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Changes in histological structure and NOS expression in aorta of rats supplemented with bee pollen or whey protein

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Changes in histological structure and NOS expression in aorta of rats supplemented with bee pollen or whey protein.

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Introduction. Various protein-based supplements are at least periodically consumed by 30-40% of sportspeople. The current study compares cardiovascular effects of diet supplementation with two different protein-rich products – bee pollen and whey proteins.

Material and methods. 30 Wistar rats were divided into two parts, one subjected to daily moderate physical activity and one not. Each part consisted of three groups: one control group, one whey-protein-supplemented and one bee-pollen-supplemented. After eight weeks rats were decapitated and proximal parts of thoracic aortas were collected, and embed in paraffin blocks. Histological slides were stained according to standard H&E, Masson's trichrome and Verhoeff-Van Gieson stainings. Special immunohistochemical stains against nNOS, eNOS and α -SMA were also prepared.

Results. Histological evaluation has revealed noticeable changes in all supplemented groups – disturbances in elastic laminae, slight increase in collagen deposition and significantly lowered nNOS and eNOS expression. The prevalence of micro atherosclerotic plaques has been the highest in not-running supplemented groups, while in running supplemented groups it has resembled the prevalence in control groups. Both running groups have had also thinner *tunica media* than control.

Conclusions. Both supplements exerts visible effects on aortic structure, but the difference between them is far less evident. In some aspects, however; the bee pollen seems to be even slightly more harmful which may be probably related to various possible contaminants like mycotoxins or pesticides.

Keywords: eNOS, NOS3, nNOS, NOS1, aorta, rat, bee pollen, whey protein, moderate physical activity.

Introduction

Sportspeople supplementation

More than 40% of Polish athletes admitted to at least periodical consumption of various protein-based supplements according to Frączek B. et al. (Frączek et al. 2016). In a 2012 study on a group of various sportspeople from Poland about one third of them have declared using protein-based diet supplements (Frączek et al. 2012).

Bee pollen

Bee pollen is one of bee products composed of flower pollen and bee saliva or nectar. It is a rich source of nutrients, including essential amino acids, unsaturated fatty acids and numerous vitamins (de Arruda et al. 2013; Yang et al. 2013; Ares et al. 2018; Kocot et al. 2018). Bee pollen exhibits various biological effects e.g. inhibitory activity against lipoxygenase or lipase and has potent antioxidant activity, mainly due to high flavonoid and polyphenol content (Araújo et al. 2017). On the other hand bee pollen may contain also residues of various pesticides (Böhme et al. 2018; Calatayud-Vernich et al. 2018). According to a recent studies by Rzepecka-Stojko A. et al., the polyphenol-rich ethanol extract of bee pollen has potent anti-atherogenic activity (Rzepecka-Stojko et al. 2017, 2018).

Whey protein

Whey protein is a known and effective diet supplement in both aerobic (Huang et al. 2017) and resistance training (Mori and Tokuda 2018). Whey protein may possibly have anti-oxidative properties (Elia et al. 2006). Despite high methionine content it most probably does not cause an increase in serum homocysteine level (Deminice et al. 2015). A recent study has shown that whey protein may also exert anti-atherogenic (Zhang et al. 2018) and anti-hypertensive activity (Figuerola et al. 2014).

Hypothesis and aim of the study

The aim of the current study was to evaluate the cardiovascular safety of bee-pollen supplementation in comparison to whey protein concentrate in both running and not-running setting. The main hypothesis of the study was that bee pollen is a safer alternative to whey protein concentrate supplementation.

Material and methods

The study protocol

The current study involved 30 young, male Wistar rats. At the onset of the experiment they were approximately 8-weeks old with the body mass of approximately 330 grams at the beginning and 400g at the end of the experiment. They were divided into six groups as shown in Table 1. During 8 weeks of the experimental phase all rats in running groups were running 5 times a week, 5 minutes each time, with the average velocity of 6km/h, no electrical shock was needed. Each rat received water and standard rodent food *ad libitum*, supplemented groups had also unlimited access to either bee pollen or whey protein concentrate. The consumption of water, standard food and supplements per cage was measured on a daily basis. Each rat had its blood pressure measured once a week by means of LE-5001 (Panlab Harvard Apparatus, Cornella, Spain) with LE5160R cuff (Panlab Harvard Apparatus). The standard tail cuff method was used. After 8 weeks all rats were decapitated on the same day. The study protocol has been approved by the Bioethical Committee at the Medical University of Lublin (No. 24/2015). Animals were cared for in accordance with Guide for the Care and Use of Laboratory Animals (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals 2011).

