Cardio- and cerebrovascular effects of vitamin D deficiency and androgens on male rat coronary arteries and on female rat cerebral arteries

Doctoral thesis

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Budapest
2021

1. Introduction

Gender differences between men and women have existed since the beginning of the world. The reasons of this difference - among other things - are male and female sex hormones and the effects of androgens and estrogens.

Vitamin D can be produced by almost every living thing on Earth, it is an evolutionary well-preserved prohormone, which has known classic skeletal and non-classic, extraskeletal actions.

Optimal vitamin D intake, synthesis of active form in appropriate quantity and adequate metabolism are needed to develop the diverse effects of vitamin D. All in all: to maintain the balance of 'vitamin D homeostasis'. The actual vitamin D supply of an organization can be determined by measuring 25-hydroxyvitamin D_3 ($25(OH)D_3$) levels. The imbalance can be led to too low or too high vitamin D levels – the former is extremely common, the latter is extremely rare in the general population.

Suboptimal vitamin D supply and vitamin D deficiency are important public health problems worldwide already.

Deaths from cardio- and cerebrovascular diseases have been leading the global mortality statistics for decades. The cardiovascular risk shows gender differences: women are relatively more protected, while men have higher risk. The reason for this difference is attributed to sex hormones: proven protective effect of estrogens in women, while in men, the influence of androgens can be presumed, because hyperandrogenic conditions may be associated with an increased cardiovascular risk in both women and men (for example, polycystic ovary syndrome in women and exogenous androgen-anabolic steroid use in men).

Therefore, investigation of the effects of vitamin D on the cardiovascular system has been highlighted among the extraskeletal effects. However, currently we have little knowledge and data about the possible gender differences in the effects of vitamin D, further the interactions of these complex effects and different cardiovascular regions and vasculature of vital organs (heart and brain).

2. Objectives

There is currently little knowledge on the relationship of vitamin D, androgens and cardiovascular risk, especially gender differences, our aim was to investigate the endocrinological and circulatory mysteries of male heart and female brain:

During our research, we aimed to directly investigate the adaptation, possible changes and damages of small vessels from different vascular regions which are responsible for the blood supply of our two most important vital organs – the heart and brain – in response to different vitamin D supply. Moreover, to examine the interactions of vitamin D deficiency and effects of testosterone in female and male animal model.

Based on these, we aimed to answer the following questions:

1) ***May the different vitamin D supply – especially vitamin D deficient state – have effects on geometrical, biomechanical and viscoelastic characteristics and adaptational mechanisms of smaller segments from arteries which are responsible for the blood supply of vital organs (heart, brain)?***

2) ***What kind of initial pathomechanisms are there in the background of these possible effects, changes and damages and are there any differences between organs, genders or between the effects of sex hormones?***

3) ***Because of the higher cardiovascular risk of male gender, in case of males may the oxidative-nitrative stress play a role in the development of possible changes and damages of coronary arterioles?***

4) ***To induce vitamin D deficiency in different animal models and to investigate interactions of the androgen-effects and vitamin D deficiency in female animals: In an additional Polycystic Ovary Syndrome animal model, may the hyperandrogenism and vitamin D supply/deficiency have affect the morphological-biomechanical-histological characteristics of female cerebral vessels?***

3. Methods

3.1. Animals

All procedures conformed to the relevant national and European Union Guidelines (ethical approval: IRB:8/2014 (PEI/001/1548-3/2014), (PEI/001/820-2/2015)).

During our chronic, 8-week-long animal experiment, 4-week-old, 100-140 g initial weight 22 male and 90-110 g initial weight female Wistar rats were used. Animals were supplied with their special rat chow and tap water ad libitum and lived in physiological conditions. During the experiment we did everything possible to minimize the suffering of the animals and at the end of the experiment we provided the human endpoint.

