Cytoarchitecture of steroid dependent target tissues after testosterone administration compared to nandrolone decanoate in castrated rats in the aim of Hershberger bio test

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Abstract
The objective was the cytoarchitecture evaluation of known steroid dependent target tissues after administering of testosterone, compared to action of its more active ester, nortestosterone (nandrolone decanoate) in castrated rat males in the aim of Hershberger bio test. Study was performed on 30 castrated male Wistar rats, aged between 35 and 39 days, in peripubertal period, divided into five groups. Androgen doses administration begun at the rats’ age of 49 days. Animals were injected i.m., daily, for 10 consecutive days as follows: Aquatest (Balkan Pharmaceuticals Ltd., Moldova) testosterone aqueous solution: Testosterone I group (0.4 mg/animal); Testosterone II (0.8 mg/animal); (Deca-Durabolin, Balkan Pharmaceuticals); nandrolone decanoate oily solution: Nortestosterone I (1.5 mg/kg body weight); Nortestosterone II (7.5 mg/kg body weight) and Control (White sesame oil, Manicos, Romania, 0.1 mL/animal). Gonadectomy (GDX) induced modifications of target tissues wet weight accompanied by important modifications in cytoarchitecture. Changes following exogenous administration of testosterone and nortestosterone decanoate were found in: liver (granular dystrophy, mega-mitochondria, tubular intumescences), prostate (increasing of the structural elements), seminal vesicles (hyalinosis, thickening of cell walls and the hyaline presence), levator ani–bulbo-cavernosus muscle (muscle fibbers dilacerations), bulbourethral glands (muscular fibbers rarefaction by fluid accumulation) demonstrating the disruptor activity especially for overdosed nandrolone decanoate.

Keywords: andrology, cytoarchitectonics, dependent tissues, steroids, Hershberger.

Introduction
The endocrine-disrupting chemicals are widely studied in the recent years. The essential role played by the endogenous and especially of exogenous steroids in male gametogenesis has opened a vast research area into the human andrology. In this aim, numerous conceived models in castrated lab animal males proven that the main changes to the androgen action can be found especially in the weight and cytoarchitecture of five sexual target organs and tissues, namely: prostate, seminal vesicles, levator ani–bulbo-cavernous muscle, Cowper (bulbourethral) glands and glans penis [1–5]. Following described methodologies, ineditied morphologic and cytohistological aspects can be revealed yet about the disrupting activity of androgenic substances with relevance both to human and animal reproduction [6, 7].

All these experimental models appeared as a need to evaluate the threat for public health (as abusive use in the human subjects and in the livestock animals) that androgens can constitute. In fact, anabolic steroids are controlled substances in numerous EU countries. Even so, great amounts of steroids there are put up to sale for medical, but also for non-medical purposes (like bodybuilding), often are followed by many undesired consequences [8, 9–13].

Due to the complexity of mammalian biology, testing yet in animal models, currently, and in near future, will remain the key to chemical hazards to human reproduction assessment [11, 14–17].

Our study wants to follow cytoarchitectonics aspects for sex tissues accessory following the administration of testosterone aqueous solution and nandrolone decanoate, anabolic steroids largely used in medical and non-medical
purposes in the light of one of the most used protocols: the Hersherberger. The methodology described can be helpful in similar evaluations of newer or classical endocrine disruptors, the evaluation of testosterone action effects compared to the nortestosterone decanoate’s upon cytoarchitectonics of steroid dependent target tissues may be considered as an element of interest in andrology.

Materials and Methods

The research respected the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, adopted in Strasbourg on 18 March 1986 (published in Romania’s Official Monitor 685 of 10.08.2006) [18], the Directive 2010/63/EU (adopted on 22 September 2010) [19] and has the agreement of the Ethics Commission of the Faculty of Veterinary Medicine, Banat’s University of Agriculture and Veterinary Medicine, Timișoara, Romania.

