The effects of $\Delta^9$-Tetrahydrocannabinole treatment on gonadal micro-vascularization and affected fertility examined by SEM and 3D-morphometry

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Abstract. The present study focuses on the effects of $\Delta^9$-tetrahydrocannabinol (THC) on the reproductive system in nude rats with special emphasis on how $\Delta^9$-THC impacts the vascularization of testes which in turn indirectly influences fertility. Basically, $\Delta^9$-tetrahydrocannabinol (THC) causes not only negative (psychoactive) effects in the human body as cannabinole administration in medical use (dose-dependent) offers multiple new treatment opportunities such as pain relief or containment of various cancers. Concerning the reproductive system it strongly influences CB-receptors along the hypothalamic-pituitary-gonadal axis resulting in reduced plasma testosterone levels. There is also altered sperm quality parameters reported such as sperm motility or sperm count. On the other hand $\Delta^9$-THC effects endothelial growth factors (VEGF, Ang-1 etc.) respectively acts on their specific receptors which in turn modify angiogenesis and vascularization of tissues and organs (e.g. tumorous tissues). This leads to new therapeutic strategies in the suppression of various cancers by inhibiting (neo-)vascularization and in turn famishment of tumorous tissues (lack of nutrition supply). Here we studied the micro-vascularization of gonads in a long-term THC-treated nude rat model by vascular corrosion casting, SEM and 3D-morphometry.

1. Introduction

Cannabinoids inhibit tumour angiogenesis in vivo and at least two mechanisms may be involved in this cannabinoid action: direct inhibition of vascular endothelial cell migration and survival as well as the suppression of proangiogenic factors (VEGF, Ang-2) and matrix metalloproteins (MMP) expression in tumours [1]. By inhibiting vascular endothelial cell migration and -survival, cannabinoids would directly prevent blood vessel formation. By targeting tumour cells, cannabinoids would induce their apoptosis [2] and would also suppress proangiogenic factor- and MMP production, further blocking tumour growth and angiogenesis. Cannabinoid-based antiangiogenic treatments thus constitute the most promising antitumoral approaches currently available. Beside these positive angiogenic inhibiting effects, $\Delta^9$-THC may also act in a negative way on gonadal neovascularization and thus may reduce fertility by reduced sperm production and quality. Here we quantitatively studied the micro-vascularization of long-term cannabinole treated nude rats and compared it with plasma serum concentrations of testosterone.

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2. Material and Methods

2.1 Animals
CR (Charles River) hairless mutation rat (Crl: CD-Prss8) were subdivided into two groups: (i) Δ⁹-Tetrahydrocannabinole (THC) treated adult nude rats (n=30 males), and (ii) healthy control adult nude rats (n=30 males). Animals attending the THC group received 3mg/kg THC i.p. once a day for a period of 182 days.

2.2 Methods
2.2.1 Animal preparation
Adult nude rats were anesthetized using an overdose of Ketavet (130 mg/kg bodyweight diluted in 10 mg/kg Xylazin and 0.9% saline). After anaesthesia the adult animals were pinned with their four ends onto a wax bin. The skin of the animals was opened with a median cut at the dorsum of the abdomen to the dorsum of the episternum and pinned on the side. Next, the abdomen was opened and stretched with a wound retractor. Following, the right Arteria testicularis was laid open and ligatured (Ethicon 6-0). After that an injection of 0.1 ml heparin (50 I.E.) and 2-3 minutes later a saturated potassium chloride solution was injected distally of the ligature of the right Arteria testicularis. At this point the right Vena testicularis was opened.

2.2.2 Vascular Corrosion casting
The vascular system of the testes was rinsed blood-free with phosphate buffered saline (body temperature). Once clear reflux returned from the opened veins the injection was continued with 5-10 ml of the polymerizing resin Mercox (CL-B2-Mix) through the same injection site. At the end of the resin injection the animals were curing about 30 minutes at room temperature and thereafter the cast organs were transferred into a water bath at 60°C over night (tempering) followed by maceration of all organic tissues in potash solution (7.5 %) at 40°C for 2-3 days (changing solution daily). The corroded casts were then rinsed with distilled water and cleaned with a jet of distilled water and HCOOH, thereafter frozen in distilled water and finally freeze-dried. Dry casts were fixed on specimen stubs and sputtered with a thin layer of gold to allow investigation in the SEM [3] (figure1).

Figure 1. Superficial view of a vascular corrosion cast of a rat’s testis examined in the SEM.

