THE EFFECTS OF VITAMIN D DEFICIENCY ON RENAL AND CAROTID ARTERIES

Ph.D. thesis

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Budapest, 2021

1. Introduction

Recently one of the most significant health problems is cardiovascular (CV) morbidity and mortality, which is still the leading cause of death worldwide. The different types of arteries are the main contributors to the pathogenesis and severity of CV disease, of which the most relevant are the renal arteries that play a role in the pathomechanism of hypertension and the carotid arteries, which are involved in the development of stroke. It has been known for a long time that there is a significant difference in the development and severity of cardiovascular disease between men and women to the benefit of women that disappears after menopause. Furthermore, Vitamin D deficiency is common, but it is also involved in the development and severity of many diseases, while its supplementation is beneficial for most of our organs. Gender differences in the effects of Vitamin D are also supposed.

Functional adaptation and gender differences in renal and carotid arterial rings in a rat model of Vitamin D deficiency and supplementation have not been studied in details yet. Therefore, we aimed to explore the adaptations of contraction and relaxation of such arteries to Vitamin D deficiency and supplementation in male and female rats.

2. Objectives

Vitamin D deficiency is a significant public health problem, associated with hypertension and cardiovascular target organ damages. Gender difference in cardiovascular risk between men and women has been known for a long time, clearly due to the protective effect of estrogen. Furthermore, also sex difference can be found in the effect of Vitamin D supplementation and deficiency on cardiovascular events. This raises the question whether there are gender differences in the effects of Vitamin D deficiency and supplementation on the function of two key blood vessels, the renal and carotid arteries

in the terms of cardiovascular diseases. To find the answer for the following questions we used a Vitamin D deficient and Vitamin D supplemented rat model:

1) Are there any sex related differences in the function of renal and carotid artery of Vitamin D supplemented young adult male and female rats?
2) Are these characteristics changing by Vitamin D deficiency?
3) Are there gender differences in Vitamin D deficiency induced changes?

3. Methods

3.1. Animals

The study was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8^{th} edition, 2011) and the EU conform Hungarian Law on Animal Care (XXVIII/1998). All procedures and handling of the animals during the study were approved by the Animal Care Committee of Semmelweis University as well as by state authorities (IRB: 8/2014 PEI/001/1548-3/2014, PEI/001/820-2/2015). The animals had access to tap water ad libitum. Rats were houses in a room with constant temperature (22 ± 1°C) with a 12-hour light-dark cycle. 48 (21-28 day old) male and female Wistar rats were delivered to the Animal Facility of Semmelweis University in agreement with Charles River (Charles River LTd., AnimaLab, Vác, Hungary). After one week of acclimatization, rats were randomly divided into four groups: male Vitamin D supplemented groups (MD+), male Vitamin D deficient group (MD-), female Vitamin D supplemented group (FD+), female Vitamin D deficient group (FD-), N=11-13 in each group.

3.2. Chronic Treatment of the Rats

In order to induce vitamin D deficiency via reduced intake, rats in the corresponding groups, were fed ad libitum with vitamin D Free Lab rat/mouse chow (Ssniff Spezialdiaten GmbH, Soest, Germany) containing less than 5 IU/kg vitamin D for eight weeks resulting in vitamin D deficiency (below 10 ng/ml). Rats in vitamin D supplemented groups were fed ad libitum with a normal chow containing 1000 IU/kg of vitamin D. Furthermore, oral administration of additional vitamin D through a gavage cannula were applied to ensure the targeted plasma vitamin D levels; 500 IU cholecalciferol on the second week and a weekly maintenance dose of 140 IU/100g on the fourth, fifth, sixth and seventh weeks (Vigantol (cholecalciferol) 20000 IU/ml, Merck/Merck Serono, Darmstadt, Germany).

3.3. Myography

After 8 weeks, rats were anesthetized with Nembutal (45 mg/kg intraperitoneal (i.p.)), perfused with heparinized nKR solution for 2 minutes. Renal and carotid arterial segments were cut into 5-5 equal rings (2mm long), 4-4 of which were placed on a conventional wire myograph setup (610-M MultiMyograph System; Danish Myo Technology), while the 5th-5th vascular rings was fixed in formaldehyde and embedded in paraffin (N=4-6 in each group).

