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ORIGINAL ARTICLE

The Effect of Testosterone, Nandrolone and Their Combination on the Structure and Ultrastructure of Muscle Fibres and Myofibrils in Pigs

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Keywords: anabolic steroids | electron microscopy | histology | muscles | pigs

ABSTRACT

There is increasing pressure on meat producers worldwide due to the need for higher yields and improved meat quality. This is why anabolic androgenic steroids (AAS) have been widely used in most countries, due to their ability to accelerate animal muscle growth. However, out of concern for their side effects, EU states have banned their use and implemented control mechanisms. But they are reaching their limits, and therefore, it is necessary to look for new ways and investigate the mechanism of action of AAS on muscle tissue. This study replicated the administration of banned AAS (testosterone, nandrolone and their combination) and observed their effect on pig muscle. The pig model was purposely chosen for the study, as no such research has been carried out on this species. At the same time, pork is one of the most consumed meats in Europe. It focused on histological changes in muscle structure, specifically the size of muscle fibres and the number of satellite cells per muscle fibre. Furthermore, ultrastructural changes in muscle fibres, the diameter of myofibrils, the number of myofibrils per area, the distance between myofibrils and the size of sarcomeres were examined. The results using the techniques of histology, fluorescent labelling and transmission electron microscopy showed that, after the application of AAS, there is an increase in the diameter of muscle fibres, an increase in the diameter of myofibrils, a decrease in the number of myofibrils per surface area and, in the case of testosterone, an increase in the distance between myofibrils and an increase in the length of sarcomeres. There was also a significant increase in the number of satellite cells per muscle fibre. The detected statistically significant differences between control and experimental groups provide evidence that selected histological parameters could be additional mechanisms for detecting the presence of AAS in pork meat in the future.

1 | Introduction

Meat consumption increased year-on-year by 0.8–84.0 kg per inhabitant. The most consumed meat is pork, with a share of 51.7%, followed by beef at 10.5% and poultry at 35.5% (European Commission 2023). With the growing demand for meat, the pressure on producers is also increasing, so the illegal use of growth-accelerating substances is becoming more and more frequent. Anabolic androgenic steroids (AAS) are most often used. Most world legislation allows using several AAS-based preparations for cattle and sheep fattening (up to 90% of cattle in fattening outside the EU, at least one type of anabolic growth

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stimulator is used). However, the European Union does not agree with the statement about the harmlessness of AAS for human consumers. Therefore, in EU member states, the use of AAS for fattening is strictly prohibited by law. However, they are often misused illegally to gain a competitive advantage in farm animals, in unauthorised doses and combinations (Official Journal of the European Union 2022; Official Medicines Control Laboratories 2022).

Anabolic steroids are chemically synthetic derivatives of the male sex hormone testosterone with increased anabolic activity and reduced androgenic activity. The effect is the acceleration of animal growth and, above all, an increase in the amount of muscle mass (Cole et al. 2019). However, AAS also have a large number of serious side effects. They cause a reduction in the quality and number of sperm, atrophy of the testes and hypertrophy of the clitoris, or complete infertility (Cole et al. 2019; Scarth et al. 2009; Šťastný et al. 2020). AAS cause damage to liver tissue, disorders in the formation and outflow of bile and dilation of blood vessels and their rupture (Mohammed et al. 2017; Navarro et al. 2017). In the kidneys, atrophy, deformation of glomeruli and rupture of glomerular walls occur. Damage to the urinary tubules and their necrosis is visible, which can lead to progressive kidney failure (Kahal and Allem 2018; Yarrow et al. 2011). In the heart tissue, after the administration of higher doses of AAS, vasocongestion, retraction of muscle fibres and rupture of muscle fibres occur. Reduced pumping efficiency of the heart is reported (Kahal and Allem 2018; Seara et al. 2017). Another negative effect of AAS has been demonstrated on brain development, causing brain atrophy and changes in behaviour (Cole et al. 2019; Šťastný et al. 2020).

The topic of AAS is very current worldwide. However, most studies on this topic have been performed in animal models such as mice (Carson and Manolagas 2015; Fontana et al. 2013), rats (Abeed et al. 2022; Elgendy et al. 2018; Hassan et al. 2023; Mohamed and Mohamed 2021; Sretenovic et al. 2018), broilers (Elmajdoub et al. 2016), guinea pigs (Appel et al. 1983) and rabbits (Tousson 2013). A large number of studies are also realised on the control of AAS in doping in sports where horses (Hyyppä 2001) or humans (Conceição et al. 2018; Eriksson et al. 2005; Hartgens et al. 2001) are studied. However, it is interesting that although in most European countries pork is consumed to a greater extent than beef and lamb, there are studies on cattle (Clancy et al. 1986; Johnson et al. 1998; Kellermeier et al. 2009) and sheep (Gerber et al. 2012). If it can be summarised, the effect of AAS on muscle structure is mainly solved by studies on laboratory animals (Sessa et al. 2022).

Most studies agree that AAS causes an increase in the diameter and area of muscle fibres (Clancy et al. 1986; Conceição et al. 2018; Elgendy et al. 2018; Eriksson et al. 2005; Fontana et al. 2013; Hyyppä 2001; Kellermeier et al. 2009; Mohamed and Mohamed 2021; Yu et al. 2014). Furthermore, the increasing proportion of collagen fibres in the intercellular space is described (Sretenovic et al. 2018). Also hydrophobic changes in myofibrillar structure and nuclear pyknosis (Tousson 2013) were observed. Articles by Mohamed and Mohamed (2021) and Abeed et al. (2022) describe drastic changes in the structure and also the ultrastructure of muscles, after the application of AAS nandrolone decanoate to rats. For example, they describe apparent muscle fibre hypertrophy, wide spacing between fibres and impaired transverse striations. Furthermore, the articles describe migrating satellite cells that are preparing for regeneration. Even a large number of human studies generate similar results. After the application of testosterone in humans, very pronounced hypertrophy of muscle fibres, a higher number of nuclei in the fibres (Eriksson et al. 2005) and severe degenerative changes with nuclear pyknosis of the sarcoplasm are described (Hassan et al. 2023).

