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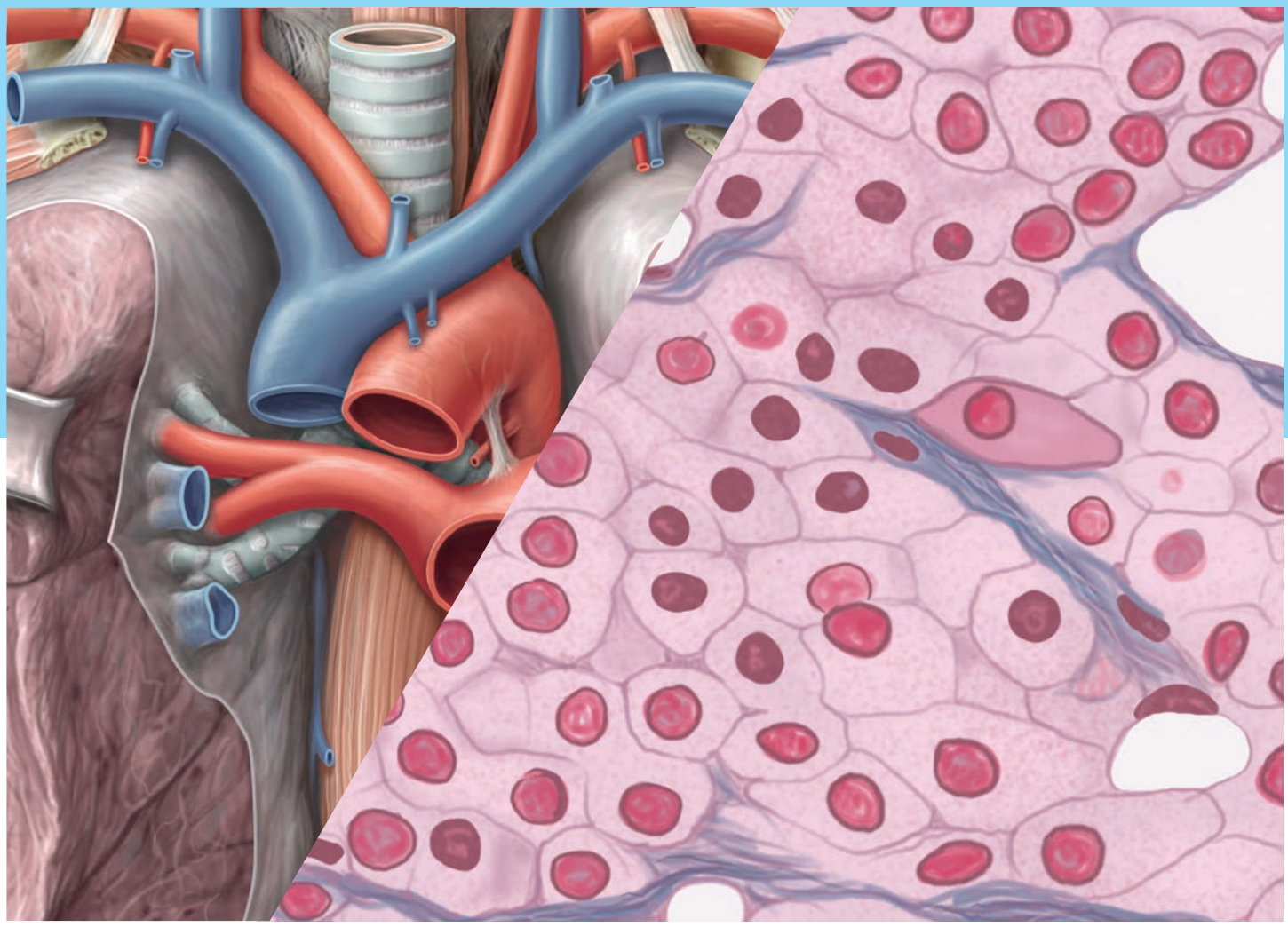

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Editorial

The new face of the Brazilian Journal of Morphology

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J Morphol Sci 2019;36:1.

Dear authors and readers,

The Journal of Morphological Sciences is facing changes on the editorial board to accomplish the international demands for higher indexations of the journal. This is a challenge for the new editorial board that will follow the new trends in science publications preserving the peer review process and keeping up with the high quality of the published manuscripts.

Editors and editorial board members were chosen based on criteria including not only the high standard scientific indexes and specific areas of expertise, but also the strong will of working hard to improve the Journal of Morphological Sciences in many different ways. We understand the importance of morphology as the foundation for the further understanding of several other biological and biomedical sciences, and we will work to keep the morphological sciences stronger as other sciences develop together with us.

We will broaden the areas of interest of the Journal of Morphological Sciences, and manuscripts dealing with imaging, neurosciences, locomotor system, dental anatomy, veterinary pathology, and histology will be strongly encouraged. We will continue to encourage publications on structure, functional morphology, animal development, as well as all levels of structural organization, from the submicroscopic to the macroscopic levels.

Finally, but most important, we would like to thank the herculean job done by the previous editors, together with the editorial board, who worked very hard to keep our journal running well and smoothly up to date.

Valéria Paula Sassoli Fazan, MD, PhD
Editor-in-Chief
Journal of Morphological Sciences

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Effects of Supraphysiological Doses of Anabolic Androgenic Steroids on the Left Ventricles of Male and Female Mice Submitted to Swimming

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Abstract

The use of anabolic androgenic steroids (AAS) has grown into a worldwide substance abuse problem over the last decades, with the doses taken by illegal users being 10 to 100 times higher than the therapeutic ones. In the present experiment, 60 mice were divided into 3 groups of 20 animals. Group 1 received testosterone cypionate (Deposteron [EMS, São Bernardo do Campo, SP, Brazil]); group 2 received stanozolol (Stanozolol Depot, Landerlan, Lambaré, Paraguay), and group 3 received saline solution), each one composed by 10 males and 10 females, treated once a week and put to swimming thrice a week for 2 months. After euthanasia, their chests were opened, the hearts removed and processed histologically for morphometric analyses. The specimens were cut into 6 different sections and each one was measured with the help of an optical microscope with a 40-fold magnification. For such analyses, the Axiovision Rel. 4.8.2 (Carl Zeiss Microscopy LLC, Peabody, MA, USA) and Axiovision 4 Module Interactive Measurement (Carl Zeiss Microscopy LLC) software were used. The results showed that there was an increase in the diameter of the left ventricles in the male mice treated with Deposteron while in the female animals treated with Winstrol, there was a decrease in the left ventricular diameter in relation to the other two groups. Thus, one can conclude that the use of supraphysiological doses of the given AAS significantly alters the ventricular diameter in both male and female animals, which can cause a considerable change in both heart rate and blood pressure, and potentially induce disorders that are very relevant to the organism.

