed consent. A total of 10 patients (males, mean age of 48.6 ± 14.1 years), who had been operated on due to herniated lumbar intervertebral disc and 9 patients suffered from lower segment spondylolisthesis, Pfirrman`s score 4-5 (2 males and 7 females; average age 50.7 ± 10.2 years) as verified on magnetic resonance images (MRI), were included in the study. The patients underwent primary spinal surgery at the Neurosurgery Clinic, JFM CU and University Hospital Martin. The patients did not have any severe systemic chronic disorders (rheumatological, endocrine, cardiovascular and oncologic) and were not administered any epidural drugs, such as analgesics, steroids or anesthetizing agents, during general anaesthesia and at least 12 week before the surgical treatment of spondylolisthesis. Intact FJ bone and IVD samples, used as a control group, were obtained from 6 adult males (average age 44.2 ± 13.2 years) during a multiorgan procurement procedure at the Transplant and Vascular Surgery Clinic, JFM CU, Martin University Hospital.

After removing the disc tissue, the nucleus pulposus (NP) and annulus fibrosus (AF) were separated by the surgeon. The subchondral bone tissue (B) was obtained from the adjacent FJ. Then all the collected tissue samples were immediately transported to the laboratory in sterile polypropylene storage tubes and kept frozen at - 80°C.

Preparation and determination of proteins: Tissue was removed from IVD and facet joints bone (FJB) and lysed using a lysis kit (Bio-PlexTM Cell Lysis Kit, Bio-Rad, USA). After that the tissue was prepared using 3x3 mm segments and washed using cell wash buffer. Tissue segments were transferred to a tube containing 500 µl of lysis solution (40 µl Factor 1, 20 µl Factor 2 and 9.9 ml Cell Lysis Buffer) incubated on ice prior to adding 40 µl phenylmethylsulfonyl fluoride (PMSF). This mixture was homogenized (Homogenizator; Stuart SHM2, Germany) for 4 min at 4000 rotations per minute (RPM) stage 1. The homogenate was centrifuged for 4 min at 5000 RPM (2370 G), and the supernatant was collected and frozen at -80°C. Final homogenate of proteins were solubilized by the addition of 10% SDS solution to final concentration of SDS 5%. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

The assessment of cytokine levels in tissue samples:The Bio-Plex® 200 System and Bio-PlexTM Human Cytokine Standard 27-Plex, Group I (Bio-Rad, Hercules, California, USA) were used to assess the cytokine levels. The method was previously described in detail by Franova et al. 2016. The total amount

*Table 1. Summary of the data obtained from the Bio-Plex® assay of nucleus pulposus (NP) tissue samples. The cytokine, chemokine and growth factors levels are expressed in pg/ml. *p≤0.05 and **p≤0.01 Control samples vs. lumbar DS (Listhesis) or herniated discs (Hernia) specimens, # p≤0.05 and ##p≤0.01 lumbar DS (Listhesis) vs herniated discs (Hernia) (one-way ANOVA with Bonferroni post-hoc test).*

	Nucleus pulposus (NP)		
	Hernia	**Listhesis**	**Control**
IL-1β	4.35 ± 0.45*	4.25 ± 2.04	2.11 ± 0.81
IL-1ra	34.50 ± 3.85*	47.55 ± 10.95*	86.22 ± 13.41
IL-6	795.36 ± 140.71**/ ##	110.66 ± 7.73*	41.64 ± 15.43
IL-8	90.26 ± 57.56*	86.82 ± 27.05*	22.79 ± 1.75
IL-10	52.21 ± 1.23	62.89 ± 2.35#	51.38 ± 6.70
IL-17	11.64 ± 3.31	14.61 ± 2.57*	8.53 ± 2.96
Eotaxin	562.87 ± 142.77**	476.90 ± 214.06**	36.70 ± 13.78
GM-CSF	105.84 ± 3.78	145.81 ± 46.72	116.40 ± 35.06
IFN-γ	51.61 ± 6.75	73.98 ± 14.38*	55.66 ± 12.57
IP-10	7021.12 ± 1642.28	8272.19 ± 3608.63#	8482.56 ± 3191.22
MCP-1	262.85 ± 50.99#	137.52 ± 44.11	309.54 ± 98.70
RANTES	246.85 ± 157.22	420.04 ± 165.34#	315.30 ± 193.99
TNF-α	11.52 ± 1.49*	19.24 ± 2.47*/ #	5.16 ± 0.88
VEGF	2277.02 ± 205.05	4328.05 ± 44.32*/ #	2251.67 ± 1142.14

of cytokines in individual samples was recalculated on their protein content.