Male animals were randomly divided into two groups and we provided different vitamin D supply:

- ♂ VD+: male vitamin D supplemented, control group (n=11)
- ♂ VD-: male vitamin D deficient group (n=11)

Female animals were divided randomly into four groups. We also induced different vitamin D supply in them and modeled hyperandrogenic state by creating additional subgroups:

- ♀ VD+/T-: female vitamin D supplemented, non testosterone-treated double control group (n=12)
- ♀ VD+/T+: female vitamin D supplemented, testosterone-treated group (n=12)
- ♀ VD-/T-: female vitamin D deficient, non testosterone-treated group (n=11)
- ♀ VD-/T+: female vitamin D deficient, testosterone-treated group (n=11)

3.2. Treatment protocol

Optimal vitamin D intake was achieved with a normal vitamin D-containing diet and additional vitamin D supplementation. Vitamin D deficiency was achieved with vitamin D-free diet. Hyperandrogenic state was induced by transdermal testosterone treatment.

3.3. In vitro pressure microarteriography and calculations

At the end of the experiment the final weight of the animals was measured: weight gain of males was greater than that of females', while in female groups testosterone-treatment resulted in greater weight gain. However, vitamin D deficiency did not affect weight gain neither in males nor in females. In general anaesthesia invasive blood pressure measurements were done. In case of male animals, after chest-opening small intramural arteriole segments of the left anterior descendent coronary artery were microprepared, while in case of female ones, after brain-removal segments of anterior cerebral artery were removed. The segments were placed into an organ chamber filled with 37 °C normal Krebs-Ringer (nKR) solution (composition in mM/L: NaCl 119; KCl 4,7; NaH_2PO_4 1,2; $MgSO_4$ 1,17; $NaHCO_3$ 24; $CaCl_2$ 2,5; glucose 5,5 and EDTA 0,034), bubbled with gas mixture containing % CO_2, 20% O_2 and 75% N_2. They were cannulated at both ends with microcannulas and pressure-servo pumps were connected to both cannulas. By changing the intraluminal pressure, pressure-diameter curves were recorded. Then, nKR solution was changed to calcium-free Krebs solution (composition in mM/L: NaCl 92; KCl 4,7; NaH_2PO_4 1,18; $MgCl_2$ 20; $MgSO_4$ 1,17; $NaHCO_3$ 24; glucose 5,5; EGTA 2,0 and EDTA 0,025) and the registration of pressure-diameter curves was repeated. Pictures of the transilluminated segments were taken during the measurement by a digital video camera then the outer and inner diameters (D_o and D_i) of the vessels were measured with image analysis software.

From the diameters and other parameters, the following morphological, geometrical and biomechanical properties of the segments were calculated:

- Outer radii/R_o (μm): $R_o = \frac{D_o}{2}$

- Inner radii/R_i (μm): $R_i = \frac{D_i}{2}$

- Wall thickness/h (μm): $h = R_o - R_i$

- Wall thickness/Lumen diameter ratio: $h - D = \frac{h}{D_i}$,

- Wall cross-sectional area/A_w (μm^2): $A_w = \left(R_o^2 - R_i^2\right) * \pi$

- Tangential stress/σ_{Tang} (kPa): $\sigma_{Tang} = \frac{p * R_i}{h}$,

p: intraluminal pressure

- Incremental elastic modulus/E_{Inc} (LogkPa): $E_{Inc} = \frac{2R_i^2 * R_o}{(R_o^2 - R_i^2)} * \frac{\Delta P}{\Delta R_o}$,

ΔP: change in intraluminal pressure
$ΔR_o$: outer radius change in response to *ΔP*

- Distensibility/D (1/kPa): $D = \frac{\Delta V}{V * \Delta P}$,

ΔV: change in lumen volume relative to the initial volume *V* in response to *ΔP*

- Myogenic tone: $\text{Myogenic tone } (\%) = \frac{R_{Ca-free} - R_{nKR}}{R_{Ca-free}} * 100$

3.4. Histology and Immunohistochemistry

Other parts of the segments have been histologically processed: Hematoxylin-Eosin (HE), Resorcin-Fuchsin (RF) stained and native slides were made. For examining oxidative-nitrative markers on male coronary vessels immunohistochemical (IHC) 4-hydroxi-2-nonenal (HNE), Poly(ADP)-ribose (PAR), 3-nitrotyrosine (NT) stainings were done. For investigating smooth muscle layer α-smooth muscle actin (α-SMA) staining were performed.