Conventional wire myograph system was used to measure the isometric tension of isolated renal and carotid arterial rings. The organ chambers were filled with 8 mL nKR and kept at 37°C. The 15 mN pre-tension was reached progressively. After the development of stable pre-tension, 124 mmol/L K+ was applied (3 min) to test the contractility of the vessels and to serve as the reference value for contraction force.

Renal arteries protocol:

Vascular rings were equilibrated in nKR and cumulative doses of phenylephrine (Phe), which is an α1-adrenergic receptor

agonist (10^{-9}–10^{-6} mol/L) or angiotensin II (10^{-9}–10^{-7} mol/L) was administered to induce contraction. Acetylcholine (Ach) induced vasodilation was examined after Phe precontraction (10^{-6} mol/L) by incubating the vessels with increasing doses of Ach (10^{-9}–10^{-6} mol/L). Phe-induced contraction and Ach-induced vasodilation was also examined after 30 min incubation with the cyclooxygenase-2 (COX-2) inhibitor NS398 (10^{-5} mol/L) or the COX inhibitor indomethacin (10^{-4} mol/L), or their vehicle dimethyl-sulfoxide (DMSO).

Carotid arteries protocol:

Acetylcholine-induced vasodilation was examined by incubating the vessels with increasing does of Ach (10^{-9}–10^{-6} mol/L), subsequent to Phe-precontraction (10^{-6} mol/L). Measurement of Ach-induced vasodilation was repeated in the presence of the nitric oxide synthase inhibitor N(G)-Nitro-L-arginine methyl ester (L-NAME) (10^{-4} mol/L), and the general COX inhibitor indomethacin (10^{-5} mol/L), or DMSO.

3.4. Immunohistochemistry

Renal arteries

Paraffin-embedded tissue sections were stained with hematoxylin-eosin (HE) and resorcin-fuchsin (RF). Immunohistochemistry was performed against α-smooth muscle actin (α-SMA), endothelial nitric oxide synthase (eNOS) and angiotensin II receptor-1 (AT1R). After deparaffinization antigen retrieval was performed by heating the slides in citrate buffer (pH = 6). Endogenous peroxidase activity was blocked by 3% H2O2 in dH2O. 2.5% normal horse serum (Vector Biolabs, Burlingame, CA, USA) was used to avoid non-specific labeling. Primary antibodies (-SMA: 1:10,000; eNOS: 1:1000, (Abcam, Cambridge, UK), AT1R: 1:500 (Sigma-Aldrich, St. Louis, MI, USA)) were applied overnight at 4°C Horseradish peroxidase-linked anti-mouse or anti-rabbit polyclonal horse antibody (Vector Biolabs, Burlingame, CA, USA) was used for secondary labeling.

Brown colored diamino-benzidine (DAB) was used for the visualization of specific labeling (Vector Biolabs, Burlingame, CA, USA). Blue colored hematoxylin served as counterstaining (Vector Biolabs, Birmingham, CA, USA).

Carotid arteries

Immunohistochemistry was performed labeling endothelial nitric oxide synthase (eNOS). Antigen retrieval was performed by heating the slides in citrate buffer (pH=6) following deparaffinization. 3% H_2O_2 in dH2O was applied to block endogenous peroxidase activity. Non-specific labeling was prevented via utilization of 2.5% normal horse serum (Vector Biolabs, Burlingame, CA, USA). Primary antibodies (eNOS: 1:50 (Abcam, Cambridge, UK)) were applied overnight at 4°C. In case of eNOS horseradish-peroxidase-linked anti-mouse (Vector Biolabs, Burlingame, CA, USA) was used for secondary labeling. Visualization of specific labeling was accomplished by brown-colored diamino-benzidine (DAB) (Vector Biolabs, Burlingame, CA, USA), while blue-colored hematoxylin served as counterstaining (Vector Biolabs, Birmingham, CA, USA).