Lab animals

Study was performed on 30 Wistar male rats, aged between 37 and 38 days, purchased from the authorized Animal Facility of the “Victor Babes” University of Medicine and Pharmacy, Timișoara. Animals with body weight between 255 and 290 g were divided into five groups as follows: Testosterone I (TI); Testosterone II (TII); Nortestosterone I (NTI); Nortestosterone II (NTII) and Control (C). Prior the study, the animals were acclimatized for seven days, being maintained in standard cages at controlled temperature and humidity. For this purpose, animals were housed in polycarbonate cages with 750×720×360 mm (L×w×h) dimensions, as bedding wood shaving being used. The environmental temperature was maintained at 20±2°C and relative humidity of 55±10%. During acclimatization period, the light cycle was 12 hours light and 12 hours dark. Non-sterile pelleted diet (code 140–501, Biovetimix, Romania) and water were offered ad libitum.

Androgens administration

Bilateral orchidectomy very similar to the rabbit castration procedure was performed after the technique described by Mateş [20].

The commercial nortestosterone used in experiment were testosterone aqueous suspension (Aquatest) and nandrolone decanoate (Deca-Durabolin), purchased from Balkan Pharmaceuticals Ltd., Moldova. Androgen doses administration begun at the rats’ age of 47–48 days, the individual differences in body weight being considered a source of variability in target tissues. The animals were injected daily at 14.00 P.M. for 10 consecutive days, following within 24 hours after the administration protocol presented in Table 1.

Table 1 – Protocol of substances administration in castrated rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Subjects No.</th>
<th>Administered substance</th>
<th>Dose</th>
<th>Time/administration way</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone I</td>
<td>6</td>
<td>Aquatest (Balkan Pharmaceuticals Ltd., Moldova) testosterone aqueous solution (50 mg/mL)</td>
<td>0.4 mg/animal</td>
<td>Daily, for 10 days/i.m.</td>
</tr>
<tr>
<td>Testosterone II</td>
<td>6</td>
<td>Aquatest (Balkan Pharmaceuticals Ltd., Moldova) testosterone aqueous solution (50 mg/mL)</td>
<td>0.8 mg/animal</td>
<td>Daily, for 10 days/i.m.</td>
</tr>
<tr>
<td>Nortestosterone I</td>
<td>6</td>
<td>Deca-Durabolin (Balkan Pharmaceuticals Ltd., Moldova) nandrolone decanoate oily solution (200 mg/mL)</td>
<td>1.5 mg/kg body weight</td>
<td>Daily, for 10 days/i.m.</td>
</tr>
<tr>
<td>Nortestosterone II</td>
<td>6</td>
<td>Deca-Durabolin (Balkan Pharmaceuticals Ltd., Moldova) nandrolone decanoate oily solution (200 mg/mL)</td>
<td>7.5 mg/kg body weight</td>
<td>Daily, for 10 days/i.m.</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>White sesame oil (Manicos, Romania)</td>
<td>0.1 mL/animal</td>
<td>Daily, for 10 days/i.m.</td>
</tr>
</tbody>
</table>

Organs and tissue collecting

In 24 hours after the last administration and accordingly to the standard known procedures rats were euthanized. Euthanasia method used was standard, by overdosing anesthetic agents using association: Ketamine (300 mg/kg body weight) + Xylazine (30 mg/kg body weight) [21]. After sampling and fresh wet target organ weight registration the histological sections were accomplished.

Cytohistological exam

Tissue fragments for cytohistological investigations were taken from the ventral prostate, seminal vesicles, levator ani–bulbocavernous muscle, Cowper’s bulbourethral glands and respectively liver (as metabolizing organ) and were fixed in ethanol 80%, after which they were washed, dehydrated and included in paraffin. Paraffin blocks containing tissue fragments were sectioned on microtome, resulting in 5-µm thick sections, after which they were mounted on glass slides with Mayer’s albumin. Sections were stained using the Hematoxylin–Eosin (HE) method. Microscopy was performed according to the tissue, to the objectives ×10 and ×20, the images being processed to an Olympus CX41 microscope, with image capture and data interpretation software.

Results

Clinical observations

The treated animals supported very well the small amounts of administrated substances. Though the administration duration was of 10 days any swelling, other general or local modifications were not observed to the animals from this study. The appetite and normal behavior was present in all situations and even weight gain was ascertained in all research groups. In Table 2 is presented the weight evolution in the studied groups before and after administrations.