Keywords
- anabolic steroids
- left ventricle
- mice
- morphometric analysis

Introduction

The use of anabolic androgenic steroids (AAS) has grown into a worldwide substance abuse problem over the last decades. Today, the majority of AAS users are not competitive athletes, but, instead, they are typically young to middle-aged men who use them primarily for personal appearance.[1] The use of AAS is a public health concern for adolescent boys who have suffered bullying by being labeled gay/bisexual.[2]

The doses taken by these users are usually 10 to 100 times higher than the therapeutic ones, bringing forth hyperandrogenism. Although these doses promote increased strength and muscle development, they concomitantly develop hormonal disorders that lead to a variety of harmful consequences.[3]

Among the most striking AAS side effects are the increase in hematocrit and coagulation, causing thromboembolism, intracardiac thrombosis and stroke, as well as other cardiac

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disturbances including arrhythmias, cardiomyopathies, and, possibly, sudden death, adenomas and carcinomas.[4]

The use of anabolic agents causes adverse effects on the musculoskeletal system, increasing the risk of tendon rupture; this is due to the increase of strength and muscle mass.[5]

The anabolic activity of testosterone and its derivatives manifests primarily by its myotrophic action, which results in increased muscle mass by rising protein synthesis in the muscle.[6]

Krieg et al[7] analyzed cardiac changes by echocardiogram and observed an increase in the ventricular mass index and in the interventricular septum thickness in AAS users compared with non-users, and also a loss of diastolic function associated with a reduction in peak velocity during the initial phase initial of diastolic filling.

Studies performed with powered athletes by means of echocardiogram examination demonstrated that the cardiac remodeling that occurs as an effect of the use of anabolic steroids is irreversible.[8]

This paper intends to analyze the possible morphometric changes in the left ventricular diameter of male and female mice submitted to swimming that received supraphysiological doses of two types of AAS.

Material and Methods

In this work, we used 60 Swiss mice (30 males and 30 females) from the Universidade Federal de Alfenas (UNIFAL-MG) bioterium, housed in boxes with 10 animals each, treated with commercial ration and water "ad libitum" (at will) and kept in a light-dark cycle of 12 hours. The present experiment was analyzed and approved by the Ethics Committee for Research and Animal Experimentation (ECRAE) of the University (protocol n° 414/2012).

The treatment with AAS consisted of intraperitoneal injections of two types of AAS, as follows: group 1 (10 male and 10 female animals) received a dose of 0.8 mg/kg of Deposteron (EMS, São Bernardo do Campo, SP, Brazil); group 2 (10 male and 10 female animals) received a dose of 1.8 mg/kg of Winstrol (Stanozolol Depot, Landerlan, Lambaré, Paraguay), and group 3 (10 male and 10 female animals) received 1.8 mg/kg saline solution. The animals were treated for 2 months, with the doses being administered twice a week at 2-day intervals. On each of these interposed days, all mice were submitted to swimming for 10 minutes.

After euthanasia by inhalation of isoflurane, the chests of the mice were opened, and the hearts were entirely removed. Finally, they were stored in glass containers immersed in a buffered paraformaldehyde solution (pH 7.4) and remained in this fixative solution for 24 hours. Thus, the specimens were processed following the standardized sequence for a conventional histological procedure: alcohol dehydration, xylol diaphanization, and paraffin inclusion. Each heart was put in a paraffin block and cut into 7 µm-thickness sections in Jinhua YIDI Medical Appliance CO., LTD (Jinhua City, Zhejiang Province, China) microtome and stained with hematoxylin and eosin. For the morphometric analysis, 6 distinct sections were selected and measured using an optical microscope with 40-fold magnification, and for the morphometric analysis of the ventricular cavity we used the Axiovision Rel. 4.8.2 (Carl Zeiss Microscopy LLC, Peabody, MA, USA) and Axiovision 4 Module Interactive Measurement (Carl Zeiss Microscopy LLC) software.[9]

To evaluate the mean values of the left ventricle areas, according to mice's gender and treatment imposed (research groups), the variance analysis of variance was used. When a significant difference ($p < 0.01$) was observed among the groups while comparing different variables, the Tukey test was used to discriminate differences and/or similarities among the evaluated means.[9]

Results

According to the graph and photomicrographs (►**Fig. 1A** and **2E**), it can be observed that in the male mice treated with

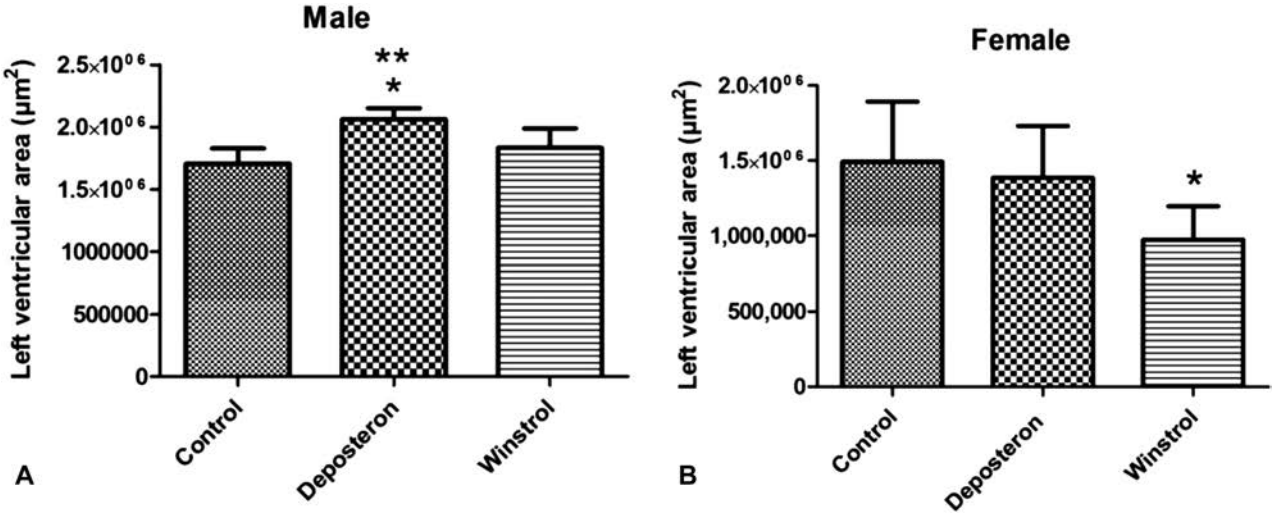

Fig. 1 Comparative graphs of left ventricular diameter in male and female animals, respectively. (**A**) * Statistically significant differences of control group animals compared with Deposteron animals ($p < 0.001$) and ** Statistically significant differences of Winstrol animals compared with Deposteron animals ($p < 0.01$). (**B**) Statistically significant differences of control group animals in relation to Winstrol animals ($p < 0.01$).