Light microscopy images were taken with Nikon ECLIPSE NI-U microscope and Nikon DS-Ri2 camera (Nikon, Minato City, Tokyo, Japan). The intima-media ratio of the vessels was calculated based on the measurement of intimal and media areas using the resorcin-fuchsin stained sections by ImageJ software (National Institutes of Health (NIH), Bethesda, MA, USA). The number of smooth muscle cell nuclei in the media layer of arteries was measured and their density per 1000 μm^2 was calculated. In order to assess the density of elastic fibers, the non-calibrated optical density of the media layer of resorcin-fuchsin stained vessels was assessed. In case of immunohistochemical labeling, non-calibrated optical density

of specific staining was measured in the intima or media layers of the vessel walls using the ImageJ software.

3.5. Statistical evaluation

Vascular function curves were analyzed by repeated measures two-way ANOVA using Bonferroni's post hoc test by Prism 8 (GraphPad Software, San Diego, CA, USA). At certain agonist concentration, vascular reactivity was analyzed by two-way ANOVA (gender, Vitamin D status). Histological evaluations of the experimental groups were compared using Kruskal-Wallis test with Dunn's multiple comparison test. $p < 0.05$ was uniformly accepted as the threshold for statistical significance.

4. Results

4.1. Vascular Function of Renal Arteries - Contraction

Vitamin D deficient female rats showed significantly increased contraction at Phe concentration of 10^{-7} mol/L compared to their Vitamin D supplemented female counterparts. Furthermore, Vitamin D deficient female rats showed significantly increased contraction compared to male groups – independently of Vitamin D status ($p < 0.001$ FD+ vs. FD−; $p<0.001$ FD− vs. MD+; $p < 0.001$ FD− vs. MD− . Repeated measures two-way ANOVA with Bonferroni's post hoc test, figure is not shown).

In case of Vitamin supplementation in females, both general COX, and specific COX-2 inhibition led to decreased contraction that was more pronounced in case of specific COX-2 inhibition. However, in case of Vitamin D deficiency in females, both indomethacin and NS398 pretreatment resulted in reduced contraction force **(Fig. 1)**. In case of Vitamin D supplementation in males, similarly to female Vitamin D supplemented group, both general COX, and specific COX-2 inhibition led to reduced contraction with a more pronounced effect of NS398. In male Vitamin deficient experimental group, both indomethacin and NS398 caused

reduced contraction with a significantly bigger inhibition by COX-2 blocker **(Fig. 2)**. In case of Angiotensin II-induced contraction, Vitamin D deficient male and female rats showed significantly decreased contraction at angiotensin concentration of 10^{-7} mol/L compared to Vitamin D supplemented males ($p < 0.05$ FD−vs. MD+; $p < 0.05$ MD+ vs. MD−. Repeated measures two-way ANOVA with Bonferroni's post hoc test, figure is not shown).

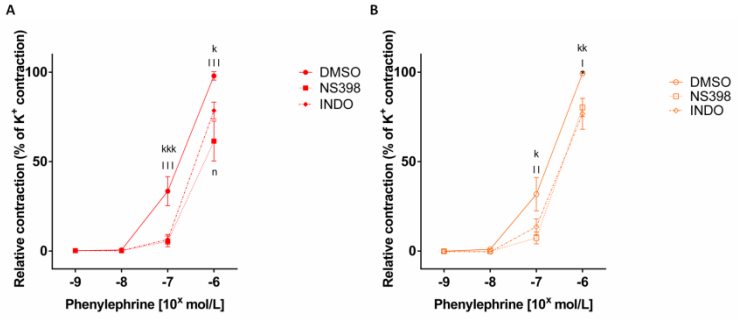

Figure 1. Phenylephrine (Phe) induced contraction

A) Female Vitamin D supplemented group; B) Female Vitamin deficient group. Phe-induced contraction in the presence of COX-2 inhibitor (NS398) or general COX inhibitor (indomethacin; INDO), or their vehicle DMSO. Repeated measures two-way ANOVA with Bonferroni's post hoc test. Data are shown as mean ± SEM; kkk: $p < 0.001$, kk: $p < 0.01$, k: $p < 0.05$ INDO vs. DMSO; lll: $p < 0.001$, ll: $p < 0.01$, l: $p < 0.05$ NS398 vs. DMSO; n: $p < 0.05$ NS398 vs. INDO.