Statistics:The results of the cytokine level assessment are presented as the mean ± S.E.M. Data were evaluated with one-way analysis of variance (ANOVA) with the post hoc Bonferroni test using GraphPad Prism software version 6.01. Findings of p≤0.05 and lower were considered to be statistically significant.

RESULTS AND DISCUSSION

A total of 10 patients, who underwent lumbar discectomy due to herniated lumbar IVD and 9 patients suffering from lower segment SL who underwent neural structure decompression, IVD replacement and transpedicular stabilization, fulfilled inclusion criteria. The difference in age between patients and controls was found not to be statistically significant (p=0.562). This study investigated the involvement of inflammatory mediators in DSD pathogenesis through the study of IVD and FJB tissues obtained from patients suffering from degenerated and herniated IVDs, lumbar SL and healthy controls during a multiorgan procurement procedure. Statistical analysis of the obtained data showed meaningful variations in the levels of several pro- and anti-inflammatory cytokines in the IVD and FJB tissue. The significantly elevated IL-1β, IL-6, IL-8, eotaxin andTNF-α were measured in NP of herniated IVDs, whereas the same structure of patients with SL produced significantly higher levels IL-6, IL-8, IL-17, eotaxin, TNF-α and vascular endothelial growth factor (VEGF). The production of anti-inflammatory interleukin-1 receptor antagonist (IL-1ra) was suppressed in both DSD (Table 1).

External AF of herniated IVDs contained significantly higher levels of IL-6, IL-8, eotaxin and chemokine monocyte chemoattractant protein-1 (MCP-1) as well. The levels of eotaxin were not changed significantly in AF samples of patients with SL, but these samples contained higher concentration of granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α and VEGF in comparison to healthy subjects (Table 2).

Previously it was proposed that DSD involves complex interactions in the immune system to mediate inflammation, remodelling and degeneration of IVD. Several studies have already reported the elevated levels of IL-1β, IL-6, IL-8, IL-17 or TNF-α in patients with DSD (Le Maitre et al., 2007; Risbud and Shapiro, 2014). Despite similar pathogenesis, the inflammatory and immune activation profile exhibited by a degenerated IVD differs from that observed in lumbar SL. Our study, for the first time, demonstrated statistically significant differences between samples of patients suffering from degenerated and herniated IVDs and from lumbar SL, as it is apparent in Table 1 and 2. These variances together suggest that pro-inflammatory changes of both NP and AF dominated in herniated IVDs, whereas the same tissue of lumbar SL patients exhibited much more complex changes in cytokine levels suggested ongoing inflammation (IL-6, IL-8, MCP-1, TNF-α), anti-inflammatory processes (IL-ra, IL-10) or remodelling (PDGF-bb, IL-17, VEGF).

Furthermore, the Bio-Plex® assay revealed significantly elevated levels of the following cytokines, chemokines and growth factors in the FJB samples of patients suffering from lumbar SL compared to the controls: IL-6 with p≤0.01; IL-7, IL-13, IFN-γ, TNF-α and PDGF-bb with p≤0.05. These finding are

Table 2. Summary of the data obtained from the Bio-Plex® assay of annulus fibrosus (AF) tissue samples. For further explanation see also legend to Table 1.

	Anulus fibrosus (AF)		
	Hernia	**Listhesis**	**Control**
PDGF-bb	17.83 ± 5.31	37.82 ± 4.43 #	41.09 ± 22.26
IL-1ra	31.16 ± 4.02	89.79 ± 4.59 #	79.54 ± 28.13
IL-6	394.52 ± 147.88**	352.94 ± 135.75**	71.44 ± 30.21
IL-8	114.79 ± 16.33*	89.20 ± 14.08*	27.41 ± 6.30
IL-10	72.48 ± 2.58	150.00 ± 92.68 #	69.17 ± 28.53
IL-17	8.82 ± 0.80	15.82 ± 8.21 #	15.92 ± 4.57
Eotaxin	298.70 ± 55.56*/ #	101.81 ± 52.14	62.61 ± 16.64
GM-CSF	135.18 ± 35.35	189.18 ± 29.47*	109.42 ± 19.64
IP-10	5744.85 ± 298.47	11744.14 ± 3026.78 #	6704.94 ± 1450.79
MCP-1	329.94.46 ± 42.65*	731.46 ± 275.68*/ #	96.43 ± 20.34
RANTES	1328.98 ± 167.72	2022.98 ± 1263.72 #	1054.33 ± 648.45
TNF-α	10.47 ± 1.37	22.04 ± 5.45 */ #	9.55 ± 1.40
VEGF	2288.78 ± 169.99	5975.74 ± 258.99*/ #	3021.25 ± 296.80