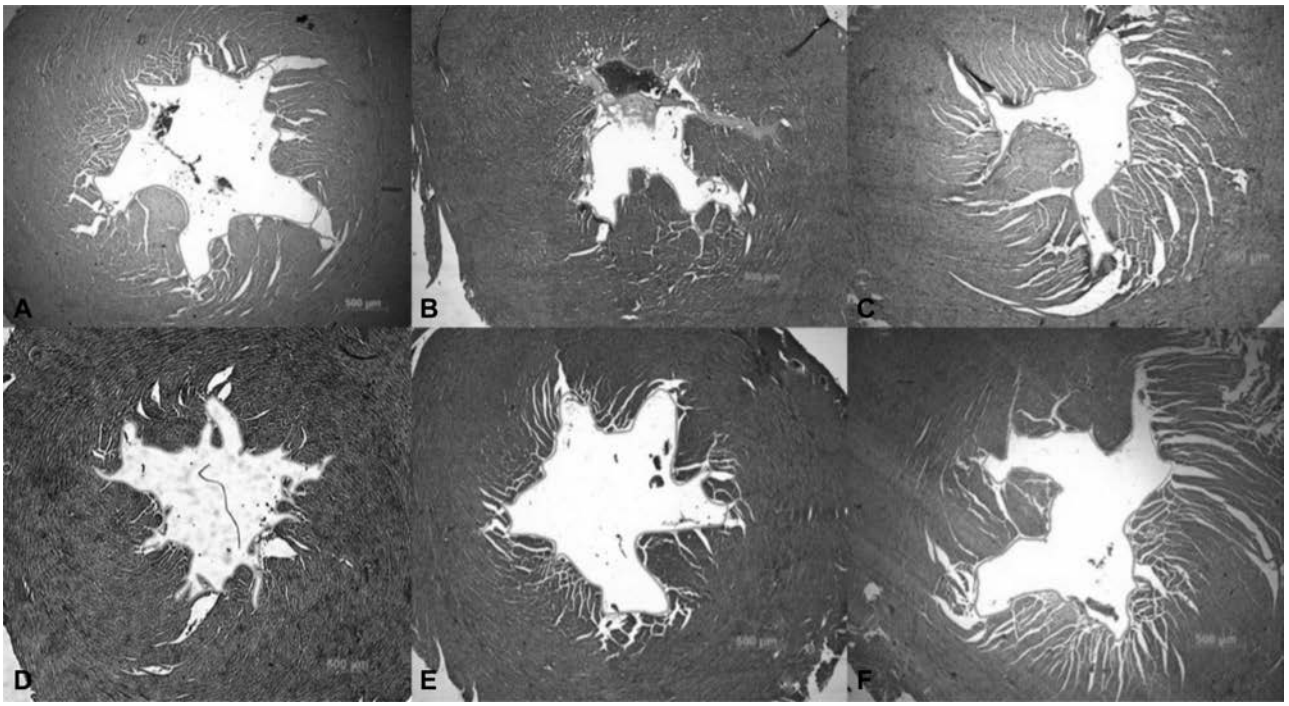

Fig. 2 Photomicrography of the cross-sections of the left ventricle of mice in both sexes and in different groups studied. A: Female control group; B: Deposteron female; C: Winstrol female; D: Male control group; E: Deposteron male, and F: Winstrol male.

Deposteron there was a significant increase ($p < 0.001$) in the diameter of the left ventricle in relation to the control group (►**Fig. 2D**) and the group treated with Winstrol ($p < 0.01$) (►**Fig. 2F**).

In the females, the results show that the group treated with Winstrol (►**Figs. 1B** and **2C**) presented a significant decrease in ventricular diameter ($p < 0.01$) in relation to the control (►**Fig. 2A**) and the Deposteron (►**Fig. 2B**) groups.

Discussion

Differences between genders are often ignored and underestimated when studying the cardiovascular system, and these cause biases and losses in the performed researches.[10] However, previous clinical and epidemiological studies have corroborated and acknowledged gender differences in cardiovascular function and disease.[11] The causal link between the use of AAS and the occurrence of cardiovascular diseases has been increasingly evidenced through researches, which demonstrate the frequent use of these substances associated with the rise in the occurrence of death due to cardiac arrest among the users.[12–14] Such studies corroborate the findings in the present study, which show that AAS use may cause morphological changes in the left ventricle and that they can lead to the appearance of cardiovascular diseases.

Cardiac changes in women and men may have been influenced by both dose and time period of administration of the drugs used, and these factors, isolated or together, may have led to different effects in both sexes. However, there are limitations and scarcity of data in the literature regarding the relevance and peculiarities of the different types of AAS.

Neto et al[15] suggest that the AAS dose is directly related to the aromatization of the testosterone; that is, the higher the dose, the greater the aromatization and the greater cardiac compromise. Pirompol et al[16] concluded that cardiac hypertrophy is not related to the dose, but to exposure to the induction of maladaptative heart responses. Therefore, although it was not possible to measure the interference of the dosage, period of use and active principle of the AAS used, the results presented here may induce and contribute to the interest and awakening for future researches.

A second hypothesis consists in the association of AAS action and the activation and increase of the sympathetic autonomic nervous system action. When present in the bloodstream, AAS reach the hypothalamus through the vascular organ of the terminal lamina or through the subfornical organ, structures that do not present a blood-brain barrier, facilitating the absorption and interaction by specific cellular groups of neurons acting on the control of viscera, blood osmolarity, angiotensin II levels and blood pressure. These negative influences of AAS use on the sympathetic modulation have already been evidenced and recorded by Neto et al,[15] whose study contributes to and strengthens the results observed here.

The consequences of non-therapeutic and abusive use of testosterone (AAS and its derivatives) are associated with an increase in blood pressure and induction of left ventricular changes, with consequent cardiac hypertrophy, as shown in some studies already performed.[17–20]

Initially, it was expected that there would be no change in the ventricular diameter in females because they have a greater amount of estrogen as a differential characteristic. This hormone is a protective factor for the cardiovascular system, and such concept has already been evidenced in

some previously published papers.[21–23] Nonetheless, some authors suggested that the decrease in the diameter of the left ventricle in female animals under supraphysiological doses of the AAS could be due to an increase in the left ventricular wall, leading to a decrease in the ventricular chamber volume with consequent hypertension, resulting in heart failure and left ventricular hypertrophy.[24,25]

Another hypothesis that would lead to an increase of muscle mass in females would be based on the same reason observed by Hayward et al,[26] who administered AAS to women and consequently observed an increase in cardiac muscle mass. This could explain the findings of increased muscle mass with a consequent decrease in diameter in females, in this study, in addition to the fact that the drugs themselves lead to an increase in ventricular mass. Thus, although females have to modulate hormonal protection (estrogen) in the cardiovascular system, such hormone would not have been able to prevent ventricular changes nor would it attenuate the androgenic actions of supraphysiological doses of AAS.

In male animals, it is suggested that the reason for finding the opposite result to that observed in female subjects was due to the drugs investigated in the present experiment, for they increased the left ventricular lumen because of an atrophy of the cardiac muscle and supposed decrease of left myocardial thickness. These effects can lead to chronic ischemia, which drives to fibrosis, reducing heart fiber nutrition, exactly as demonstrated in other studies.[27,28]

Other studies demonstrate that another reason that could lead to an increase in left ventricular diameter in male animals, as found in this study, is that AAS would induce dilated cardiomyopathy, primary heart muscle disease with dilation and change in the contractile function of the left ventricle, which is more prevalent in men than in women.[29,30]

Another factor that could contribute to the increase in left ventricular diameter is that, physiologically, males have a greater amount of endogenous testosterone when compared with females; this factor, added to supraphysiological doses, could lead to toxicity of the cardiac muscle tissue by inducing pro-oxidative actions on the cardiovascular system.[10]

This wide variety of hypotheses may be a reflection of the limitations pertinent to AAS studies and of the morphological consequences to the cardiovascular system caused by the use of AAS. This is due to several reasons, such as the manifestation of the effects, that do not appear in a short-term period and make it difficult to provide an early diagnosis, as well as the intrinsic factors of the AAS (dose, duration of treatment and active principle) that interfere in their action.[1,31–33]

Conclusion

Thus, one can conclude that the use of supraphysiological doses of the administered AAS significantly and differently alters the ventricular diameter in male and female animals. Such findings may contribute to elucidate the possible effects and consequences of the indiscriminate use of these drugs concerning the cardiovascular system.