Where the studies differ greatly is the role of satellite cells in muscle. Most of the original hypotheses assume that, after the application of AAS, satellite cells will be activated, their number will increase and they will subsequently fuse into new muscle fibres. Conceição et al. (2018) reported that hypertrophy induces an increase in the number of nuclei in muscle. A study by Yu et al. (2014) agrees with this conclusion, claiming that AAS use is associated with the activation of satellite cells and their fusion with pre-existing muscle fibres, leading to a higher number of myonuclei. Other studies report only a numerical increase in satellite cells, which would imply that the growth is not accompanied by the formation of new myonuclei (Asfour et al. 2021). Other results describe an invariant number of myonuclei and the activity of satellite cells after AAS stimulation (Almeida et al. 2016; Brack and Rando 2012; Chen et al. 2020). Horwath et al. (2020) outline the possibility that there might be a limit to where muscle fibres can grow even without the participation of satellite cells. Thus, the clear role of satellite cells remains unclear.

In general, very few studies describe the effect of AAS on pig muscle, as mentioned above. However, the effects of the combination of testosterone and nandrolone have not yet been studied in a pig model, although this combination is widely used worldwide. This study aims to confirm the hypothesis that AAS application leads to changes in the skeletal muscle of pigs at the level of structure and ultrastructure. Therefore, the long-term effects of the administration of testosterone, nandrolone and their combination on the muscles of pigs, specifically on the parameters of muscle fibres and myofibrils, were studied.

2 | Materials and Methods

2.1 | Ethics Statement

The experiment, involving the administration of steroids and feeding the pigs, ran at the Veterinary Research Institute in Brno, Czech Republic. The slaughter of animals and collection of samples was carried out at a slaughterhouse in Skalice nad Svitavou, Czech Republic. The laboratory work was carried out at Mendel University in Brno, Czech Republic. All experimental procedures were approved by the Central Commission for Animal Protection of the Czech Republic, serial number MZe 2081, no. 6931/2019-MZE-17214 on 7 February 2019.

2.2 | Animals and Protocol

Overall, 24 hybrid pigs of the OL 48 line (Bioprodukt Knapovec Corp., Czech Republic), that is, hybrids of Large White ×

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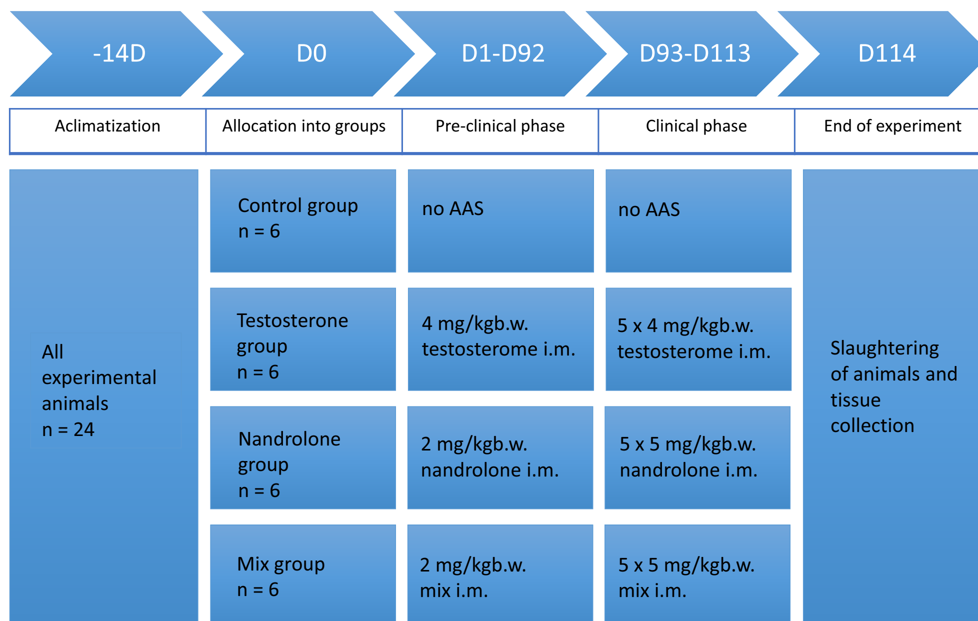
TABLE 1 | Schematic diagram of the experimental model.



Landrace (sow) × Duroc (boar), were used for the experiment. The pigs were divided into four groups of six based on the different applications and dosages of steroid drugs. The first group was administered testosterone—Sustanon preparation at 250 mg/mL (30 mg Testosterone propionas; 60 mg Testosterone phenylpropionas; 60 mg Testosterone isocaproas; 100 mg Testosterone decanoas propionate and a total amount of 17β-testosterone at 176 mg/mL, Organon, CZ, Reg. no. 56/357/91-C); the second group was administered nandrolone—the veterinary preparation Myodine at 25 mg/mL (ester-laureate of 19nor-17beta-testosterone, Le Vet Beheer B.V., The Netherlands, CZ Reg. no. 96/030/17-C); a mixture of the previous two preparations was administered to the third group (Sustanon and Myodine in a ratio 1:1); and the fourth group was a control group without the application of any anabolic steroid hormones. The groups were designated as the control, testosterone, nandrolone and mixed groups. To verify the pharmacokinetics, the first 92 days 4 mg/kg bw testosterone im, 2 mg/kg bw nandrolone im and 2 mg/kg bw mix im were administered to 2-month-old pigs. In the second phase of the experiment, which lasted from days 93 to 114, the amount of administered steroid hormones was increased to obtain positive samples of all tissues. Pigs were given 5 × 4 mg/kg bw of testosterone im, 5 × 5 mg/kg bw nandrolone im and 5 × 5 mg/kg bw mix im, always after 4 days. AAS concentrations were selected based on the pharmacokinetic results of the first phase of the experiment. Pigs were slaughtered at the age of approximately 6 months, after reaching a slaughter weight of 120–130 kg bw Muscle tissue samples of a size of approximately 1 cm^3 were taken from the *Musculus longissimus dorsi* from each animal immediately after slaughter and were histologically processed. Animal carcasses were handled by applicable national legislation (Act No. 266/1999 Coll.) and European Union legislation (Regulation (EC) No. 1069/2009). For greater clarity, the experimental scheme is shown in a diagram (Table 1).