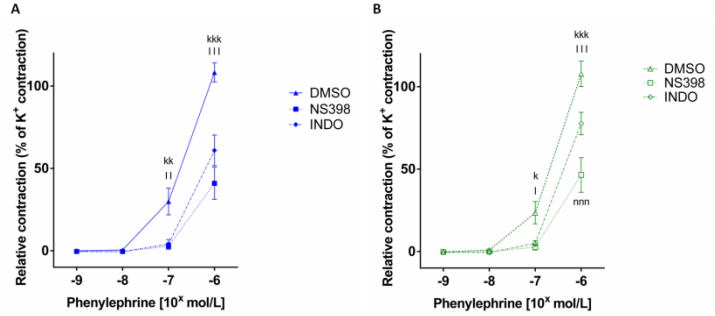

Figure 2. Phenylephrine (Phe) induced contraction

A) Male Vitamin D supplemented group. B) Male Vitamin deficient group. Phe-induced contraction in the presence of COX-2 inhibitor (NS398) or general COX inhibitor (indomethacin; INDO), or their vehicle DMSO. Repeated measures two-way ANOVA with Bonferroni's post hoc test. Data are shown as mean ± SEM;

kkk: $p < 0.001$, kk: $p < 0.01$, k: $p < 0.05$ INDO vs. DMSO; lll: $p < 0.001$, ll: $p < 0.01$, l: $p < 0.05$ NS398 vs. DMSO; nnn: $p < 0.001$ NS398 vs. INDO.

4.2. Vascular Function of Renal Arteries - Relaxation

Vitamin D deficient female rats showed significantly decreased relaxation at 10^{-7} mol/L compared to Vitamin D supplemented females. In addition, significantly lower relaxation was observed in male deficient group compared to the female supplemented rats at 10^{-7} mol/L Ach concentration ($p < 0.05$ FD−vs. MD+; $p < 0.05$ MD+ vs. MD−; $p < 0.001$ FD+ vs. FD−; $p < 0.01$ FD+ vs. MD−. Repeated measures two-way ANOVA with Bonferroni's post hoc test, figure is not shown). In case of Vitamin supplementation in females, COX-2 inhibition resulted in increased relaxation. In case of Vitamin D deficiency in females, there was no significant difference in relaxation in the presence of general COX or specifical COX-2 inhibitor **(Fig. 3)**. In case of Vitamin D supplemented group in males, general COX inhibition led to enhanced relaxation compared to specific COX-2 inhibition. In case of Vitamin D deficient group in males, more pronounced relaxation occurred in the presence of indomethacin **(Fig. 4)**.

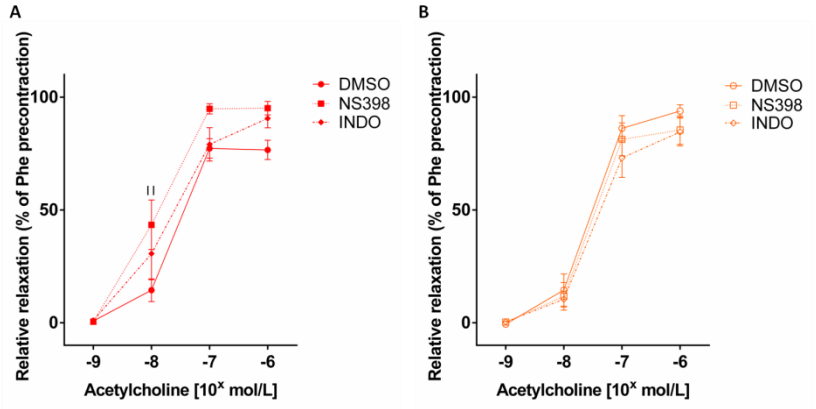

Figure 3. Ach-induced relaxation

A) Female Vitamin D supplemented group. B) Female Vitamin D deficient group. Ach-induced relaxation in the presence of COX-2 inhibitor (NS398) or

general COX inhibitor (indomethacin; INDO), or their vehicle DMSO. Repeated measures two-way ANOVA with Bonferroni's post hoc test. Data are shown as mean ± SEM; ll: $p < 0.01$ NS398 vs. DMSO.