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Conflicts of Interest
The authors have no conflicts of interest to declare.

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Comparative Anatomy of the Forearm and Hand of Wildcat (*Leopardus geoffroyi*), Ocelot (*Leopardus pardalis*) and Jaguar (*Panthera onca*)

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Abstract

Introduction The thoracic limbs of cats facilitate jumping and represent one of their main ways for pursuing and capturing prey. The main muscles and nerves involved in these activities are present in the region of the forearm and of the hand. The scant anatomical reference availabe on South American cats species justifies the present comparative study.

Materials and Methods The forelimbs of wildcat, ocelot and jaguar wild felines were fixed. Images of the dissected limbs were captured using a digital camera. Measurements were made using a caliper.

Results The long and short heads of the extensor carpi radialis muscle of the ocelot and of the jaguar showed a great development in comparison with those of the wildcat. The flexor digitorum profundus muscle in the three felines is formed by five heads. In the jaguar, the radial or deep head presented two sesamoid bones. The brachioradialis muscle of the jaguar and of the ocelot is inserted medially at the distal end of the radius and at the proximal row of the carpus by a thick and flattened tendon. The pronator teres muscle of the jaguar extended to the carpal region. In the wildcat and in the ocelot, this muscle was less developed

Conclusions The main variations observed between the forearms and the hands of these South American cats were found between the supinator and the pronator muscles, presenting a variation in the size of their bellies and tendons. Our study of the muscular characteristics represents a contribution to the homologies and differences of the forearm and hand muscles of each of these species of felines.

Keywords

- South American felines
- felines
- forearm muscles
- hand muscles
- jaguar
- ocelot
- wildcat

Introduction

Argentina is the only country in the American continent that has all the species of felines present in South America. With the exception of the red lynx (*Lynx rufus*) and of the Canada lynx (*Lynx canadensis*), which only inhabit North America, the remaining 10 species that inhabit the continent have part of their distribution in Argentina. However, very little is known about most of these autochthonous species. From the jaguar (*Panthera onca*) to the wildcat (*Leopardus geoffroyi*), the largest and the smallest of the American felines, respectively, all of the South American wild cats are almost unknown to science.[1] Currently, most of these native felines are protected in Argentina. However, the deep ignorance that exists about their anatomy could hinder actions tending to

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their effective preservation and management in the areas destined to their preservation.[2]

For a long time, the domestic cat has been the biological model used for the study of different feline species, mainly due to the greater availability of specimens. The lower availability of material from the other species significantly limits their study.[3] From the agreement of collaboration maintained by the Facultad de Ciencias Veterinarias of the Universidad Nacional de la Plata with the zoo of the municipality of La Plata, which cedes the dead specimens to our Instituto de Anatomia, we began to study the locomotor apparatus and the peripheral nervous system of the jaguar and of the puma.[4,5]

Although some aspects related to ecology, reproduction and nutrition are known, there is not much information related to the anatomy of these autochthonous species, especially to their locomotor system. The description of the muscles of these feline species is particularly useful for phylogenetic studies.[6] Comparative studies of these taxa and homologies show the evolution of their muscles, as well as a very careful review and clarification of the nomenclature used based on the Nomina Anatomica Veterinaria.[7,8]

Knowledge about the anatomical region of the forearm and of the hand is of high importance to understand the hunting and movement habits of these animals.[9] Thus, some species of felines, such as the serval (*Leptailurus serval*), have developed long and thin thoracic limbs, to which is added the great development of auricular pavilions, which they use to detect the prey in tall grass.[5,10] In contrast, other species of felines, such as the jaguar, have developed shorter thoracic limbs, with well-developed arms and forearms that allow them to grab the prey strongly.[11]

The lack of knowledge about the muscles of the forearm and of the hand of these felines poses difficulties for the discussion not only of their morphology, but also of their function and of the evolution of these native felines. The objective of the present study was to carry out a descriptive analysis of the forearm and hand muscles of wildcats, ocelots and jaguars and to compare them with the data recorded for the domestic cat. Likewise, the accuracy of the homologies, as well as of the nomenclature used among these feline species for the description of the muscles, was critically examined and discussed.

Materials and Methods

Muscles of the right and left thoracic limbs of 1 adult male jaguar (body weight [BW]: 78 kg) and of 1 young female ocelot (BW: 7 kg) that died of natural causes in the zoo of the municipality of La Plata were dissected. A total of 3 wildcats, 1 young female (BW: 3 kg) and 2 adult males (BWs between 3.6 and 4 Kg), found dead in the field or on the route of Mount Berisso, in the province of Buenos Aires, were also used.

The animals were frozen and stored in the Instituto de Anatomia of the Facultad de Ciencias Veterinarias of the Universidad Nacional de la Plata. They were then fixed with a fixing solution composed of 10% formaldehyde, 1 L of carbolic acid, 1 L of glycerin, and 5 g of thymol. The common carotid arteries were channeled, and an amount of fixative liquid equivalent to 10% of the BW of each animal was injected. At each stage of the dissection, the muscles were photographed with a Sony Cyber-Shot DSC-P10 digital camera (Sony Corporation, Tokyo, Japan). The Adobe Photoshop CS6 software (Adobe Inc., San Jose, CA, USA) was used to process the images.

The following variables were analyzed: presence of muscles, length of the muscles, length of the muscular bellies, length of the tendons from their origin to their insertion, and some morphological variations of the muscles were compared between the three studied feline species. The measurements were made using a 300 mm Vernier caliper (200 mm, Isard, China).

Results

The extensor carpi radialis muscle of the ocelot and of the jaguar showed a great development of the long and short heads, compared with that of the wildcat. Both muscular bellies originated in the lateral supracondylar ridge, distal to the humerus bone. The long head was inserted into the dorsal surface of the second metacarpal, and the short head at the base of the third metacarpal (►**Fig. 1A**, **2A**). The extensor digitorum communis muscle of the three species was formed by two bellies: medial and lateral. However, the lateral digital extensor muscle of the jaguar had only one belly, whereas in the wildcat and in the ocelot, it was clearly divided into two bellies, of which the medial belly was divided again into two tendons (►**Fig. 3A**, **C**). The abductor digiti longus muscle of the first finger showed a more proximal origin in the jaguar and in the ocelot than in the wildcat. It originated along the entire length of the lateral edge of the ulna, in the proximal two thirds of the radius and in the interosseous membrane of the forearm. In the jaguar and in the ocelot, the extensor digiti I and the extensor digiti II muscles originated in the proximal third of the diaphysis of the ulna and were divided into two bellies. The belly of finger I was continued with two tendons, which then fused, forming an expansion in its insertion in the distal phalanx. The belly of finger II had a single tendon, which fused with the tendon of the extensor digitorum lateralis muscle of finger II (►**Fig. 2B**).