2.3 | Light Microscopy

Tissue samples were fixed in a 4% formaldehyde solution. Samples were washed under running water, dehydrated in ethanol and cleared with xylene and paraffin-embedded. Sections with a thickness of 5 μm were cut and stained for haematoxylin (Bamed, Czech Republic) and eosin (Merck KGaA, Germany). Histological preparations were observed on an Olympus BX51 microscope (Olympus, Tokyo, Japan) and scanned with a Promicam 3–5 CP camera (Promicra s.r.o., Czech Republic). Muscle fibre diameter was evaluated by the QuickPHOTO MICRO 3.2 program (Promicra s.r.o., Czech Republic).

2.4 | Transmission Electron Microscopy (TEM)

Samples were then prefixed in 3% glutaraldehyde in Millonig's buffer (Serva, Germany) for 24 h. They were washed with Millonig buffer and fixed in a 1% buffered osmium tetroxide solution (Serva, Germany). After osmium fixation, the samples were washed and dehydrated in 30%, 50%, 70%, 96% and 100% acetone for 30 min each. They were saturated in a mixture of acetone/epon + durcupan 1:1 for 1 h, 1:3 for 1 h and then twice for 12 h in a pure mixture of Epon 812 + Durcupan (Serva, Germany). The polymerisation took place for 96 h at 60°C. Ultrathin sections with a thickness of 60 nm were made on a Leica UC7 ultramicrotome (Leica, Austria). Sections were contrasted with 2% uranyl acetate and 1% lead citrate. The samples were observed under a Philips 208S Morgagni transmission electron microscope (FEI, Czech Republic) at a magnification of 3500–10,000×, and an accelerating voltage of 80 kV. The size of sarcomeres (distance between two Z-lines), the thickness of myofibrils and the distance between myofibrils were evaluated by the QuickPHOTO MICRO 3.2 program (Promicra s.r.o., Czech Republic).

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2.5 | Immunofluorescence Analysis

Muscle tissue samples were embedded in Tissue Tek cryoprotectant (O.C.T. Compound, Sakura Finetek, Torrance, CA, USA). They were frozen at −80°C. Sections with a thickness of 3 μm (5 sections from each sample) were made. Protein block (Baria, Prague, Czech Republic) was used for protein blocking for 30 min at room temperature. Samples were incubated for 60 min in the dark with primary antibodies Anti-Hu CD56 (Exbio, Prague, Czech Republic, at a dilution of 1:100) to label satellite cells and Laminin Rat Monoclonal Antibody (OriGene Technologies, Rockville, MD, USA, at dilution 1:800) to label the basement membrane of muscle fibres. Samples were washed in PBS + 0.1% Tween 20 (Merck KGaA, Germany), 3 × 4 min. Secondary antibodies were applied—goat anti-rat IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 488 (Thermo Fisher Scientific, Brno, Czech Republic, dilution of 1:1000) and goat anti-mouse IgG2a cross-adsorbed secondary antibody, Alexa Fluor 594 (Thermo Fisher Scientific, Czech Republic, dilution of 1:1000), and the slides were incubated for 60 min in the dark. The samples were washed again for 3 × 4 min. To stain myonuclei, samples were incubated for 10 min in the dark with DAPI (Merck KGaA, Germany). The samples were washed again for 3 × 4 min. Slides were mounted using ProLong Diamond Antifade Mountant (Thermo Fisher Scientific, Czech Republic). Slides were imaged using an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan) and scanned with a Promicam 3–5 CP camera (Promicra s.r.o., Czech Republic). The number of satellite cells per muscle fibre was determined using the QuickPHOTO MICRO 3.2 program (Promicra s.r.o., Czech Republic).

2.6 | Semi-Thin Sections

Tissue samples were fixed in a 4% formaldehyde solution. Samples were washed under running water, dehydrated in ethanol and cleared with xylene. The specimens in stocks were used for semi-thin sections were used the specimens in stocks, in the mixture of epoxy/durcopan resin embedded. The stocks were sliced into semi-thin sections of thickness 0.5 μm under an ultramicrotome Leica EM UC 7 (Leica, Austria) and stained with 0.5% Toluidine blue (Sigma-Aldrich, Germany). Histological preparations were observed on an Olympus BX51 microscope (Olympus, Tokyo, Japan) and scanned with a Promicam 3–5 CP camera (Promicra s.r.o., Czech Republic). The number of myofibrils per area (100 μm^2) was evaluated by the QuickPHOTO MICRO 3.2 program (Promicra s.r.o., Czech Republic).

2.7 | Statistical Analysis

The Kolmogorov–Smirnov test was used to determine whether the distribution of data was normal. Based on the normality of the data, an unpaired Student's *t*-test or one-way ANOVA test was performed for parametric data and the Mann–Whitney test for non-parametric data. The results were expressed as the mean ± SEM (standard error of the mean) and $p < 0.05$ was considered statistically significant. GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California USA) statistical software program was used for all statistical analyses. To confirm our hypotheses, a multivariate statistical analysis was performed using the PCA (principal component analysis) method in the program Statistica v. 13.3 (DataBon s.r.o., Praha, Czech Republic).

3 | Results

In general, AAS used in this study caused significant changes in muscle at both structural and ultrastructural levels. Structurally, there was an acceleration of the growth of muscle fibres and an increase in the number of satellite cells. Furthermore, inside the muscle fibres, there was an increase in the size of myofibrils and other changes.

3.1 | Changes in Muscle Fibres

Control group samples corresponded to the physiological description of muscle fibres. The longitudinal section of muscles demonstrated the appearance of regularly arranged, well-organised parallel muscle fibres with multiple peripheral oval vesicular nuclei located beneath the sarcolemma. Moreover, the transversely striated pattern of muscle fibres was visible. On the transverse histological section, compact muscle fibres with obvious nuclei distributed around the fibre were visible, and exceptionally, a centrally located nucleus was observed, both under the optical microscope and under fluorescence. Isolated satellite cells were seen beneath the sarcolemma. The fibres were separated by a small amount of endomysium. If we compared the control group of pigs with the experimental ones, statistically significant differences were found in fibre diameter and the number of satellite cells per fibre (as is evident in Table 2).