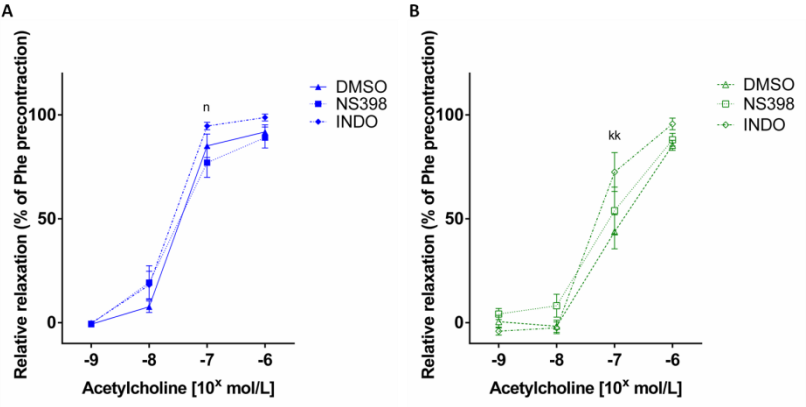

Figure 4. Ach-induced relaxation

A) Male Vitamin D supplemented group. B) Male Vitamin D deficient group. Ach-induced relaxation in the presence of COX-2 inhibitor (NS398) or general COX inhibitor (indomethacin; INDO), or their vehicle. Repeated measures two-way ANOVA with Bonferroni's post hoc test. Data are shown as mean ± SEM; kk: $p < 0.01$ INDO vs. DMSO; n: $p < 0.05$ NS398 vs. INDO.

4.3. Histology of Renal Arteries

The structural changes of the vessels were examined on paraffin-embedded, resorcin-fuchsin stained tissue sections. The intima/media ratio did not differ between groups **(Fig. 5A)**. However, the optical density of media layer of Vitamin D deficient female animals increased significantly compared to both Vitamin D supplemented females and males **(Fig. 5B)**. However, the staining intensity of α-smooth muscle actin (α-SMA) was significantly higher in vitamin D supplemented males compared to both female groups - that was not associated with any measured vascular function **(Fig. 5C)**. The smooth muscle cell nuclei density in the media layer was similar in each group **(Fig. 5D)**. The intensity of eNOS immunohistochemical staining was significantly lower in female Vitamin D deficient animals compared to Vitamin D

supplemented females **(Fig. 5E)**. Finally, the intensity of special staining of angiotensin II receptor 1 (AT1R) was significantly higher in Vitamin D deficient males than in Vitamin D deficient females **(Fig. 5F)**.