Supplements

The current study utilised bee pollen from the vicinity of Lublin. An enriched whey protein

concentrate (Olimp Laboratories Sp. z o.o., Dębica, Poland) was used, 100g of this supplement contained: 77g protein, 6g carbohydrates and 7g lipids. Total methionine content: 0.8g per 100g of whey protein. On average 100g of bee pollen contains approximately 23g of protein, 31g of carbohydrates, 5g of lipids and total of about 0.8g of vitamins (A, E, D, B1, B2, B3, B5, B6, B7, C) (Komosinska-Vassev et al. 2015). An average of 0.3g of methionine can be found in 100g of bee pollen collected in Poland (Szczęsna 2006).

Slides preparation

After decapitation the proximal parts of thoracic aortas were collected and fixed in formalin, and afterwards embed in paraffin blocks. Five μm thick slides were prepared and stained according to standard H&E, Masson's trichrome and Verhoeff-Van Gieson stainings. Additional slides underwent immunohistochemical staining. Monoclonal antibodies against α -SMA (Abcam, Cambridge, UK), NOS1/nNOS (Elabscience, USA) and NOS3/eNOS (Elabscience, USA) were used. Antigenic sites were exposed with Proteinase K (Sigma-Aldrich, Saint Louis, USA) for 5 minutes. Endogenous peroxidase activity was blocked by 0.3% solution of perhydrol in methanol. Non-specific binding was prevented by the addition of normal serum. The primary antibody was diluted as proposed by the manufacturer. The material was incubated with primary antibody for 60 minutes and afterwards for another 30 minutes with HRP-conjugated secondary antibody. The reaction was visualised with diaminobenzidine, hematoxylin was used to counter-stain nuclei.

Evaluation and analysis

Slides were evaluated under light microscope. All measurements were performed with Olympus-BX4 and CellSens Software. The mean arterial blood pressure was calculated with the equation: $\text{MBP} = \text{DBP} + [(\text{SBP}-\text{DBP})/3]$, MBP (mean blood pressure), DBP (diastolic blood pressure), SBP (systolic blood pressure). The extent of fibrosis was measured with Fiji (Schindelin et al. 2012) by colour deconvolution and subsequent optical density calculation. Background

subtraction was used before colour deconvolution to provide neutral background. The statistical analysis was performed with Statistica 12 (StatSoft, USA). Kruskal-Wallis test was used to calculate statistical significance. The effect size was calculated with η^2 as proposed by Tomczak M. & Tomczak E. for Kruskal-Wallis test (Tomczak and Tomczak 2014): $\eta^2 = (H - k + 1) / (n - k)$, where k is the number of groups and n is the total number of observations.

Results

Daily supplement consumption

The mean daily whey protein concentrate consumption was approximately 5.18g (1.33g per 100g body weight) for not-running and 5.19g (1.59g per 100g body weight) for running rat, which contained an average of 41.47mg and 41.55mg of methionine respectively. Simultaneously, the average bee pollen consumption was 13.45g (3.58g per 100g body weight) per rat in the not-running and 11.96g (3.35g per 100g body weight) per rat in the running group, it corresponded to the mean methionine consumption of 40.36mg and 35.88mg respectively. The mean food and water consumption, total caloric intake and mean rat weight is presented in Supplementary Table S1.

Blood pressure

No significant differences in MBP, DBP and SBP were noted between groups. The difference between not-running and running groups are, however; easily noticeable and statistically significant in case of SBP with lower value observed in non-running animals (Table 2).

Aortic endothelium

Visual analysis of endothelium revealed significant differences between groups (Fig 1). Only one case of atherosclerotic plaque was noted in both control groups, while five cases in not-running supplemented groups (3 in whey-protein-supplemented and 2 in bee pollen receiving group). Both running supplemented groups had an endothelium similar to control group and only

one case of atherosclerosis was noted among them. Nevertheless, the thickest endothelium was observed in both running supplemented groups (Table 3).