After digitalization, on HE-stained slides smooth muscle cell nucleus counting were done and on RF-stained slides we measured optical density or elastic fiber density of tunica media layer. On IHC slides, positively-stained area percentage was calculated, nucleus counting was done and non-calibrated optical density was measured in image analysis program.

3.5. Statistical analysis

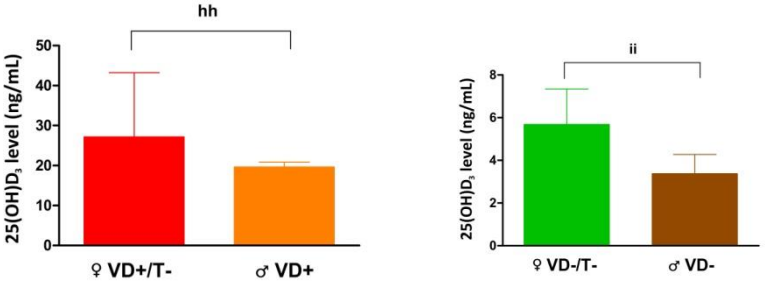
After checking normality tests, depending on the distribution unpaired T-test with F-test or Mann-Whitney-U-test was used. Additionally, analysis of variance (ANOVA) with Bonferroni's or Tukey's post hoc tests or Kruskal-Wallis with Dunn post hoc test was applied. Repeated measures ANOVA with Bonferroni's post hoc test was used in case of increasing intraluminal pressures. $P < 0.05$ was considered statistically significant difference. Data are expressed in mean ± SEM or in median [IQR].

4. Results

4.1. Treatment validation, Blood pressure measurement

Our treatment modeled successfully the vitamin D deficient state in both gender ($25(OH)D_3$ levels in ng/mL: ♂ VD+: 19.66 ± 0.81; ♂ VD-: 3.59 ± 0.21; ♀ VD+/T-: 32.328 ± 4.49; ♀ VD+/T+: 33.106 ± 4.46; ♀ VD-/T-: 6.044 ± 0.63; ♀ VD-/T+: 6.006 ± 0.68). Transdermal testosterone treatment resulted in biochemical hyperandrogenism in females (total-T levels in ng/mL: ♀ VD+/T-: 0.311 ± 0.16; ♀ VD+/T+: 4.292 ± 0.56; ♀ VD-/T-: 0.720 ± 0.16; ♀ VD-/T+: 5.495 ± 0.56).

Identical vitamin D supplementation protocol resulted in significantly higher $25(OH)D_3$ levels in females than in males, while vitamin D-free diet caused significantly lower $25(OH)D_3$ levels in males than in females [***Figure 1 (a) and (b)***].



(**a**) (**b**)

Figure 1. $25(OH)D_3$ levels (ng/mL) at the 8th week of experiment in vitamin D supplemented (VD+) and vitamin D deficient (VD-) female and male groups. (**a**) $25(OH)D_3$ levels of female and male vitamin D supplemented groups (n=10-11, y-axis: 0-50 ng/mL), ♀ VD+/T- vs. ♂ VD+: **hh**: p<0,01. (**b**) $25(OH)D_3$ levels of female and male vitamin D deficient groups (n=10-10, axis: 0-10 ng/mL), ♀ VD-/T- vs. ♂ VD-: **ii**: p<0.01. Mann-Whitney-U-test. Median [IQR].

There was no significant difference in the measured blood pressures either in male or female groups.