Statistically significant increases in muscle fibre diameter (hypertrophy) were observed between the control group and all experimental groups. The greatest apparent hypertrophy was noted in the group with nandrolone; the group with a mixture

TABLE 2 | Average measured values of structural parameters ± SEM.

	C	*T*	*N*	*M*
Fibre diameter (μm)	72.16 ± 0.89	95.54** ± 1.21	106.50** ± 1.10	94.42** ± 0.96
Number of satellite cells per fibre	0.14 ± 0.01	0.29** ± 0.01	0.45** ± 0.02	0.21** ± 0.01

Note: The groups were labelled as control (*C*), testosterone (*T*), nandrolone (*N*) and a mix of testosterone and nandrolone (*M*). Significant differences between control and AAS-administrated groups are marked with asterisks.
**$p < 0.01$.

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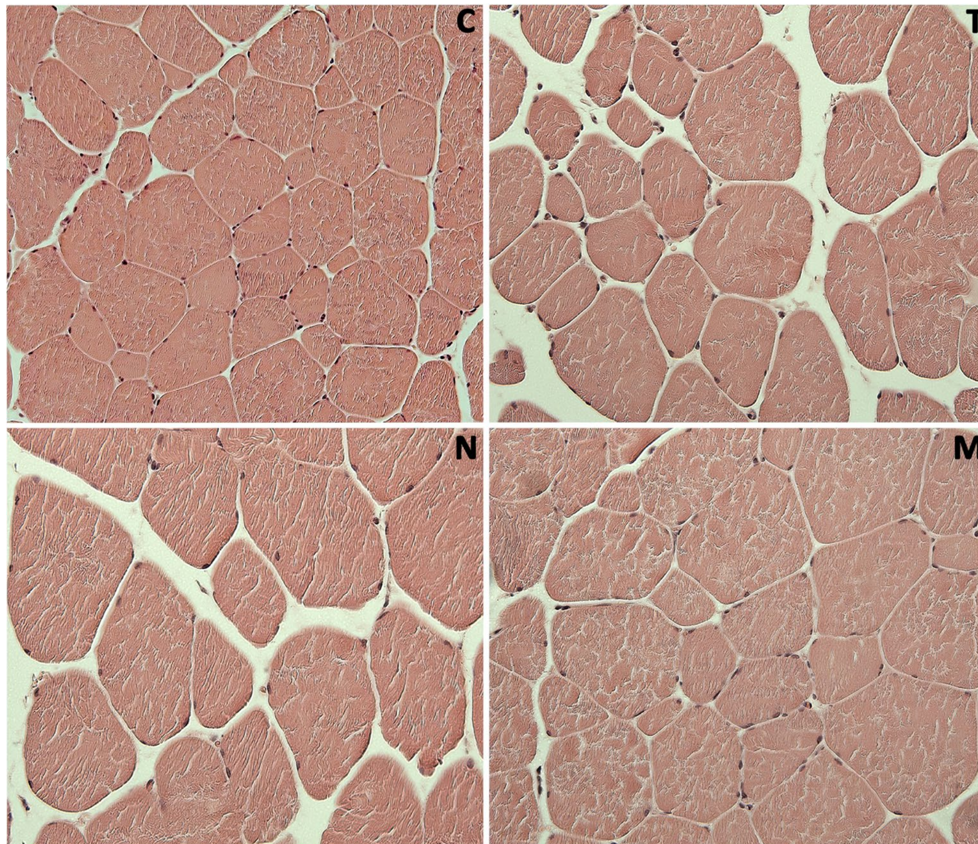

FIGURE 1 | Light microscopy of histological cross section of muscle fibres in control group without anabolic steroids (*C*), and in groups administrated by testosterone (*T*), by nandrolone (*N*) and with mixture of testosterone and nandrolone (*M*). Magnification 40×.

of testosterone and nandrolone differed the least from the control (Figure 1). The same pattern of changes was noted for the number of satellite cells. In the nandrolone group, the amount of satellite cells per fibre increased the most, more than three times compared to the control (Figure 2).

3.2 | Changes in Myofibrils

Analysis by light and transmission electron microscopy of semithin and thin sections revealed the physiological ultrastructure of myofibrils. These muscle fibre units were oval or rounded in cross section. The longitudinal section showed myofibrils as elongated structures with clearly distinguishable sarcomeres. At higher magnification, sarcomere components such as *Z* lines, actin and myosin filaments, intermyofibrillar mitochondria, *T* tubules and sarcoplasmic reticulum cisternae were distinguishable. Mostly, no changes in ultrastructure were noted compared to the control. However, wide spaces between myofibrils and slight fragmentation of myofibrils after testosterone and enlarged terminal sarcoplasmic reticulum cisternae after nandrolone administration were noted only in some samples (Figure 3).

In addition, at the ultrastructural level, we focused on myofibrils, specifically on their number, width, spacing and sizes of the basic sarcomere units. AAS has been found to cause changes at both the muscle fibre level and the myofibril level as is evident in Table 3.

There was a statistically significant increase in the diameter of myofibrils in all groups with applied AAS. The number of myofibrils per unit area is statistically significantly correlated with this when their number decreased in all experimental groups. The greatest changes were noted in the group with a mixture of testosterone and nandrolone when the number of myofibrils per $100\,\mu m^2$ was reduced compared to the control by 57.8% (Figure 4) and the diameter of myofibrils was reduced by 49.1% (Figure 5). The reason for the not entirely accurate correlation between the values could be the mutual distance of myofibres from each other due to AAS and thus the increased amount of intercellular mass between them. However, although the distance between myofibres was measured in the mix group, it was not statistically significant. The reason will most likely be the biological variability of the samples. The distance between the myofibres turned out to be statistically significant only in the testosterone group, by 6.1%. In all groups with applied AAS, there was an increase in the length of the sarcomere, but it was statistically significant again only in the testosterone group, where there was an increase compared to the control by 11.6% (Figure 6).