Table 2 – Body weight of rats from experimental and control groups, initially and after exposures [g]

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial</th>
<th>After</th>
<th>Initial</th>
<th>After</th>
<th>Initial</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>253.83</td>
<td>270.5</td>
<td>287.83</td>
<td>293.33</td>
<td>285.66</td>
<td>306.83</td>
</tr>
<tr>
<td>TI</td>
<td>±27.93</td>
<td>±29.22</td>
<td>±29.47</td>
<td>±33.46</td>
<td>±23.02</td>
<td>±26.48</td>
</tr>
<tr>
<td>TII</td>
<td>±27.93</td>
<td>±29.22</td>
<td>±29.47</td>
<td>±33.46</td>
<td>±23.02</td>
<td>±26.48</td>
</tr>
</tbody>
</table>
Cytoarchitecture of steroid dependent target tissues after testosterone administration compared to nandrolone...

<table>
<thead>
<tr>
<th>Control</th>
<th>TI</th>
<th>TII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>After</td>
<td>Initial</td>
</tr>
<tr>
<td>Weight gain [%]</td>
<td>Weight gain [%]</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>NTI</td>
<td>NTII</td>
</tr>
<tr>
<td>Initial</td>
<td>After</td>
<td>Initial</td>
</tr>
<tr>
<td>6.56</td>
<td>1.91</td>
<td>7.41</td>
</tr>
<tr>
<td>253.83 ±27.93</td>
<td>270.51 ±20.11</td>
<td>309.33 ±18.62</td>
</tr>
</tbody>
</table>

TI: Testosterone I; TII: Testosterone II; NTI: Nortestosterone I; NTII: Nortestosterone II. Each value is mean ± standard deviation of six animals.

Cytohistological observations

The main changes following administration of exogenous testosterone or nortestosterone decanoate, in different doses, observed in castrated male rats are represented in Figures 1–4.

Important histological changes identified by us were present in: liver (Figure 1), prostate (Figure 2), seminal vesicles (Figure 3), levator ani muscle and bulbourethral glands (Figure 4), all being followed by the modification on weight of fresh (wet) accessory sex tissues from the castrated rats as presented in Table 3.

Figure 1 – Liver’s histological section in castrated males from NTII, TI and Control (C) groups (HE staining, ×100). NTII: Liver’s granular dystrophy: hydroprotidic dystrophy and steatosis, hepatocytes with pale cytoplasm and larger than normal, due to micro- and macrovesicular steatosis, thickening of intima tunic of the central vein (arrow). TI: Liver cells hypertrophied and cytoplasm numerous reddish fine granules, due to increased mitochondria volume and a blurred appearance (tubular intumescences), numerous nuclei in karyopyknosis in the area near the central vein. C: Normal aspect of the hepatocytes in size and shape; hepatic lobule with interlobular branches: central vein (arrow).

Figure 2 – Prostate histological section in castrated males from NTII, TI and C groups (HE staining, ×200). NTII: Increased volume of the alveolar lumen, in detriment of epithelial cells height (atrophy of structural elements), associated with enptiness (arrow) of the great majority of alveoli content. TI: Slight histological changes due to testosterone treatment, increased volume of prostate alveoli, but with slight modification of the secretorial function and without epithelial atrophy (normal height). C: Normal histological aspect of the prostate with alveolar epithelia integrity and secretion activity (arrow – normal secretion fluid).

Figure 3 – Seminal vesicle histological section in castrated rat males from NTI, TI and C groups (HE staining, ×200). NTI: Very thick primary folds and a visible decrease of them number, even the absence of tertiary folds. TI: Thick primary folds and well-expressed secondary and tertiary folds with slight hyalinosis, characterized by the excessive storage of hyaline substance (arrow). C: Normal histological aspect of the seminal vesicles where the glandular folds and all cytoarchitecture, including secretory features were totally preserved.
Figure 4 – Levator ani muscle and bulbourethral gland histological sections in castrated males from NTII group (HE staining, ×200). Massive dilacerations and ruptures of striated muscular fibers in levator ani muscle samples and muscular fibers rarefaction and fluid accumulation increase in volume and weight to bulbourethral glands.