4.2. Examined characteristics of coronary arteriole segments

4.2.1. Arteriole geometry

In vitamin D deficient group, inner radii was significantly lower *[**Figure 2 (a)**]*, significantly larger wall thickness and wall thickness/lumen diameter ratio were shown [***Figure 2 (b) and (c)***], while the cross-sectional area of the wall did not change [***Figure 2 (d)***].

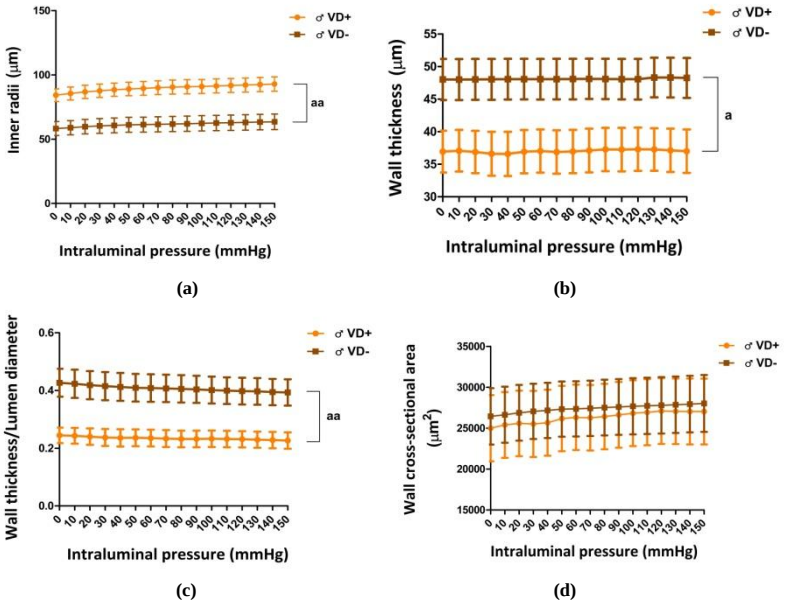


Figure 2. Geometrical properties of male coronary arteriole segments. **(a)** Inner radii (μm, n=8-8); **(b)** Wall thickness (μm, n=8-8); **(c)** Wall thickness/lumen diameter ratio (n=8-8) and **(d)** Wall cross-sectional area (μm^2, n=7-7) in calcium-free solution on 0-150 mmHg intraluminal pressures. Repeated measures ANOVA, Bonferroni. Mean ± SEM. ♂ VD+ vs. ♂ VD-: **a**: $p<0.05$; **aa**: $p<0.01$.

4.2.2. Arteriole elasticity

Tangential stress and incremental elastic modulus were significantly decreased in male vitamin D deficient group [***Figure 3 (a), (b) and (c)***].