Elastic laminae

Elastic laminae were assessed in both the H&E and Verhoeff-Van Gieson staining. Elastic laminae in both control groups were regularly arranged and nearly no disruptions were observed. On the contrary, several disruptions and slight irregularity in arrangement was seen in all experimental groups with no noticeable differences between them (Fig 2). Elastic laminae were also counted in *tunica media* and their thickness was measured. The mean number of laminae differed – running bee-pollen-supplemented group had the lowest number (Table 3). Not-running whey-protein-supplemented group had slightly thinner while the remaining three experimental groups had slightly thicker elastic laminae than both control groups (Table 3). At last, the thickness of the whole *tunica media* was measured. Lower values were noted in both running supplemented groups than in running control. Among the not-running animals the whey-protein supplemented group exhibited lower values and the bee-pollen-supplemented group higher values than the not-running control (Table 3).

Smooth muscles

An immunohistochemical staining against α -SMA (smooth muscle actin) was performed for the assessment of smooth muscle cells. The intensity of staining was slightly higher in control groups than in any of the experimental groups, but the regularity of fibres was mostly preserved with only slight variations in experimental groups. Nevertheless, perinuclear halo, round nuclei and signs of smooth muscle degeneration were easily noticeable in experimental groups. The highest intensity of those changes was observed in both bee-pollen supplemented groups (Fig. 3). The nuclei in experimental groups tended to be less elongated than in control groups.

Fibrosis

The extent of fibrosis was assessed by both the traditional visual analysis and computed analysis with calculation of optical density score (Table 3). The results of both methods were coherent. All not-running groups exhibited similar rate of collagen deposition, but there is a significant difference between both the non-running and running and between running-control and both running-experimental groups. Both the bee-pollen- and whey-protein-supplemented running groups exhibited significant fibrosis with easily noticeable numerous collagen fibers interconnecting the *elastic laminae* (Fig. 4).

nNOS(NOS1) and eNOS(NOS3)

For assessment of the endothelial function two independent immunohistochemical stainings were performed – one against eNOS and another against nNOS. The intensity of anti-eNOS reaction in both running experimental groups was similar to that of control, while in non-running supplemented groups it was slightly lower (Fig. 5). nNOS endothelial expression was lower in both not-running experimental groups while similar to control in both running experimental groups. On the other hand, nNOS expression in smooth muscle in whey-protein-supplemented non-running group was similar to control, the remaining three experimental groups exhibited lower expression than control one (Fig. 6).

Tunica advenitia

The tunica advenitia was poorly preserved in numerous specimen, therefore, no firm conclusions can be drawn. Nevertheless, the remaining parts of *tunica advenitia* had shown no important differences between groups.

Discussion

The initial evaluation has revealed noticeable changes in aortic structure in all supplemented

groups, most notably the increased collagen deposition in *tunica media* and disturbed architecture of elastic laminae. The IHC stains have showed subsequent significant differences, most notably in the expression of eNOS and nNOS. Most probably those changes in both nitric oxide synthases is the reason for observed alternation in aortic architecture – this is schematically depicted in Fig. 7.

Both bee pollen and whey protein concentrate slightly lowers eNOS and nNOS expression in aorta. Although bee pollen is a rich source of antioxidants, including flavonoids and polyphenols, it seems to exert no positive influence over eNOS or nNOS in rat aorta. Pomegranate is an example of remarkably well tested polyphenol-rich antioxidant which is known to significantly improve vascular endothelial function (Schmitt and Dirsch 2009). Its action, however, is based purely on NO-protection against oxidative factors and it does not cause increase in eNOS expression or catalytic activity (Ignarro et al. 2006). An influence of red wine polyphenols on eNOS expression is a partial explanation to the current observations. Wallerath et al. have demonstrated that different polyphenols and other compounds of red wine exert different effects on eNOS expression and activity (Wallerath et al. 2005). Therefore, a potential future study of bee-pollen influence on the aortic tissue should also include blood NO measurement to assess NOS activity and also in-depth study of composition of bee pollen used.