Table 3 – Effects of testosterone and nandrolone decanoate on weight of accessory sex tissues from castrated rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Ventral prostate [mg]</th>
<th>Glans penis [mg]</th>
<th>Cowper’s glands [mg]</th>
<th>Seminal vesicles [mg]</th>
<th>LABC [mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>21.03±2.43</td>
<td>50.23±3.67</td>
<td>6.96±0.89</td>
<td>54.83±4.39</td>
<td>153.13±18.45</td>
</tr>
<tr>
<td>TI</td>
<td>171.60±11.16</td>
<td>76.83±7.28</td>
<td>41.13±3.12</td>
<td>443.35±45.36</td>
<td>559.08±33.56</td>
</tr>
<tr>
<td>TII</td>
<td>235.53±15.61</td>
<td>91.08±3.79</td>
<td>43.81±3.58</td>
<td>757.65±39.97</td>
<td>710.13±34.89</td>
</tr>
<tr>
<td>NDI</td>
<td>178.46±15.12</td>
<td>90.15±3.9</td>
<td>57.48±2.65</td>
<td>750.05±46.15</td>
<td>930.62±50.53</td>
</tr>
<tr>
<td>NDII</td>
<td>164.26±8.54</td>
<td>87.68±2.51</td>
<td>62.25±1.19</td>
<td>1003.76±58.57</td>
<td>1053.00±34.83</td>
</tr>
</tbody>
</table>

C: Control; TI: Testosterone I; TII: Testosterone II; NTI: Nortestosterone I; NTII: Nortestosterone II; LABC: Levator ani–bulbocavernous muscle. Each value is mean ± standard deviation of six animals.

In Figure 1 are presented liver sections (HE staining, ×100) in groups NTII, TI and C. The cytohistological images revealed the liver’s granular dystrophy; hydroprotic dystrophy and steatosis (NTII group) characterized by the entry and storage of serum proteins in mitochondria, causing the mitochondria swelling (mega-mitochondria). Hepatocytes presented a pale cytoplasm and larger than normal (revealing the hypertrophy) due to micro- and macrovesicular steatosis as a following of the endocrine disruptor activity. Also, was observed the thickened of the intimae tunic of the central vein. Comparatively to Control, in the case of TI group, liver cells appeared hypertrophied and in its cytoplasm numerous reddish fine granules were present due to increased mitochondria volume and a blurred appearance resulting (tubular intumescences). In the area near the central vein, we observed also numerous nuclei in karyopyknosis.

Figure 2 is revealing the prostate sections (HE staining, ×200) for NTII, TI and C groups. Normal histological aspect of the prostate (C) with alveolar epithelia integrity and secretion activity (arrow – normal secretion fluid) was in great contrast with a very well expressed growth by volume and the massive increase of the structural elements found by us in NTII group. Here, the increased volume of the alveolar lumen, in detriment of epithelial cells height (atrophy of structural elements), associated with emptiness (arrow) of the great majority of alveoli content may justify the prostate’s hypofunction, due to the activity of nandrolone decanoate was clearly observed comparatively with only slight histological changes due to testosterone treatment in TI group. Here were observed also the increased volume of prostate alveoli, but with slight modification of the secretorial function and without epithelial atrophy (normal height).

In Figure 3 are depicted the images of seminal vesicles (HE staining, ×200) sections in groups NTI, TI and C. The image of seminal vesicles, because of nandrolone decanoate activity, revealed very thick primary folds and a visible decrease of them number, even the absence of tertiary folds, comparatively with the normal histological aspect of the seminal vesicles where the glandular folds and all cytoarchitecture, including secretory features were totally preserved. In the TI group, we observed thick primary folds and well expressed secondary and tertiary folds with slight hyalinosis, characterized by the excessive storage of hyaline substance (arrow), in our opinion, due to testosterone activity.

In Figure 4 are presented the modifications for levator ani muscle and bulbourethral glands (HE staining, ×200) that we have ascertained only in the case of NTII group. Massive dilacerations and ruptures of striated muscular fibers, due to nandrolone decanoate activity which impairing the organ’s function were found in levator ani muscle samples from group NTII. Similarly, muscular fibers rarefaction and fluid accumulation increase in volume and weight was detected also to bulbourethral glands.

All these findings are confirming the important deleterious activity as endocrine disruptor on the target tissues for androgens of nandrolone decanoate (nortestosterone) comparatively with testosterone. The important changes in histoarchitecture of these target organs that we have ascertained, only after 10 days administration, are followed with certainty by functionality impairment due to this more powerful and newer testosterone ester.
Discussion

Reproductive and developmental disorders can be considered as a significant source of health detriment. In this respect, anabolic steroids are accepted as reference substances in terms of endocrine disruption, because they largely remain the most bioactive compounds, exerting their actions by different general mechanisms [22–25].