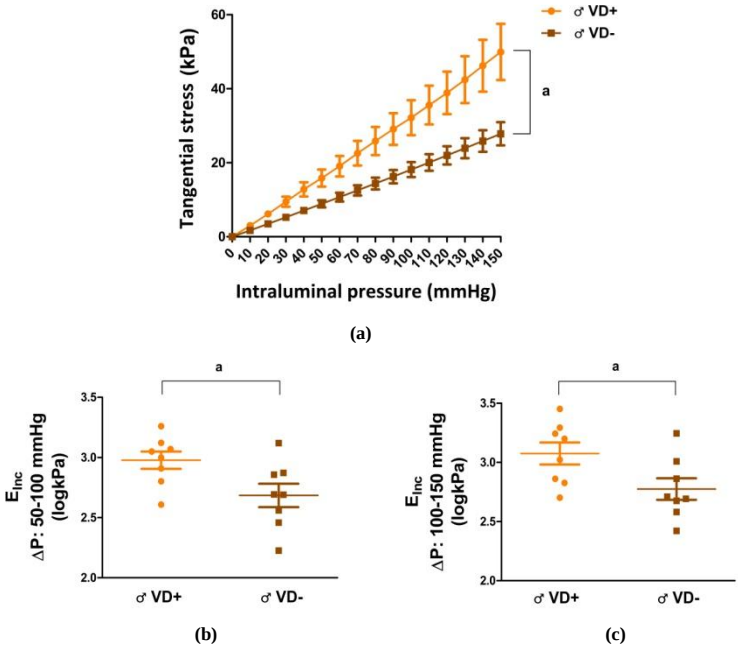


Figure 3. Elasticity of male coronary arteriole segments. **(a)** Tangential stress (kPa) on 0-150 mmHg intraluminal pressures; **(b-c)** Incremental elastic modulus (logkPa, n=8-8) **(b)** on 50-100 and **(c)** 100-150 mmHg intraluminal pressures in passive state. Repeated measures ANOVA, Bonferroni. Unpaired t-test. Mean ± SEM. ♂ VD+ vs. ♂ VD-: **a**: $p<0.05$.

Distensibility did not change significantly between the two groups.

4.2.3. Myogenic tone

Myogenic tone of coronary arterioles from vitamin-D-deficient group did not seem to be able to keep the tone at higher pressures; however, this difference did not reach the level of statistical significance, but a downward tendency can be seen ***[Figure 4]***.

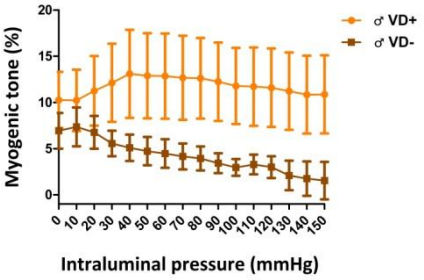
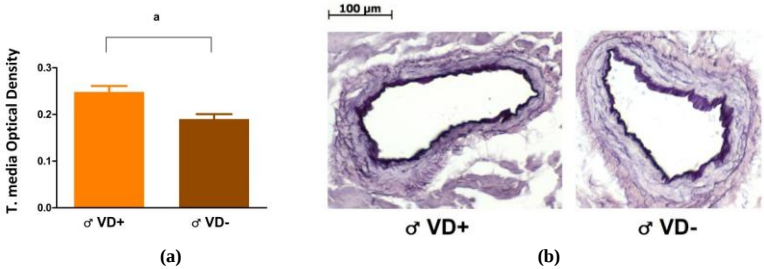


Figure 4. Spontaneous tone of male coronary arteriole segments (%, n=6-6). Repeated measures ANOVA, Bonferroni. Mean ± SEM. n. s.

4.2.4. Histology

There were no significant differences either in the number of smooth muscle cell nuclei counted in the medial layer on HE-stained sections, or in tunica intima/tunica media area percentage measured in RF-stained sections.

Optical density of RF elastica stain in the medial layer was significantly lower in vitamin D deficient vessels [***Figure 5 (a) and (b)***].



(a) **(b)**

Figure 5. Histological characteristics of male coronary arterioles. ***(a)*** Tunica media optical density (n=4-4) investigated in RF-stained slides. Mann-Whitney-U-test. Median [IQR]. ♂ VD+ vs. ♂ VD-: **a**: $p<0.05$; (***b***) Representative images of Resorcin-Fuchsin-stained male rat coronary arteriole segments from control and vitamin D deficient groups. Scale bar, 100 µm.

4.2.5. Immunohistochemistry

The oxidative stress and lipid peroxidation marker, HNE level was significantly elevated in male vitamin D deficient group *[**Figure 6 (a)**, **(b)**]*. There were no statistically significant differences in the levels of other oxidative-nitrative markers (PAR, NT) and in the expression of α-smooth muscle actin (α-SMA).