3.3 | PCA Analysis

The results of PCA analysis of five measured parameters (diameter of muscle fibres, diameter of myofibrils, number of myofibrils per area, size of sarcomere and number of satellite cells per muscle fibre) show that there was a clear differentiation into two clusters. The first cluster represents the control

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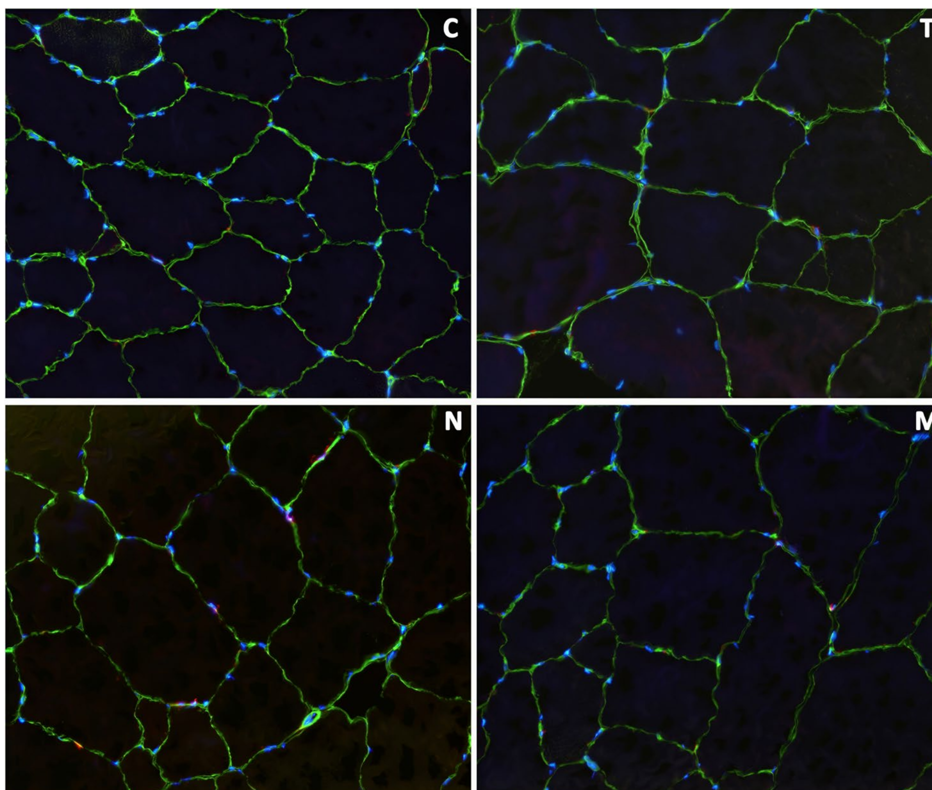

FIGURE 2 | Immunofluorescence analysis of satellite cells in muscle fibres in control group without anabolic steroids (*C*) and in groups administrated by testosterone (*T*), by nandrolone (*N*) and with mixture of testosterone and nandrolone (*M*). Sections were stained with an antibody against CD56 in satellite cells (red), laminin (green) and DAPI nuclear stain (blue). Magnification 40×.

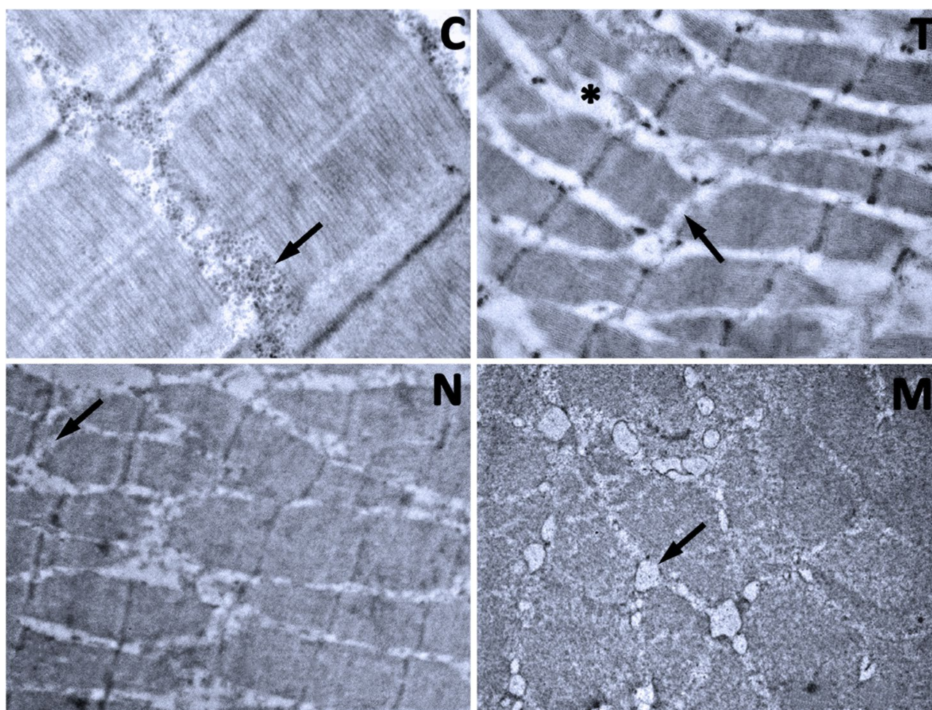

FIGURE 3 | Ultrastructural changes in myofibrils in control group without anabolic steroids (*C*) and in groups administrated by testosterone (*T*), by nandrolone (*N*) and with mixture of testosterone and nandrolone (*M*). Accumulation of glycogen granules between myofibrils in control (*C*, arrow). Disruption and myofibrillar loss (asterisk), disruption of *Z* lines in testosterone group (*T*, arrow). Disruption of myofibrils in nandrolone group (*N*, arrow). Dilatation of sarcoplasmic reticulum cisternae in mix group (*M*, arrow). Magnification 26,000× (*C*), 8900× (*T*, *N*), 12,600× (*M*).