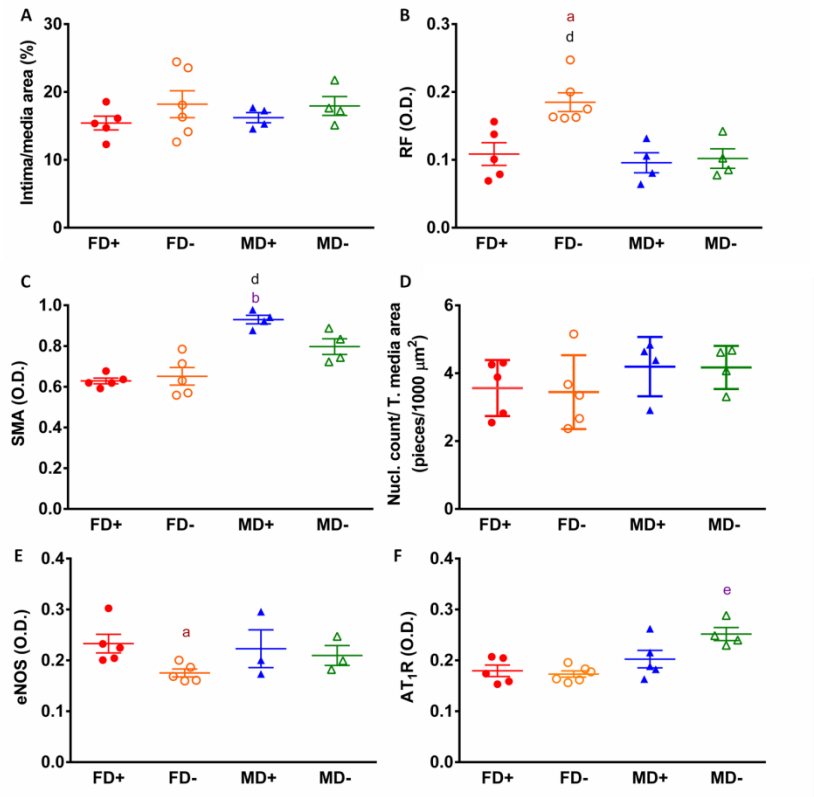

Figure 5. Histology of the renal arteries

A) Intima/media ratio of the renal arteries. B) Density of resorcin-fuchsin staining int he media layer of renal arteries. C) α-smooth muscle actin (α-SMA) immunohistochemistry of renal arteries. D) Density of smooth muscle cell nuclei in the media layer of renal arteries. E) eNOS immunohistochemistry of renal arteries); F) Angiotensin II receptor-1 (AT1R) immunohistochemistry of renal arteries Kruskal-Wallis test with Dunn's multiple comparison test. Data are shown as median [IQR]; a: $p < 0.05$; b: $p < 0.05$ FD+ vs. MD+; d: $p < 0.05$ FD−vs. MD+; e: $p < 0.05$ FD−vs. MD−.

4.4. Vascular Function of Carotid Arteries – Relaxation

Male gender was associated with less pronounced relative Ach-induced relaxation at 10^{-6} mol/L Ach concentration ($p < 0.01$; two-way ANOVA, figure is not shown). Male rats showed significantly reduced relaxation compared to females at Ach concentration of 10-6 mol/L independently from vitamin D status ($p < 0.01$ FD+ vs. MD+; $p < 0.001$ FD+ vs. MD−; $p < 0.001$ FD−vs. MD−, figure is not shown). L-NAME blocked the vasodilation in male and female vitamin D supplemented and deficient groups, co-incubation with INDO had no additional effect **(Fig. 8 and Fig. 9)**.

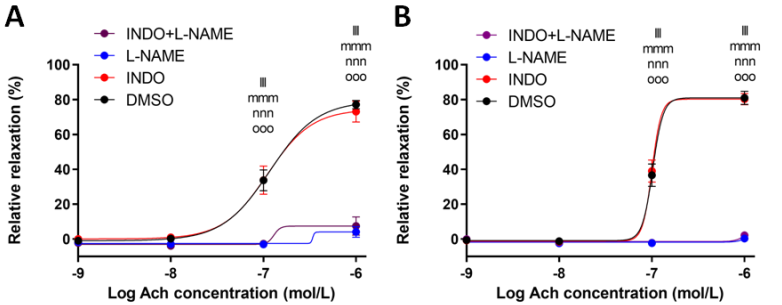

Figure 8. Ach-induces relaxation

Female Vitamin D supplemented group. B) Female Vitamin D deficient group. In the presence of nitro-L-arginine methyl ester or general COX inhibitor (indomethacin; INDO), or their vehicle DMSO. Repeated measures two-way ANOVA with Bonferroni's post hoc test. lll: $p < 0.001$ DMSO vs. L_NAME; mmm: $p < 0.001$ DMSO vs. INDO+L-NAME; nnn: $p < 0.001$ INDO vs. L-NAME; ooo: $p < 0.001$ INDO vs. INDO+L-NAME