Combined results of the current study indicate that even slight decrease of eNOS and nNOS expression in both whey-protein- and bee-pollen-supplemented non-running groups may lower plasma NO levels as signs of atherosclerosis have been slightly more evident in those groups than in control. The inflammatory conditions of the atherosclerotic plaque are known to decrease NO levels by the means of oxidation by reactive oxygen species (Napoli et al. 2006). On the other hand high-protein diet exerts a proatherogenic effect (Meeker and Kesten 1941; Kostogrys et al. 2015). This effect can be attenuated by moderate physical activity, which is proved by lowered rate of atherosclerosis in running supplemented groups in comparison to non-running supplemented groups. This is in line with the results of previous studies (Jakic et al. 2018).

Moderate physical activity has been found to improve the endothelial function by increasing

the activity of eNOS and thus the NO levels (Tanaka et al. 2015). This is coherent with the current study results as similar expression of eNOS and nNOS has been observed in control and experimental running animals while lowered in non-running experimental groups.

The degree of changes in smooth muscle as shown by IHC against α -SMA seems to be coherent with the expression of nNOS. The lower the nNOS expression in smooth muscle cells is the more pronounced the changes are. This may be related to the role of NO in regulation of vascular smooth muscle proliferation (Chen et al. 2011). It is worth to notice the nearly total lack of nNOS expression in *tunica media* in both running supplemented groups and in bee pollen supplemented not-running group. As has been observed in control groups and also previously reported (Brophy et al. 2000), there should be a noticeable expression of nNOS in vascular smooth muscle cells.

Observed changes in eNOS and nNOS expression may be related to methionine intake and homocysteine level. The mean daily dietary intake of methionine has been similar for both bee-pollen and whey-protein supplemented groups irrespective of their physical activity. It has been proved that high methionine consumption even without increased serum homocysteine level may significantly promote atherosclerosis in susceptible mice (Selhub and Troen 2016). Moreover, methionine-rich diet may significantly increase serum homocysteine level (Ditscheid et al. 2005; Pexa et al. 2008; Holstein et al. 2012). However, a recent study has showed that such diet in rats does not change plasma homocysteine level (Deminice et al. 2015). Nevertheless, homocysteine has a significant potential to induce the oxidative stress through blocking of NOS activity (Topal et al. 2004; Zhang et al. 2007; Liu et al. 2012). Methionine-rich diet has also been proved to induce media thickening, collagen deposition and elastic laminae disorganisation (Kirac et al. 2013). This is similar to the results of the current study and, therefore; further supports the methionine-homocysteine-hypothesis. B6, B12 and folic acid supplementation has been showed to lower the serum homocysteine level (DiFrancisco-Donoghue et al. 2012; Zappacosta et al. 2013). It is, therefore; probable that increased serum methionine level is itself capable of lowering NOS activity,

but this requires further studies. A novel in-depth study with direct measurement of homocysteine and methionine levels seems necessary.

Daily exercise has been found to promote eNOS expression and alleviate negative changes in hypertensive rats (Moraes-Teixeira et al. 2010). Physical activity also promotes NO activity in ovariectomized rats (Lee et al. 2012) and eNOS and iNOS mRNA expression in aorta (Yang et al. 2002). This is in line with the results of the current study as in both running experimental groups the expression of eNOS and nNOS is similar to control group while it is lower in supplemented not-running groups. There is, however, a possibility that this effect is mediated by exercise-induced stress as proposed by Chies AB et al., who reported increase in NO activity as a result of swimming-related stress in rats (Chies et al. 2003). Nevertheless, physical activity seems to alleviate or nearly completely eliminate the negative impact of bee pollen or whey protein supplementation. This suggests that regular physical activity may possibly partially protect from negative impact of protein supplementation on the structure of aorta.

The daily consumption of whey protein by sportspeople varies, but a dose of approximately 2g per 1kg body weight is not uncommon (Naclerio and Larumbe-Zabala 2016). This corresponds to rat doses of 1.24g per 100g rat body weight (Nair and Jacob 2016). The latter is similar to the doses used in the current study. Similar doses of whey protein have also been used previously by Roberts et al., Aparicio et al. and El-Desouky et al. (Roberts et al. 2014; Aparicio et al. 2014; El-Desouky et al. 2017). The mean concentration of protein is approximately 3-4 times lower in bee pollen than in whey protein concentrate, thus the dosage of bee pollen used in the current study resembles those of whey protein concentrate in terms of protein consumption.