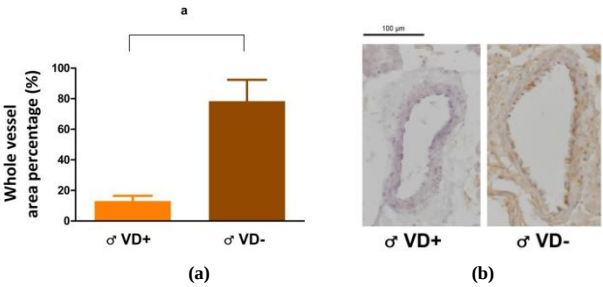
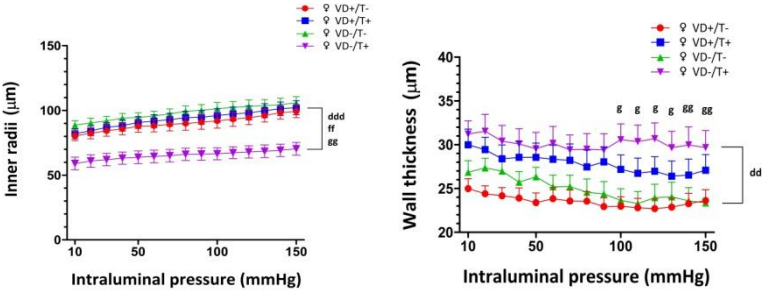


Figure 6. Results of anti-4-hydroxi-2-nonenal (HNE) immunohistochemical staining. **(a)** Percentage area of coronary arteriole cross-sections positively staining with anti-HNE antibodies. Mann-Whitney-U-test. Median [IQR], n=5-4. ♂ VD+ vs. ♂ VD-: **a**: $p<0.05$; **(b)** Representative images of HNE immunohistochemistry sections of male rat coronary arteriole segments. Scale bar, 100 µm.

4.3. Examined characteristics of cerebri artery segments

4.3.1. Artery segment geometry

Cerebral artery segments of female vitamin D deficient and testosterone-treated group (♀ VD-/T+) had significantly lower inner radii and significantly greater wall thickness compared to other groups [***Figure 7 (a) and (b)***].



(a) **(b)**

Figure 7. Geometrical properties of anterior cerebral artery segments. **(a)** Inner radii (μm, n=11/group); **(b)** Wall thickness (μm, n=11/group) in calcium-free solution on 10-150 mmHg intraluminal pressures Repeated measures ANOVA, Tukey. Mean ± SEM. ♀ VD-/T+ vs. ♀ VD+/T-: **dd**: p<0.01; **ddd**: p<0.001; ♀ VD-/T+ vs. ♀ VD+/T+: **ff**: p<0.01; ♀ VD-/T+ vs. ♀ VD-/T-: **g**: p<0.05, **gg**: p<0.01.

4.3.2. Artery segment elasticity

In female vitamin D deficient and testosterone-treated group (♀ VD-/T+) significantly lower tangential stress was shown compared to all three other groups [***Figure 8.***], while there weren't any significant differences in incremental elastic modulus and in distensibility between the groups.

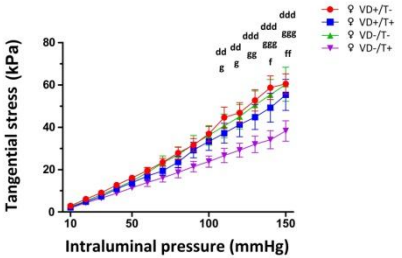


Figure 8. Tangential stress of anterior cerebral artery segments. (kPa, n=11/group) in calcium-free solution on 10-150 mmHg intraluminal pressures Repeated measures ANOVA, Tukey. Mean ± SEM. ♀ VD-/T+ vs. ♀ VD+/T-: **dd**: p<0.01, **ddd**: p<0.001; ♀ VD-/T+ vs. ♀ VD-/T-: **g**: p<0.05, **gg**: p<0.01, **ggg**: p<0.001; ♀ VD-/T+ vs. ♀ VD+/T+: **f**: p<0.05, **ff**: p<0.01.