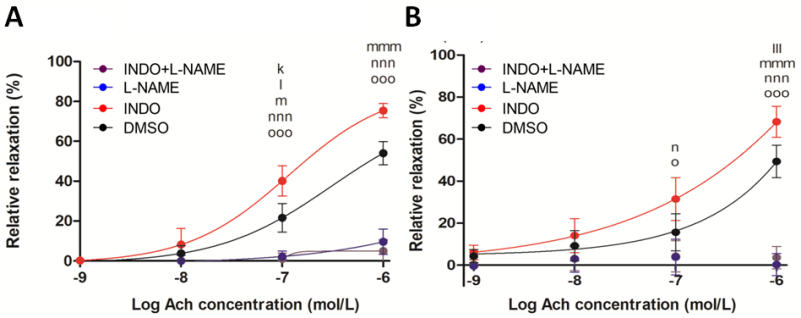

Figure 9. Ach-induces relaxation

A) Male Vitamin D supplemented group. B) Male Vitamin D deficient group. In the presence of nitro-L-arginine methyl ester or general COX inhibitor (indomethacin; INDO), or their vehicle DMSO in male Vitamin D supplemented and male Vitamin D deficient rats. Repeated measures two-way ANOVA with Bonferroni's post hoc test. k: $p < 0.05$ DMSO vs. INDO, l: $p < 0.05$ DMSO vs. L-NAME; lll: $p < 0.001$ DMSO vs. L_NAME; m: $p < 0.05$ DMSO vs. INDO+L-NAME; mmm: $p < 0.001$ DMSO vs. INDO+L-NAME; n: $p < 0.05$ INDO vs. L-NAME; nnn: $p < 0.001$ INDO vs. L-NAME; o: $p < 0.05$ INDO vs. INDO+L-NAME; ooo: $p < 0.001$ INDO vs. INDO+L-NAME

4.5. Histology of Carotid Arteries

eNOS immunohistochemical staining density was significantly higher in the FD+ group compared to the MD- group **(Fig. 10)**.

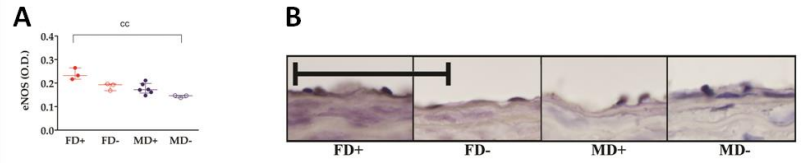

Figure 10. eNOS immunohistochemistry of carotid arteries

A) eNOS immunohistochemistry of carotid arteries. eNOS specific labeling intensity was significantly lower in Vitamin D deficient males; **B) Representative images of carotid arterial sections stained against eNOS** (scale bar is 10 μm). Kruskal-Wallis test with Dunn's multiple comparison test cc: $p < 0.01$ FD+ vs. MD-.

5. **Conclusions**

Our experiments focused on the following questions:

1) Are there gender differences in renal and carotid artery function in Vitamin D supplemented young adult male and female rats?

No gender differences were found in Phe-induced and AII-induced contraction and in Ach-induced relaxation in renal arteries of Vitamin D-treated rats. However, whereas the specific COX-2 inhibitor significantly increased Ach-induced relaxation compared to vehiculum in vitamin D-treated females, the general COX inhibitor, indomethacin significantly increased Ach-induced relaxation compared to the inhibitory effect of specific COX-2 in Vitamin D-treated males. A significant gender difference was found in Ach-induced relaxation in carotid rings, as females had more intense relaxation compared to males.

2) Are these attributes altered by Vitamin D deficiency?

Maladaptive changes occurred in vessel functions due to Vitamin D deficiency. Phe-induced contraction level increased significantly in females' renal arteries. General and special COX-2 inhibition pre-treatment significantly decreased the contraction level in both sexes. Furthermore, the level of Ach-induced relaxation decreased significantly in renal arteries of both sexes. In parallel with these findings, our histological results demonstrated that optical density of media layer of female animals increased significantly compared to Vitamin D supplemented females and males, that may have contributed to the increased contractile force and the reduced relaxation ability of these vessels. Furthermore, also in Vitamin D deficient females, the development of endothelial disfunction

was indicated by the reduced intensity of eNOS staining. The level of relaxation decreased in carotid arteries in both sexes as a result of Vitamin D deficiency.

3) Are there gender differences in the lesions caused by vitamin D deficiency?

Among gender differences in the vascular effects of vitamin D deficiency it is worth highlighting that vascular response was shifted towards vasoconstriction in renal vessels of female animals, as the Phe-induced contraction increased, while Ach-induced relaxation decreased. In males only reduced relaxation was observed, the increased constrictor response was missing. In contrast, no gender difference was detected in carotid arteries between Vitamin D deficient groups.

6. Bibliography of the candidate's publications

Publications related to the thesis:

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Publications not related to the thesis:

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