The flexor muscles showed no relevant differences between the felines studied. The palmaris longus muscle of the ocelot and of the jaguar, located medial to the forearm, ended in five tendons, while in the wildcat it was divided into four tendons (►**Fig. 1B**, **3B**). In the three wild species, they ended up attached to the tendons of the flexor digitorum superficialis muscle in the proximal extremity of the middle phalanx.

In the three South American felines, the flexor digitorum superficialis muscle was formed by two heads: one ulnar and one radial, which united to form a common belly. Particularly in the jaguar, this muscle formed a thick and flattened muscular belly that later became divided into four tendons, one for each finger (►**Fig. 1B**).

In felines, the flexor digitorum profundus muscle has five heads (►**Fig. 1B**, **3B**); the first head, or superficial head, is the ulnar. The second, third and fourth heads originate from the

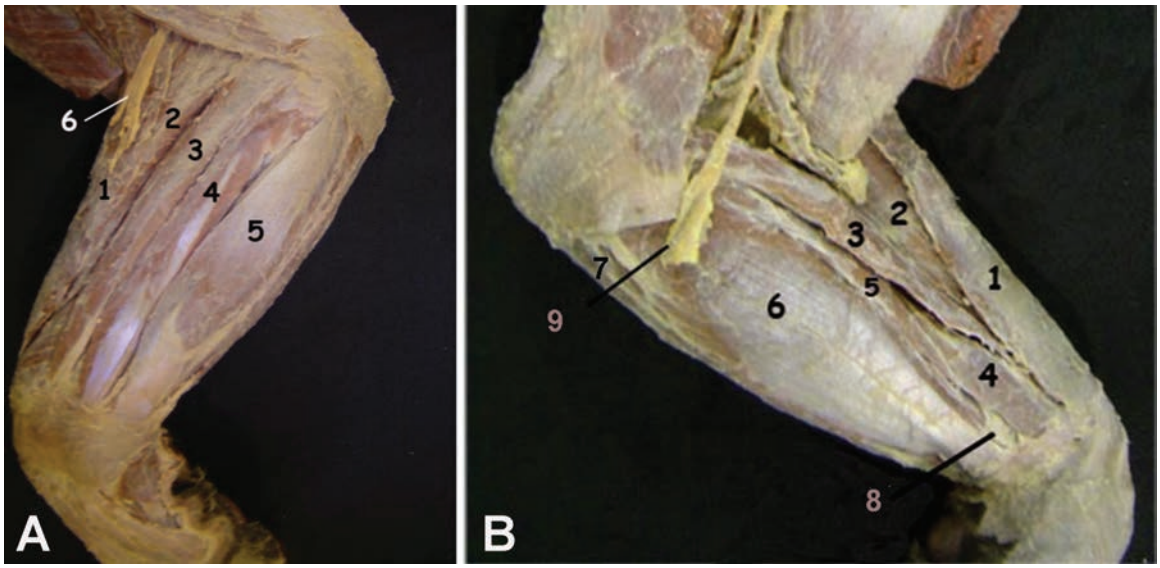

Fig. 1 (A) Lateral view of the forearm and of the hand of the jaguar. 1. Brachioradialis muscle. 2. Extensor carpi radialis muscle. 3. Extensor digitorum communis muscle. 4. Extensor digitorum lateralis. 5. Extensor carpi ulnaris muscle. 6. Radial nerve. (B) Medial view of the forearm and of the hand of the jaguar. 1. Brachioradialis muscle. 2. Extensor carpi radialis muscle. 3. Pronator teres muscle. 4. Flexor carpi radialis muscle. 5. Palmaris longus muscle. 6. Flexor digitorum superficialis muscle. 7. Flexor carpi ulnaris muscle. 8. Median nerve.

humerus, completely separated and located in a median plane. Finally, the radial or deep head originates from the distal middle third of the radius and of the ulna. In the jaguar, this muscular belly had two sesamoid bones. The second head (humeral) formed the tendon of finger I, while the remaining four heads formed the tendons of fingers II to V. Each tendon descended through the proximal annular ligament. The tendons of fingers II to V continued distally through the distal annular ligament to insert on the palmar side of the distal phalanx (►**Fig. 2B**, **3A**).

In the jaguar, a great development of the supinator and pronator muscles was observed (►**Table 1**) (►**Fig. 1A**). However, they were less bulky in the ocelot and in the wildcat (►**Table 1**). The brachioradialis muscle, located under the superficial fascia of the forearm, showed a very developed fleshy belly in the jaguar and in the ocelot, in comparison with that of the wildcat (►**Fig. 2A**, **3A**). This muscle had an origin in the proximal third of the caudal surface of the humerus and covered the cranial muscles of the forearm, and then inserted medially at the distal end of the radius and at the proximal row of the carpus by a thick and flattened tendon (►**Fig. 1A**).

Beneath the extensor muscles, the supinator muscle was located. In the jaguar, this muscle was formed by a flat muscular belly that spiraled around the proximal end of the radius. At its origin, it presented a short tendon starting from the lateral side of the annular ligament of the radius and from the radial collateral ligament to insert on the cranial surface of the distal third of the radius. In the ocelot and in the wildcat, this muscle was less bulky and ended with a tendon on the cranial surface of the middle third of the radius (►**Fig. 4A**).

The pronator teres muscle of the jaguar originated through a thick tendon starting from the medial epicondyle of the humerus, to end up being inserted in the cranial face of the distal end of the radius. Then, it extended to the carpal region, where it fused with the flexor retinaculum (►**Fig. 1B**, **4B**) muscle. In the wildcat and in the ocelot, this muscle was less developed (►**Table 1**) and inserted into the medial border of the proximal third of the radius (►**Fig. 2A**, **3D**, **4B**).

The pronator quadratus muscle was in a deeper plane. It originated at the flexor surface of the ulna and in the interosseous membrane. Its very fleshy fibers passed obliquely and distally to be inserted in the caudal surface of the radius. In the jaguar and in the ocelot, this muscle was covered by a thick tendon blade.