Authors suggested that deleterious effects of nandrolone on the reproductive tract would be due to a disruption of feedback regulation on the way of hypothalamic-pituitary-gonadal axis by the exogenous agent with palpable histostructural effects [26, 27].

Anabolic substances used by humans or in livestock destined for the human consumption, through their long-term effect, can generate neuro-hormonal signals that will transmit the information to the hypothalamic anterior-pituitary levels, to interstitial Leydig cells, Sertoli cells and to the germinative seminal epithelium.

At cellular level, the steroid-converting enzymes are responsible for the induction of the androgens within the particular target tissues. In these reproductive target tissues, testosterone is acting as a pro-hormone, being converted to the more potent androgens (e.g., dihydrotestosterone) by 5α-reductase enzyme. In addition, some synthetic substances (e.g., 5α-reductase inhibitors) inhibit the conversion of testosterone to dihydrotestosterone. Such substances have the potential to produce adverse health effects, especially, of the reproductive function or of body development [28, 29].

Consequently, cyto-architecture will be affected and consequently, fertility and fecundity can be inhibited by altering the function of any of these levels of male genitalia.

For example, Kumar et al., have investigated the endocrine disruption on reproductive processes and systemic toxicity in castrated and uncastrated male rats using Hershberger’s protocol. Results revealed that to uncastrated males from the control group, the mean testosterone obtained was of 7.5 ng/mL, but in castrated rats group treated water samples was much greater, of 10.40 ng/mL, confirming similar results obtained by us using the same protocol [30].

Nandrolone decanoate is known as a multifaceted substance with both beneficial and harmful properties [31]. For example: in case of postmenopausal osteoporosis [32]; on weight and lean body mass in HIV-infected humans [33]; to treat anemia associated with chronic kidney failure [34]; to treat prostate cancer and benign prostate hyperplasia, because nandrolone could not be converted into dihydrotestosterone, the most potent androgen [35].

On the other hand, numerous studies in the human medicine are demonstrating the effect on the testis of nandrolone, because fertility and fecundity can be inhibited by altering the cytoarchitecture, and finally, the reproduction function at any level of male genitalia and nandrolone can become easy an endocrine disruptor. It is known yet that prolonged nandrolone treatments in male, leads to a decrease of testosterone secretion [27], altered testicular morphology [30, 36], or sperm quality reduction [26, 37, 38].

All these important changes are mirrored also in cytoarchitectonics of the up mentioned tissues, investigations offering helpful information. In our study, we ascertained that the most important alterations at the cellular level were present in the experimental non-testosterone (nandrolone decanoate) group NTII (at dose of 7.5 mg/kg body weight), but in lower intensity, lesions were visible also to all experimental groups. Liver as metabolizing organ was affected granular dystrophy and mega-mitochondria observed by us, being the result of different states of androgens’ intoxication.

In this respect, Boissonneault [6] put in evidence apoptosis in the castration-induced atrophy of the rat’ levator ani muscle. In our case, due to volume and weight increase we have observed in the (bulbocavernous) levator ani muscle only slight muscle fiber dilacerations, uniquely in the case of NTII group (the greatest dosage) lesions being really significant.

Shabsigh et al. [7] demonstrated that androgens can influence prostate’s size by regulating blood flow to the prostate gland. Our observations proved the significant growth in weight and volume of this organ and of seminal vesicles. In our opinion, these tissues are reacting firstly and very quickly to the administered androgens. About the hyaline presence in ventral prostate in some castrated males from the experimental groups, we consider that is a pathological process that forms over time, but is not necessary related to the of administration androgenic substances.

Considering the experimental animal models that may be used in human andrology, we believe as great majority of authors, that experimental model using the rat is the most viable and a long-term alternative. Rats are preferable to mice, hamsters or others, due to their well studied andrologic characteristics very suitable for research in andrology [1, 4, 14, 15, 26, 36].

Conclusions

Consecutive administration of androgens to the castrated male rats we can confirm the deleterious activity after prolonged doses of testosterone and nandrolone decanoate. We observed that, in order, most obvious cyto-morphological changes occurred in the groups: NTII followed by NTI, TII and respectively TI groups, tissues weight and histoarchitecture suggesting that nandrolone has a certain and important role as endocrine disruptor.

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References