To the best knowledge of the authors, current study is the first evaluation of bee pollen supplementation on the structure of aorta.

Conclusions

The initial hypothesis has been proved to be wrong as the extent of histological changes has

been similar in both whey protein and bee pollen groups. Both supplements exerts visible effects on aortic structure, but the difference between them is far less evident. In some aspects, however; the bee pollen seems to be even slightly more harmful which may be probably related to various possible contaminants like mycotoxins or pesticides. Both supplements have caused slight vascular remodeling.

Conflict of interests: The authors have no conflicts of interest to report.

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Fig 1. The tunica intima and tunica media. **A.** Not-running control group, visible small atherosclerotic plaque (curved arrow), endothelium is thin and smooth. **B.** Running control group, thin and smooth endothelium is visible. **C.** Not-running whey-protein-supplemented and **D.** not-running, bee-pollen-supplemented, small atherosclerotic plaques (curved arrows) and binucleated smooth muscle cells (arrow heads) visible. **E.** Running whey-protein-supplemented and **F.** running, bee-pollen-supplemented, binucleated smooth muscle cells (arrow heads) and small atherosclerotic plaque (curved arrow) visible. H&E, 400x magnification.

Fig 2. Elastic laminae by groups. Not-running control group (**A**) and running control group (**B**) showing correct structure of elastic laminae. Irregularity and small disruptions (arrow) can be noted in all experimental groups: not-running whey-protein-supplemented group (**C**), not-running bee-pollen-supplemented group (**D**), running whey-protein-supplemented group (**E**) and running bee-pollen-supplemented group (**F**). Verhoeff-Van Gieson staining. 400X magnification

Fig 3. Immunohistochemical staining against α -SMA. Not-running control group (**A**) and running control group (**B**) exhibits proper structure of smooth muscle cells. Not-running whey-protein-supplemented group (**C**) and running whey-protein-supplemented group (**E**) show slight changes with limited focal changes – degeneration (curved arrow), perinuclear halo (thin arrow) and round nuclei (arrowhead). not-running bee-pollen-supplemented group (**D**) and running bee-pollen-supplemented group (**F**) shows clearly visible intensive changes – numerous round nuclei (arrowhead) and well visible signs of degeneration (curved arrows) and some perinuclear halo (thin arrow). IHC staining. 400X magnification.

Fig 4. The extent of fibrosis by groups. Asteriks (*) marks the lumen of the aorta. Not-running and (**A**) running control groups (**B**) show correct amount of collagen. Not-running whey-protein-supplemented group (**C**) and not-running bee-pollen-supplemented group (**D**) present no significant differences in comparison to the control groups. Running whey-protein-supplemented group (**E**) running bee-pollen-supplemented group (**F**) presents high intensity of collagen staining, multiple thin collagen fibres interconnects the elastic laminae, creating a dense net of fibres. Masson's

trichrome staining. 400X magnification.

Fig 5. The anti-eNOS (NOS3) stain. Asteriks (*) marks the lumen of the aorta. Not-running (**A**) and running control (**B**) groups show easily noticeable eNOS expression in the endothelium. Not-running whey-protein-supplemented group (**C**), not-running bee-pollen-supplemented group (**D**), running whey-protein-supplemented group (**E**) and running bee-pollen-supplemented group (**F**) exhibit lowered expression of eNOS in endothelium. IHC staining. 400X magnification.

Fig 6. The anti-nNOS (NOS1) stain. Asteriks (*) marks the lumen of the aorta. Not-running (**A**) and running (**B**) control groups exhibit nNOS expression in endothelium and to a lesser extent also in smooth muscle cells. Not-running whey-protein-supplemented group (**C**) shows slightly lowered endothelial expression and preserved smooth muscle expression. Not-running bee-pollen-supplemented group (**D**), running whey-protein-supplemented group (**E**) and running bee-pollen-supplemented group (**F**) show significantly lowered nNOS expression in smooth muscle cells. IHC staining. 400X magnification.