2.2.3 SEM inspection
Coated specimens were investigated in the scanning electron microscope (Philips ESEM XL-30, FEI, Eindhoven, NL) at an accelerating voltage of 5-10 kV (figure 2). This accelerating voltage is high enough to identify those details at the (luminal) surface of casted vessels which allow distinguishing...
between venous and arterial vessels (e.g. cell nuclei imprints). For consecutive the 3D morphometry 
analyses stereo-paired images (tilt angle: 5-12°; working distance: 10-30 mm; resolution: 480x480 px) 
were taken and transferred into M³ analysis software (ComServ OG, Ebenau, Austria).

2.2.4 3D morphometry

3D representations (figure 3) were generated to gain spatial orientation for point setting. Morphometric 
measurements were performed considering the third dimension in the SEM by calculating the parallax. 
Therefore, the type of projection (i.e. parallel projection [magnification (mag) > 500 x and working 
distance (wd) > 10 mm] respectively, central perspective projection [mag. ≤ 500x and wd ≤ 10 mm]) 
and the field of view (fw) were critical parameters when acquiring the stereo-paired images (tilt = 6 ± 
0.1°). Every point in 3D (x,y,z) was computed from its corresponding 2D-plane co-ordinates (s,t) and 
(u,v) obtained by point-setting using the computer’s input device. For mathematical reasons, we first 
scaled the planar stereo pair co-ordinates onto a [-1,1] interval within a Cartesian system of co-
ordinates and then used homogeneous co-ordinates (x,y,z,1) for computing the necessary 
transformation matrices and finally applied the appropriate formula (1) or (2) to calculate a point’s 3D 
co-ordinates [4]. With this spatial information geometric vector equations allowed to calculate vascular 
parameters such as intervascular distances, interbranching distances, vessel diameters and branching-
off angles (figure 4).

Figure 3. 3D-morphometric angular measurement – bifurcations of one 
supplying artery.  

Figure 4. Screenshot of the M³ morphometry software – 
vessel diameter measurement on stereo-paired SEM images.

Formulas for 3D point calculation in central perspective projection:

\[
\begin{align*}
10 \quad x &= \frac{1}{N} \cdot (4 s v (\sin(\gamma) \sin(\rho/2))^2 + s \sin(2\gamma) \sin(\rho)), \\
10 \quad y &= \frac{1}{N} \cdot ((t + v) \sin(\gamma) \sin(\rho)), \\
10 \quad z &= \frac{1}{N} \cdot (4 t v \sin(\gamma) (\sin(\rho/2))^2 + (t - v) \cos(\gamma) \sin(\rho)),
\end{align*}
\]

whereby \( N = \sin(2\gamma) (1 + \cos(\rho)) + t v \sin(2\gamma) (1 - \cos(\rho)) + (t - v) \cos(2\gamma) \sin(\rho). \)
Formulas for 3D point calculation in parallel perspective projection:

\[
x = \frac{\mu s}{2}, \quad y = \mu' \frac{t + v}{4 \cos(\gamma)}, \quad z = \mu' \frac{t - v}{4 \sin(\gamma)}
\]  

(2)

3. Results & conclusion

Regarding data in table 1 daily Δ9-THC treatment over a period of 6.5 month showed a massive change in vascular organization in every part of the testes. Intervascular distances (IVD) the main responsible factor for \(O_2\)- and nutrients supply of the surrounding tissue were increased in all cases after THC treatment. With the exception of the medial part of the testicular supply also the diameters of vessels in front of bifurcations were enlarged significantly implicating a higher demand of hormonal supply due to reduced plasma testosterone levels (see figure 5) in THC treated animals. Slightly larger branching-off angles at bifurcations in all parts of the testes in the treatment group indicate a reduced haemodynamic activity which in turn results in a lower blood supply of the sperm producing tissue in the gonads.

In conclusion, we could approve that like in tumors the vascular meshwork is also affected by longtime THC treatment in the gonads which in turn may result in lesser fertility of drug addicted people and cancer patients who receive adjuvant THC therapy.

| Segment / Treatment | VD(µm) | IVD(µm) | BA(°) |
|---------------------|--------|---------|-------|
| dist. / control     | 133.1  | 213.4   | 28.2  |
| med. / control      | 316.0  | 303.5   | 36.9  |
| prox. / control     | 299.7  | 268.3   | 39.7  |
| dist. / THC         | 236.5  | 315.6   | 31.8  |
| med. / THC          | 196.3  | 581.7   | 38.1  |
| prox. / THC         | 421.0  | 709.1   | 43.2  |

Table 1. Morphometric comparison of vascular parameters: (a) VD – vessel diameters (b) IVD – intervascular distances, and (c) BA – branching-off angles at bifurcations – controls vs. THC-treated rats’ testes divided in distal, medial and proximal portions (mean values).

Figure 5. Serum testosterone levels (ng/ml): comparison between THC treated- and control (C) animals.

References

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