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TABLE 3 | Average measured values of ultrastructural parameters ± SEM.

	C	*T*	*N*	*M*
Number of myofibrils (per 100 μm^2)	75.20 ± 1.35	40.66** ± 0.56	33.71** ± 0.54	31.72** ± 0.51
Myofibrils diameter (nm)	604.80 ± 12.68	715.20** ± 12.50	813.60** ± 10.98	1186.00** ± 33.24
Myofibril distance (nm)	153.00 ± 3.31	162.40* ± 3.94	160.30 ± 3.31	156.20 ± 3.87
Sarcomere length (nm)	1276 ± 7.84	1424* ± 8.95	1369 ± 14.28	1315 ± 16.77

Note: The groups were labelled as control (*C*), testosterone (*T*), nandrolone (*N*) and a mix of testosterone and nandrolone (*M*). Significant differences between control and AAS-administrated groups are marked with asterisks.
**$p < 0.01$.
*$p < 0.05$.

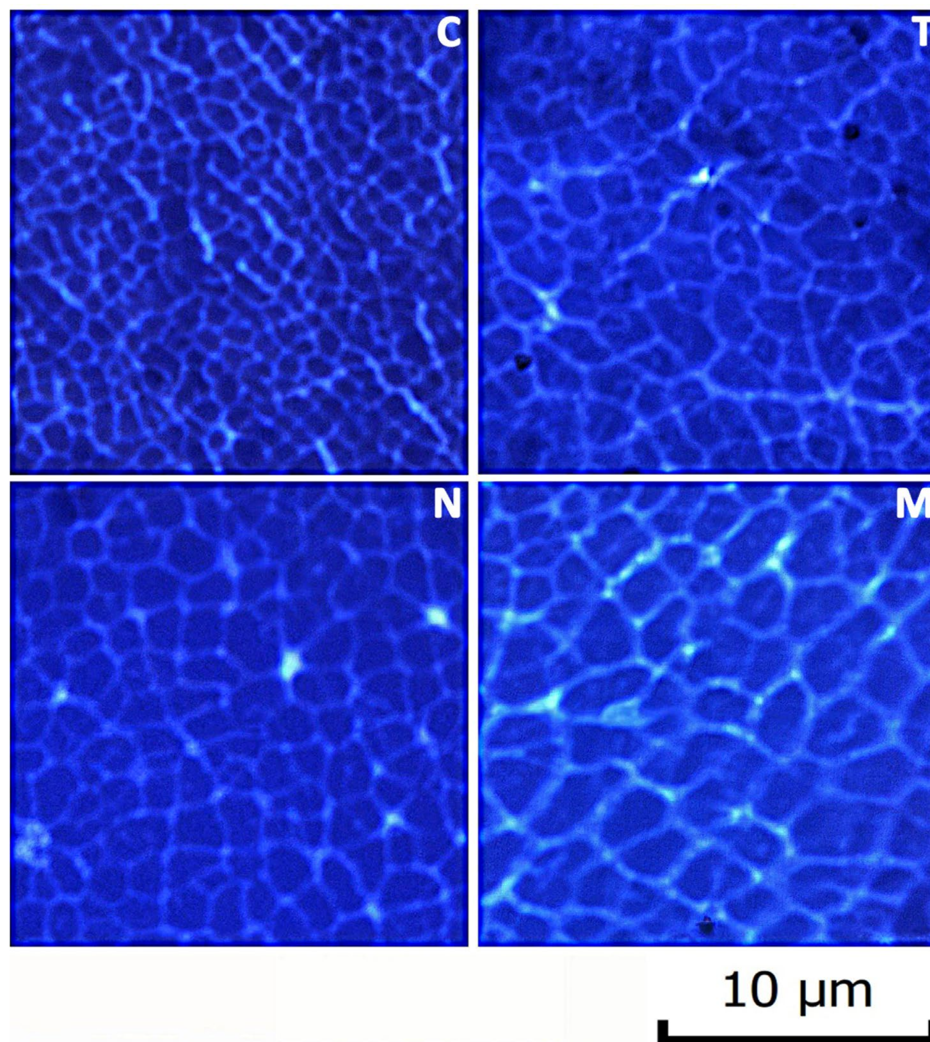

FIGURE 4 | Semi-thin cross sections of myofibrils in control group without anabolic steroids (*C*) and in groups administrated by testosterone (*T*), by nandrolone (*N*) and with mixture of testosterone and nandrolone (*M*). Magnification 1000×.

group, which was separated from the experimental groups with administered AAS (Figure 7). The two main components (Factor 1 and Factor 2) explain 40.40% and 24.33%, respectively, which together account for about 65% of the variability in the data. The PCA dot plot analysis clearly shows that AAS has a significant effect on muscle structure, and we can distinguish the experimental groups from the control based on these changes.

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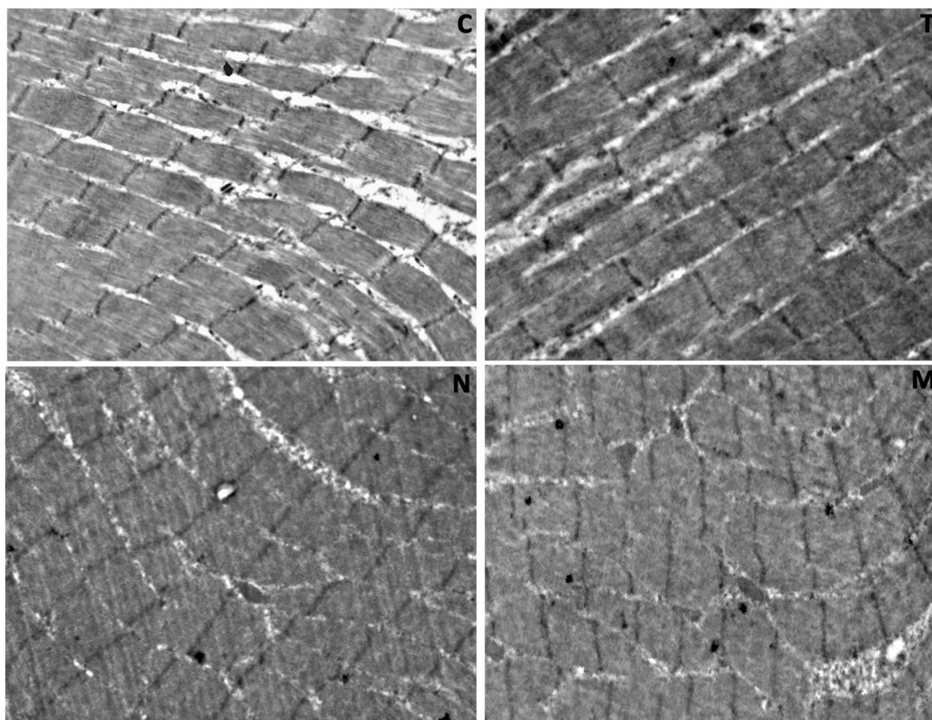