Fig. 7. Schematic representation of probable interactions between bee pollen and/or whey protein, NOS and aortic smooth muscle. Thick solid lines represent positive influence on health while thin solid lines negative. Dotted lines represent unknown nature of the interactions due to complicated chemical formula of bee pollen. Based on the results of the current study, one may assume that bee pollen exerts negative influence on both eNOS and nNOS. Protein-rich diet is representative for both bee pollen and whey protein.

Table 1. Groups of rats used in the experiment.

Group	Con-Sed	Con-Run	WP-Sed	BP-Sed	WP-Run	BP-Run
Running	no	yes	no	no	yes	yes
Supplementa tion	no	no	Whey protein	Bee pollen	Whey protein	Bee pollen
No. of rats	5	5	5	5	5	5

Group naming: Con = control, WP= whey protein, BP = bee pollen, Run = running, Sed = sedentary

Table 2. Systolic (SBP), diastolic (DBP) and mean blood (MBP) pressure by groups

Group	Con-Sed	Con-Run	WP-Sed	BP-Sed	WP-Run	BP-Run	P value ^A
DBP	114±27	121±44	112±37	111±29	121±43	121±42	0.6389
SBP	171±36	182±44	164±47	167±39	178±48	177±36	0.2297
MBP	133±38	141±57	130±41	130±41	140±53	140±55	0.8362
Group	Not running		Running		P value ^A		
DBP	112±31		121±43		0.1974		
SBP	167±41		179±46		0.0252		
MBP	131±43		140±55		0.3163		

^A – Kruskal-Wallis test

Group naming: Con = control, WP= whey protein, BP = bee pollen, Run = running, Sed = sedentary

P values presents the total p value. The p values for multiple comparisons greater than 0.05. The η^2 for all comparisons is lower than 0.02

Table 3. Measurement values by groups.

Group	Con-Sed (1)	Con-Run (2)	WP-Sed (3)	BP-Sed (4)	WP-Run (5)	BP-Run (6)	p	η^2
Elastic laminae number	8.64±1.65	9.67±1.98	8.50±0.94	8.56±0.78	8.67±0.52	7.88±0.64	0.0312 3vs5: 0.047	0.082
Elastic laminae thickness	2.57±0.65 μm	2.80±0.66 μm	2.32±0.52 μm	2.94±0.80 μm	2.94±0.53 μm	2.92±0.53 μm	<0.00001 1vs2: 0.036 1vs5: 0.047 1vs6: 0.040 2vs4: 0.00001 3vs4: 0.0005 4vs5: 0.00005 4vs6: 0.00004	0.087
Endotheliu m thickness	0.56±0.10 μm	0.56±0.12 μm	0.66±0.15 μm	0.61±0.12 μm	0.76±0.25 μm	0.71±0.20 μm	<0.00001 1vs6: 0.019 2vs3: 0.023 2vs5: 0.0009 2vs6: 0.00007	0.146
Tunica media thickness	97.01±25.1 7 μm	111.05±19. 11 μm	93.12±9.69 μm	105.38±10. 56 μm	93.23±6.87 μm	98.76±26.2 3 μm	<0.00001 1vs2: 0.011 1vs3: 0.0009 2vs4: 0.033 3vs4: 0.005 3vs5: 0.019	0.146
Masson's trichrome optical density ^A	0.11±0.03	0.12±0.04	0.10±0.02	0.14±0.05	0.19±0.04	0.20±0.06	<0.00001 1vs5: 0.0002 1vs6: 0.0005 2vs5: 0.0003 2vs6: 0.0007 3vs5: 0.000004 3vs6: 0.00001	0.362

^A – optical density is here a measurement of the intensity of fibrosis, the higher the values the more intense the fibrosis is.

Group naming: Con = control, WP= whey protein, BP = bee pollen, Run = running, Sed = sedentary
P values presents both the total p value and the values for multiple comparisons where $p < 0.05$

304x152mm (300 x 300 DPI)

304x152mm (300 x 300 DPI)

304x152mm (300 x 300 DPI)

304x152mm (300 x 300 DPI)

304x152mm (300 x 300 DPI)

304x152mm (300 x 300 DPI)

256x228mm (300 x 300 DPI)