4.3.3. Myogenic tone

Vitamin D deficient and hyperandrogenic state in female cerebral artery segments (♀ VD-/T+) resulted in significantly decreased myogenic tone compared to double control group (♀ VD+/T-). On higher intraluminal pressures, significantly lower myogenic tone was also shown in vitamin D deficient non-testosterone-treated group [***Figure 9***], the curve is similar to the myogenic tone of the male vitamin D deficient coronary segments [***Figure 9 and Figure 4***].

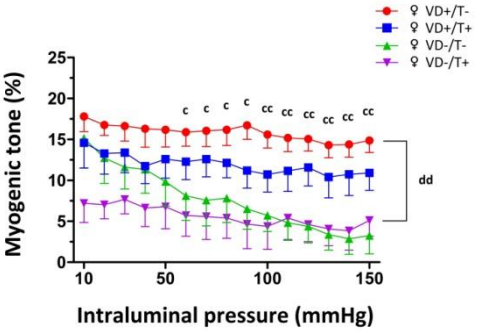
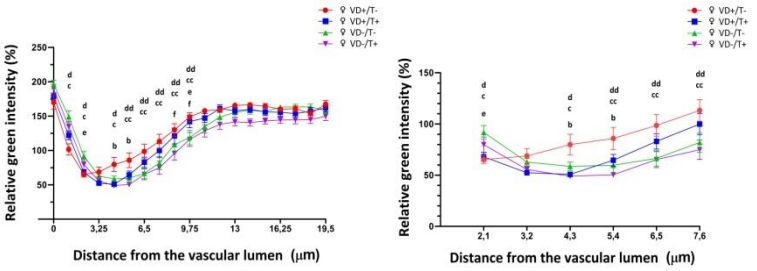


Figure 9. Myogenic tone of anterior cerebral artery segments. (%, n=11/group) in calcium-free solution on 10-150 mmHg intraluminal pressures. Repeated measures ANOVA, Tukey. Mean ± SEM. ♀ VD-/T+ vs. ♀ VD+/T-: **dd**: p<0.01; ♀ VD-/T- vs. ♀ VD+/T-: **c**: p<0.05, **cc**: p<0.01.

4.3.4. Histology

The relative green intensity investigated on RF-stained sections significantly decreased in the inner elastic membrane layer in vitamin D deficient groups, independently from testosterone treatment (♀ VD-/T+ és ♀ VD-/T-). Testosterone treatment also affected the structure of the layer: in testosterone-treated vitamin D supplemented group (♀ VD+/T+) the green intensity significantly reduced compared to double control (♀ VD+/T-), vitamin D deficient non-testosterone-treated (♀ VD-/T-) and testosterone-treated (♀ VD-/T+) groups.

The relative green intensity is the opposite of the density of the elastic fibers dyed to violet color, thus, the presence of both noxa – vitamin D deficiency and testosterone treatment – also increased the density of inner elastic membrane ***[Figure 10 (a) and (b)]***.



(a) **(b)**

Figure 10. Elastic membrane density of anterior cerebral artery segments (%, n=6/group). **(a)** Relative green intensity (%) measured in radial direction from the endothelial surface. **(b)** Relative green intensity in inner elastic membrane. Repeated measures ANOVA, Tukey. Mean ± SEM. ♀ VD-/T+ vs. ♀ VD+/T-: **d**: p<0.05, **dd**: p<0.01; ♀ VD-/T- vs. ♀ VD+/T-: **c**: p<0.05, **cc**: p<0.01; ♀ VD+/T+ vs. ♀ VD+/T-: **b**: p<0.05; ♀ VD+/T+ vs. ♀ VD-/T-: **e**: p<0.05; ♀ VD+/T+ vs. ♀ VD-/T+: **f**: p<0.05.

There was no significant difference in wall cross-sectional area of cerebral artery segments between the groups.