consistent with Raggatt and Partridge (2010), who observed significantly higher concentrations of IFNγ, IL-6, IL-7 and IL-13 cytokines in synovial fluid of joints with cartilage defects and osteoarthritis than in healthy donors.

CONCLUSIONS

We can summarize that the study results confirmed significant involvement of the immune system, as represented by several cytokines, chemokines and growth factors, in the pathogenesis of DSD. The control samples, which were obtained during the multiorgan procurement procedures, represent the main strength of this study. This method should avoid any false findings due to cytokine level changes caused by cytokine degradation after the cessation of breathing, blood circulation and death. The main limitation of this study is the gender of the subjects included in the groups: only male subjects were included in control and herniated IVDs groups, while most of the lumbar SL patients were female. Only 2 female patients were of reproductive age, therefore there is low possibility that sex hormones interfere with cytokine levels. However, there is still much to be learned about the interactions among and the influence of many components that regulate the immune cascade in DSD. We believe that improving the knowledge and understanding of immune system participation in this disease could improve future therapeutic strategies.

ACKNOWLEDGEMENTS

This study was supported by the 'BioMed' project, which is co-financed by EC sources and VEGA grant No. 1/0163/16.

References

[1] Franova S, Kazimierova I, Pappova L, Joskova M, Plank L, Sutovska M. Bronchodilatory, antitussive and anti-inflammatory effect of morin in the setting of experimentally induced allergic asthma. Journal of Pharmacy and Pharmacology. 2016;68(8):1064-1072.

[2] Friebe A, Volk HD. Stability of tumor necrosis factor alpha, interleukin 6, and interleukin 8 in blood samples of patients with systemic immune activation. Arch Pathol Lab Med. 2008;132:1802-1806.

[3] Lee AS, Ellman MB, Yan D, Kroin JS, Cole BJ, van Wijnen AJ, Im HJ. A current review of molecular mechanisms regarding osteoarthritis and pain. Gene 203;527:440–447.

[4] Le Maitre CL, Hoyland JA, Freemont AJ. Catabolic cytokine expression in degenerate and herniated human intervertebral discs: IL-1ßand TNFα expression profile. Arthritis Res Ther. 2007; 9:R77.

[5] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193:265—275.

[6] Modic MT, Ross JS. Lumbar degenerative disk disease. Radiology 2007; 245:43-61.

[7] Raggatt LJ, Partridge NC. Cellular and molecular mechanisms of bone remodeling. J Biol Chem. 2010;285:25103-25108.

[8] Risbud MV, Shapiro IM. Role of cytokines in intervertebral disc degeneration: pain and disc content. Nat Rev Rheumatol 2014;10:44-56.

[9] Wuertz K, Haglund L. Inflammatory Mediators in Intervertebral Disk Degeneration and Discogenic Pain. Global Spine 2013;J 3:175–184.

DE GRUYTER OPEN

Eur. Pharm. J. 2017, 64 (1): 30.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.1515/afpuc-2017-0011

EUROPEAN PHARMACEUTICAL JOURNAL

Retraction Note

Retraction of: Itoandon EE, Nwachukwu VA, Erukainure OL, Lasore OO, Nwagala PN : Cola millenii Leaf Ethyl Acetate Extract: Fourier Transform Infrared (FTIR) Spectroscopy; Modulatory Effect on Serum Indices and Redox Biomarkers Eur. Pharm. J. LXIII, 2016 (2): 7-11 (DOI 10.1515/afpuc-2016-0016)
10.1515/afpuc-2017-0024
The published data emanated from the undergraduate research of Miss Abisola Onaduja with matric number RUN 0910/2524, (http//run.edu.ng/convocation/index.php?active=graduate&year=2013&start=401),co-supervised by Prof Osho of the Microbiology department and Dr C.A. Otuechere (Department of Chemical Sciences, Redeemer's University, Ede, Osun State Nigeria). There were serious problems in ethic principles about authorship and uncertainity about completness of the authors list.