Discussion

The myology of the forearm and of the hand of felines was described only for the domestic cat (*Felis catus*) and for the lion (*Panthera leo*).[12] There are some studies performed on the cheetah (*Acinonyx jubatus*) and on the puma (*Puma concolor*), but the descriptions are brief and lack morphological details.[9,13] On the other hand, the description of the muscles of the domestic cat and of the lion are much more extensive, with many well-illustrated and detailed dissections.[12,14,15]

During the evolution of mammals, the supinator and pronator muscles became vestigial or disappeared, as their movement capacity was reduced or lost. Among cats, however, these movements are feasible and very broad, with a pronation of 40° and a supination of 50°.[16] These movements are possible due to the great development of the brachioradialis muscle, which performs the external rotation and the supination of the forearm and of the hand.[17–19] In felines, the movement of supination is necessary to capture, manipulate and exhaust the prey.[20] The muscles of the forearm are better developed in wild cats compared with what is described for

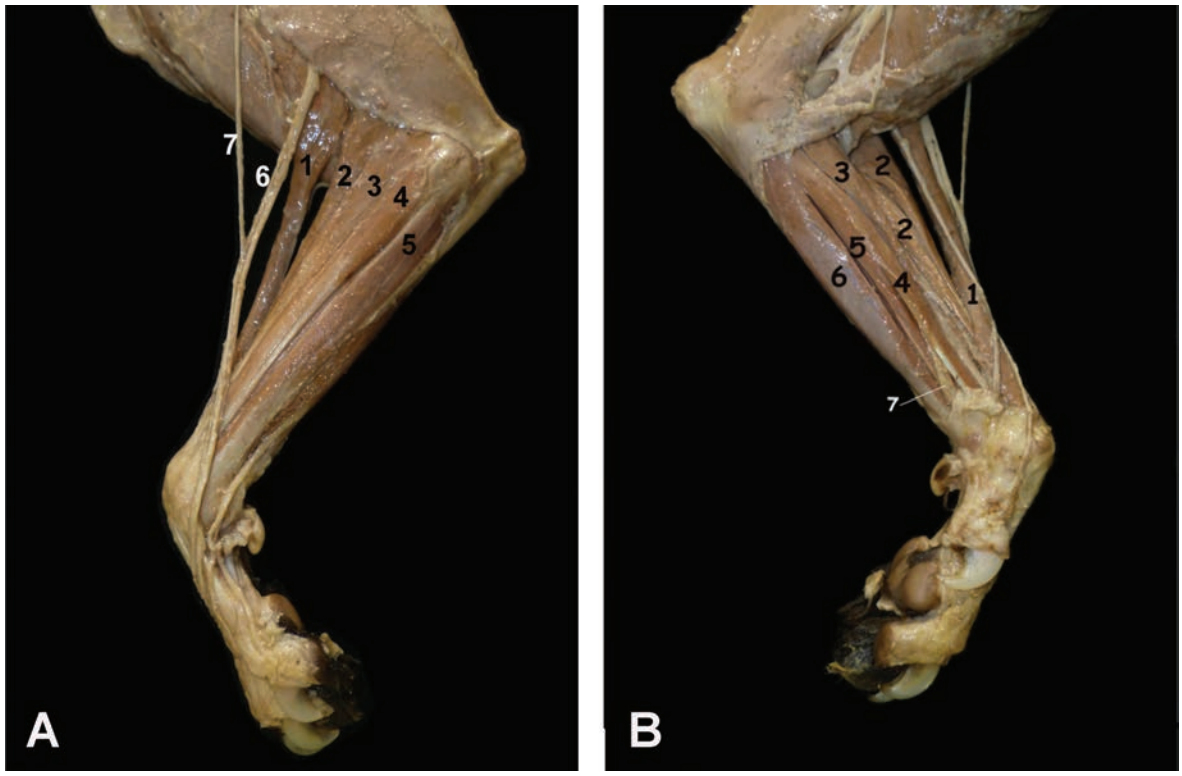

Fig. 2 (A) Lateral view of the forearm and of the hand of the ocelot. 1. Brachioradialis muscle. 2. Extensor carpi radialis muscle. 3. Extensor digitorum communis muscle. 4. Extensor digitorum lateralis muscle. 5. Extensor carpi ulnaris muscle. 6. Surface branch of the radial nerve. 7. Lateral and medial branches of the superficial branch of the radial nerve. 8. Cephalic vein. (B) Medial view of the forearm and of the hand of the ocelot. 1. Brachioradialis muscle. 2. Extensor carpi radialis muscle. 3. Pronator teres muscle. 4. Flexor carpi radialis muscle. 5. Palmaris longus muscle. 6. Flexor digitorum superficialis muscle. 7. Median nerve.

the domestic cat.[4,14] This seems to be related to the size of the prey and to the adaptation these cats have to hunt in the water or in heights and alone, not in a herd.

The greatest differences were found in the extensor muscles of the carpus and of the fingers, probably because with the distal portion of the thoracic limb rotation, supination and pronation movements are performed with greater amplitude.[21] However, this observation is unlikely to explain the variation in the number of bellies or tendons of the forearm and hand muscles.

It has been described that the extensor digitorum lateralis muscle of the ocelot has three muscular bellies, while the extensor digitorum communis muscle is formed by four muscular bellies.[22] This differs from what we have described in our dissections, in which a division of two muscular bellies in both muscles was observed. The same authors described that, in the ocelot, the long palmar muscle terminated in four tendons.[22] Nevertheless, according to our observations, the termination of the palmaris longus muscle is divided into five tendons in the ocelot and in the jaguar, and into four tendons in the wildcat.

The division of the number of tendons to the fingers is related to the degree of movement of the digits.[23] These muscular characteristics are consistent with the behavior of each species during the hunting of its prey,[24] both in the distances they travel and in the size of the different preys.

For authors describe the palmaris longus muscle is not present in the puma, but it is part of the flexor digitorum superficialis.[9] However, had already described this muscle for the domestic cat separated from the flexor digitorum superficialis,[14] as we have observed in our dissections in the three feline species studied. However, their tendons in the distal extremity of the middle phalanx ended up attached to the tendons of the flexor digitorum superficialis muscle.

Table 1 Comparative measurements of the brachioradialis, supinator, and pronator muscles

Species	Brachioradialis muscle			Supinator muscle			Pronator teres muscle		
	L	W	T	L	W	T	L	W	T
Jaguar	21	0.5	0.4	14	1.5	1.2	14.5	1.8	2.5
Ocelot	10	1	0.2	8	0.8	0.5	7	1.2	0.5
Wildcat	8	1	0.2	5	0.5	0.2	6	1	0.5

Measurements are expressed in cm.
Abbreviations: L, Lenght; W, Width; T, Thickness.