FIGURE 5 | Ultrastructural longitudinal cross section of myofibrils by transmission electron microscopy in control group without anabolic steroids (*C*), and in groups administrated by testosterone (*T*), by nandrolone (*N*) and with mixture of testosterone and nandrolone (*M*). Magnification 7100×.

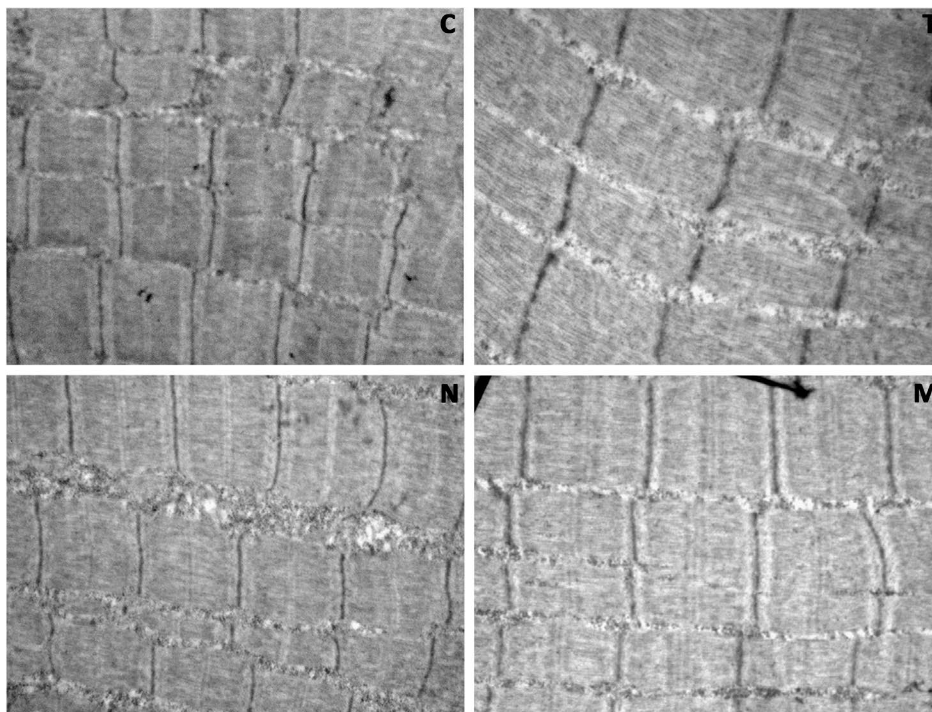

FIGURE 6 | Sarcomeres on longitudinal cross section of myofibrils by transmission electron microscopy in control group without anabolic steroids (*C*), and in groups administrated by testosterone (*T*), by nandrolone (*N*) and with mixture of testosterone and nandrolone (*M*). Magnification 8900×.

4 | Discussion

Our work hypothesis confirmed that testosterone, nandrolone and their mix cause changes in muscle fibres and myofibrils in the muscles of pigs on structural and ultrastructural levels. The results showed that AAS induces visible changes, whereas the most significant changes at the level of muscle fibre structure were caused by synthetic nandrolone. The greatest effect on

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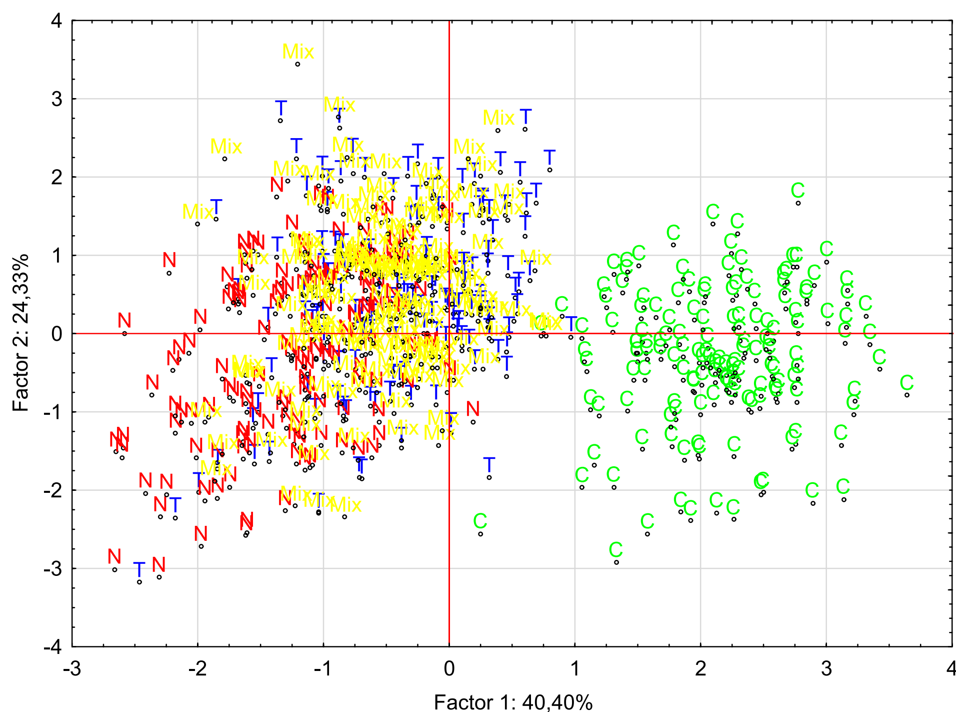

FIGURE 7 | Scatter plots built with Factor 1 (*x*-axis) and Factor 2 (*y*-axis) for principal component analysis (PCA) models calculated from measured values among the control group (*C*, green), testosterone group (*T*, blue), nandrolone group (*N*, red) and group with a mix of testosterone and nandrolone (Mix, yellow).

myofibrils ultrastructure had the mixture of testosterone and nandrolone.