5. Conclusions

Based on these, we aimed to answer the following questions:

1) ***May the different vitamin D supply – especially vitamin D deficient state – have effects on geometrical, biomechanical and viscoelastic characteristics and adaptational mechanisms of smaller segments from arteries which are responsible for the blood supply of vital organs (heart, brain)?***

Vitamin D deficiency resulted in reduced inner radii, increased wall thickness and wall thickness/lumen diameter ratio and unchanged wall cross-sectional area in male coronary artery segments. Cerebral vessel segments from female vitamin D deficient hyperandrogenic animals had smaller inner radii, enhanced wall thickness which had been accompanied by unchanged wall cross-sectional area. In terms of functional characteristics, in male coronary artery segments tangential stress and incremental elastic modulus reduced, while female cerebral artery segments had lowered tangential stress, unchanged incremental elastic modulus and lowered myogenic tone. These changes occurred partially in only vitamin D deficient female cerebral vessels.

Our results refer to the development of inward eutrophic remodeling independently from gender and vascular region, which have accompanied by partially different, gender-specific functional impairments. At constant blood pressure, these changes may be considered as pre-hypertensive changes.

2) ***What kind of initial pathomechanisms are there in the background of these possible effects, changes and damages and are there any differences between organs, genders or between the effects of sex hormones?***

According to the results of histological examinations, male vitamin D deficient coronary segments had decreased Resorcin-Fuchsin optical density in the tunica media layer, however there were no differences in the number of smooth muscle cell nuclei and α-SMA expression. In female brain vessels, vitamin D deficiency and concomitant hyperandrogenic state together and separately resulted in increased density of inner elastic membrane. Elastic components were changed differently in the two sexes: it can be assumed that the pathomechanisms differ between genders and vascular regions: in male gender the reduction of elastic components, while in females the abnormal accumulation of elastin may have caused the identified morphological and functional changes.

3) ***Because of the higher cardiovascular risk of male gender, in case of males may the oxidative-nitrative stress play a role in the development of possible changes and damages of coronary arterioles?***

Investigating the oxidative-nitrative parameters of male vitamin D deficient coronary artery segments, a lipid peroxidation marker, the HNE level significantly elevated, while there were no differences in PAR and NT levels. In the background of the identified changes, it can be assumed that in male gender, initial lipid peroxidation and oxidative damage also play a role in the pathomechanism.

4) ***To induce vitamin D deficiency in different animal models and to investigate interactions of the androgen-effects and vitamin D deficiency in female animals: In an additional Polycystic Ovary Syndrome animal model, may the hyperandrogenism and vitamin D supply/deficiency have affect the morphological-biomechanical-histological characteristics of female cerebral vessels?***

In vitamin D deficient hyperandrogenic female brain vessels, morphological remodeling and functional impairments occurred, while in only vitamin D deficient females these changes occurred partially (decreased myogenic tone, increased inner elastic membrane density). In vitamin D supplemented hyperandrogenic females, only increase in inner elastic membrane density was detected.

Based on our results, there is a relationship between vitamin D and androgen hormone effects. Co-existence of vitamin D deficiency and hyperandrogenism in female gender resulted in marked morphological-functional impairments of cerebral vessels, while in case of optimal vitamin D levels – despite the hyperandrogenic state – these changes did not or minimally occurred.

In female gender, potential negative additive effects of vitamin D deficiency and hyperandrogenic state can be assumed.

In conclusion, I would like to pay attention to the importance of the determination of vitamin D state ($25(OH)D_3$ level measurement) and resolving existing vitamin D deficiency in both genders!

6. Bibliography of the candidate's publication

Publications related to the thesis:

1) **Sziva RE**, Fontányi Z, Pál É, Hadjadj L, Monori-Kiss A, Horváth EM, Benkő R, Magyar A, Heinzlmann A, Benyó Z, Nádasy GL, Várbíró S. (2020) Vitamin D Deficiency Induces Elevated Oxidative and Biomechanical Damage in Coronary Arterioles in Male Rats. Antioxidants (Basel), 9(10): 997.
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  IF = 2.357

CUMULATIVE IMPACT FACTOR (16.04.2021.):
E IF = 25.376

(*: Expected IF values)