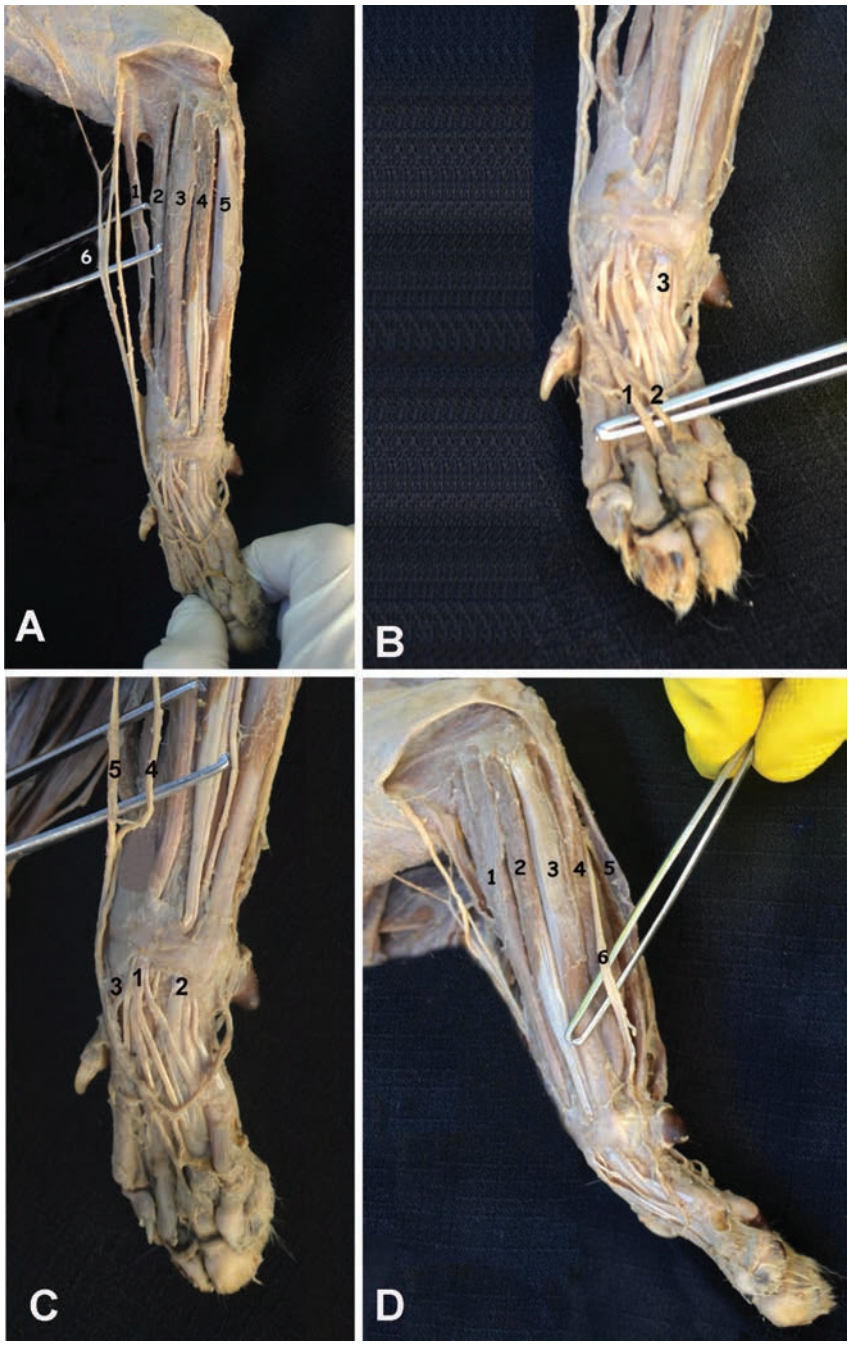

Fig. 3 (A) Side view of the forearm and of the hand of the wildcat. 1. Brachioradialis muscle. 2. Extensor carpi radialis muscle. 3. Extensor digitorum communis muscle. 4. Extensor digitorum lateralis muscle. 5. Extensor carpi ulnaris muscle. 6. Radial nerve. (B) Palmar view of the hand of the wildcat. 1. Palmar lateral branch 2. Medial palmar branch of the median nerve. 3. Tendons of the flexor digitorum profundus muscle. (C) Lateral view of the forearm and of the hand of the wildcat 1. Extensor carpi radialis muscle. 2. Extensor digitorum communis muscle. 3. Extensor digitorum lateralis muscle. 4. Extensor carpi ulnaris muscle. 5. Flexor carpi ulnaris muscle. 6. Dorsal and palmar branches of the ulnar nerve. (D) Dorsal view of the hand of the wildcat. 1. Tendons of the Extensor digitorum communis muscle 2. Tendons of the extensor digitorum lateralis muscle 3. Tendons of the abductor pollicis 4. Lateral branches of the radial nerve 5. Medial branches of the radial nerve.

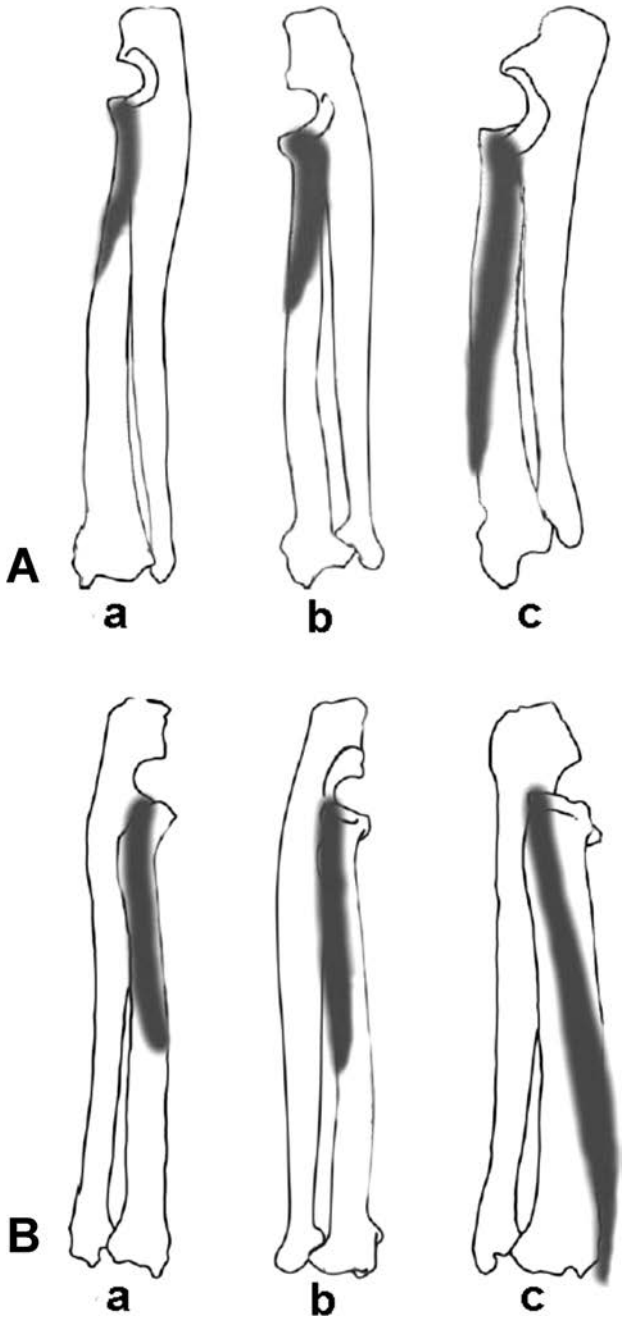

Fig. 4 A. Drawings of the radii and ulnae of the wildcat (a), ocelot (b), and jaguar (c) showing the variation in size and insertions of the supinator muscle. B. Outlines of the radii and ulnae of the wildcat (a), ocelot (b) and jaguar (c) showing the variation in size and insertions of the pronator teres muscle.

Conclusion

The most distinctive morphological adaptations that we have found between the jaguar, the ocelot, and the wildcat are the powerful supinator and pronator muscles, presenting a variation in the size of their bellies and tendons. These variations among the three species studied are related to ecomorphological variables, such as the size of their prey and their habitat.

The studies of the muscular characteristics of each species of felines and their differences will be particularly useful for future phylogenetic reconstructions. These South American cats have been little studied, and our work represents a contribution on the homologies and differences of the forearm and hand muscles.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Anthropometric Evaluation of Foramen Ovale in Adult Dry Skulls of the Mysuru-based Population

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Abstract

Introduction The greater wing of sphenoid presents various foramina, of which the foramen ovale is one important foramen through which advanced surgical therapeutic and diagnostic procedures related to the middle cranial fossa are performed.