In this study, testosterone, nandrolone decanoate and their mix were chosen because they are some of the most abused AAS. Testosterone was selected for its very easy availability and nandrolone for the highest anabolic: androgenic ratio of all AAS. Illegal use of both has often been reported in the EU in the past in livestock (Skoupá et al. 2022). However, currently, combinations of AAS are mainly abused in animal fattening due to detection limits. Therefore, we also choose a mix of testosterone and nandrolone in addition to testosterone and nandrolone alone for the study. As already mentioned above, studies carried out to determine the effect of AAS on the structure and ultrastructure of muscle were conducted on laboratory animals, mainly mice, rats, broilers and others. However, the effect of testosterone, nandrolone, and their combination has not yet been investigated in a pig model, and therefore, this is the first study to describe these effects.

Our study confirmed that AAS causes an increase in muscle fibre diameter. Testosterone, its derivatives and its metabolites induce an increased synthesis of muscle proteins in skeletal muscle through binding to androgen receptors located in the sarcoplasm (Vingren et al. 2010). Subsequently, they act as transcription factors and lead to changes in mRNA expression of up to thousands of genes (Jiang et al. 2009). Administration of supraphysiological levels of AAS thus promotes marked skeletal muscle hypertrophy (Horwath et al. 2020; Roberts et al. 2023). Studies suggest that this growth is due to satellite cell-mediated myonuclear addition to existing muscle fibres (Horwath et al. 2020; Yu et al. 2014). They are crucial in muscle tissue for growth and development and, above all, form a pool of muscle stem cells for remodelling processes after muscle damage. As muscle fibre diameter increased after AAS application, the number of satellite cells also increased in our study. After the application of nandrolone, the number of satellite cells increased almost three times; in the testosterone and mix group, the increase was approximately double compared to the control. These results agree with a large number of studies that claim that increased myogenic activity, that is, activation and subsequent fusion of satellite cells into muscle fibres, is the main mechanism of muscle growth after AAS application (Asfour et al. 2021; Horwath et al. 2020; Machek et al. 2021; Yu et al. 2014). Similar results have also been obtained in studies examining satellite cells in response to resistance training (Lundberg et al. 2020; Snijders et al. 2016; Oxfeldt et al. 2020). They describe that myoblasts derived from satellite cells associate with muscle fibres in response to muscle damage caused by resistance training. However, other studies report a statistically unchanged number of satellite cells after growth stimulation (Almeida et al. 2016; Brack and Rando 2012; Chen et al. 2020; Wozniak et al. 2005). For example, a study by Englund et al. (2019) showed that satellite cell-deprived mice show similar increases in muscle mass compared to testosterone-treated control mice, suggesting that satellite cells may not be required for AAS to induce hypertrophy.

This ultrastructure study of steroid-treated pigs showed an increase in the diameter of myofibrils by 18.25% in the testosterone group and an increase of 34.52% in the nandrolone group. The biggest increase was measured in the mix group, by 96.1%, that is, almost twice as much compared to the control. The

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number of myofibrils per area is negatively correlated with this, which decreased in all experimental groups. Increased intermyofibrillar space was noted in all groups with applied AAS, but it was not statistically significant and ranged from 2% to 6%. These observed changes are supported by the previous study on rats. However, the methodology used for processing muscle samples varied slightly in our study. Mohamed and Mohamed (2021) show that steroid-treated rats had enlarged myofibrils and wide spaces between myofibrils with localised areas of myofibrillar loss at the ultrastructural level. Also, Abeed et al. (2022) describe substantial changes in ultrastructure after the application of nandrolone decanoate to rats. They describe hypertrophy of myofibrils and the wide spacing between them. A similar hypertrophy was also described after the administration of AAS together with physical exercise, where the effect was even more obvious (Mohamed and Mohamed 2021; Mosler et al. 2012). The size of the sarcomere showed no relevant statistical significance; only in the testosterone group, there was a significant increase of 11.6%. Visible degeneration of sarcomeres was not demonstrated; only mild fragmentation of myofibrils and *Z* lines in some of the testosterone group was noted. The observation is again similar to the study by Mohamed and Mohamed (2021), where they describe that some myofibrils appeared with fragmentation and others showed disorganisation, lysis and disruption of *Z* lines. The continuity of the muscle fibres is interrupted by a large number of vacuoles and deformed nuclei, some of which move from the periphery to the centre of the fibres. At the ultrastructural level, myofibrils are also moving away from each other. Disrupted *Z*-lines of sarcomeres and congested mitochondria with degenerated cristae were observed.

5 | Conclusions

The application of AAS leads to changes in the skeletal muscle of pigs at the histological and ultrastructural levels. The application of testosterone, nandrolone and their mix for 4 months led to an increase in the diameter of muscle fibres, an increase in the diameter of myofibrils, a decrease in the number of myofibrils per area and in the case of testosterone, an increase in the distance between myofibrils and an increase in the length of sarcomeres. The mixture of testosterone and nandrolone had the greatest effect on myofibril ultrastructure, in comparison with nandrolone and testosterone alone. There was also a significant increase in the number of satellite cells per muscle fibre. Therefore, further studies are recommended looking more closely at the involvement of satellite cells in the growth process after AAS use.

Since the administration of AAS has a significant demonstrable effect on the structure and ultrastructure of skeletal muscle, it would be interesting to focus on the effect of AAS on cardiac muscle in further studies. Because the possible effect of AAS is also proven in cardiac muscle, especially retraction and rupture of muscle beams.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study can be requested from the corresponding author.

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