Materials and Methods A total of 40 dried adult skulls of unknown gender and age, obtained from the Department of Anatomy of the JSS medical College, Mysuru, Kamakata, India. The length and the width of the foramen ovale were measured using digital sliding calipers (tiny deal 150 mm SS digital caliper with LCD display, Kristeel-Shimwa industries, Bombay, India).

Results The mean length of the foramen ovale was 0.745 $\pm$ 0.31 cm on the right side (RS), and 0.68 $\pm$ 0.15 cm on the left side (LS). The mean width was 0.6 $\pm$ 0.17 cm on the RS, and 0.56 $\pm$ 0.14 cm on the LS.

Conclusion The knowledge of variations in the length and breadth of the foramen ovale is of immense importance in neurosurgery during various invasive surgical procedures, such as percutaneous trigeminal rhizotomy, and in the biopsy of cavernous sinus tumors and of Meckel cave lesions.

Keywords
- foramen ovale
- greater wing of sphenoid
- middle cranial fossa
- mandibular nerve

Introduction

The foramen ovale is one of the important foramina in the greater wing of the sphenoid bone, through which the infratemporal fossa communicates with the middle cranial fossa. It is located lateral to the foramen lacerum and medial to the foramen spinosum. It transmits the mandibular division of the trigeminal nerve, of the lesser petrosal nerve, of the accessory meningeal artery, and of the emissary vein connecting the pterygoid venous plexus with the cavernous sinus.[1]

The foramen ovale is used for various invasive surgical and diagnostic procedures.[2] The foramen ovale is one of the important routes for the spreading of nasopharyngeal carcinomas into the cranial cavity.[3]

Like other foramina of the skull, the foramen ovale also differs in shape, size and other morphological features. Sometimes, it is covered by an osseous ligament extending from the lateral pterygoid plate to the spine of the sphenoid bone.[4] Ossified pterygospinous and pterygoalar ligaments divide the foramen ovale into compartments.[5] They can compress the structures passing through it or block the passage of the needle through the foramen ovale.[6,7] A bony spur on the anteromedial side divides the foramen ovale in two.[8]

The right side (RS) foramen ovale is smaller than the left side (LS) foramen ovale.[9] The available literature reveals that the foramen ovale shows a wide range of variations. The present study aims to measure the length and the width of the foramen ovale, the knowledge of which helps in reducing complications during surgeries for neurological diseases.

Materials and Methods

The present study was conducted on 40 dried adult skulls of unknown gender and age obtained from the Department of Anatomy of the JSS Medical College, Mysuru, Kamakata, India. Ethical clearance was obtained from the institutional ethical committee to conduct this study. The measurement of the length (Anteroposterior diameter) and width (transverse diameter) of the foramen ovale was taken using sliding

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Table 1 The following observations were made

Parameters	Mean and SD
Right side length	0.745 ± 0.31 cm
Left side length	0.68 ± 0.15 cm
P-value	0.26
Right side width	0.6 ± 0.71 cm
Left side width	0.56 ± 0.14 cm
P-value	0.29

Abbreviations: SD, standard deviation.

digital calipers (Kristeel-Shimwa industries, Bombay, India) and then analyzed (►**Fig. 1** and ►**Fig. 2**).

Observations

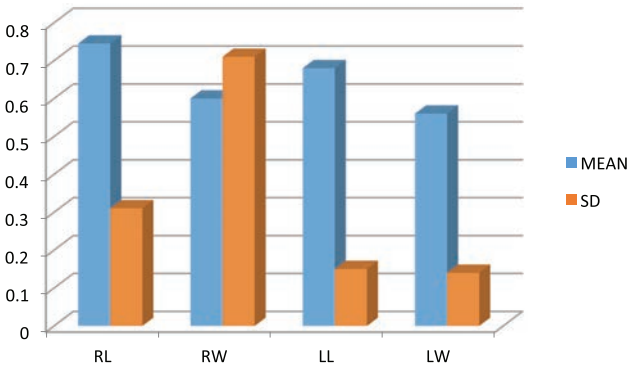
The present study was conducted on a total of 80 sides of 40 dry adult skulls. The mean length of the foramen ovale was 0.745 ± 0.31 cm on the RS, and 0.68 ± 0.15 cm on the LS. The mean width was 0.6 ± 0.17cm on the RS, and 0.56 ± 0.14 cm on the LS (►**Table 1**).

The length and the width were longer on the RS when compared with the LS. The difference between the length of the RS and of the LS was not statistically significant ($p > 0.05$). Similarly, the difference between the width of the RS and of the LS was not statistically significant (**Graph 1**).

Discussion

In the present study, the mean length of the foramen ovale was 0.745 ± 0.31 cm on the RS, and 0.68 ± 0.15 cm on the LS. The mean width was 0.6 ± 0.17 cm on the RS, and 0.56 ± 0.14 cm on the LS. Therefore, both the length and the width are longer on the RS when compared with previous studies.

In his developmental study, Yanagi[10] reported that the length of the foramen ovale in newborns is ~ 3.85 mm, and ~ 7.2 mm in adults. The earliest appearance of the foramen ovale as a ring-shaped area is seen in the 7th month of intrauterine life and lasts for 3 years after birth.[8]



Graph 1 Bar chart showing mean ± standard deviation of the length and of the width of the foramen ovale. Abbreviations: LL, left length; LW, left width; RL, right length; RW, right width; SD, standard deviation.

Fig. 1 Base of the skull showing the foramen ovale.

Fig. 2 Showing the measurement of the foramen ovale using digital sliding calipers.

Lang et al[11] reported that the average maximum length of the foramen ovale is ~ 7.48 mm, and that the average minimum length is ~ 4.17 mm in adults. The width ranges from 1.81 mm in newborns to 3.7 mm in adults.[12]

A study by Chandra Philips et al[13] conducted on 50 dry skulls showed that the mean length of the foramen ovale was 7.27 ± 1.41 mm on the RS, and 7.46 ± 1.41 mm on the LS.[14]

A study by Patel et al[12] conducted on 100 dry skulls showed that the mean length of the foramen ovale was 6.6 mm on the RS, and 6.5mm on the LS. The RS foramen ovale was longer than the LS. The mean width was 3.6 mm on the RS, and 3.5 mm on the LS.[7]

Ray et al[2] conducted a study on 35 dry skulls that showed that the mean length of the foramen ovale was 7.46 ± 1.41 mm on the RS, and 7.01 ± 1.41 mm on the LS. The mean width was 3.21 ± 1.02 mm on the RS, and 3.29 ± 0.85 mm on the LS.

In the present study, the length and the width are longer on the RS when compared with the LS, which is in line with the studies conducted by Daimi et al[6] and by Patel et al.[12]

It is through the foramen ovale that many invasive surgeries and diagnostic procedures are performed.[2] Percutaneous trigeminal rhizotomy in trigeminal neuralgia is also performed through the foramen ovale.[15] Percutaneous biopsy of cavernous sinus tumors and biopsy of deep lesions of the brain, such as Meckel cave lesions, are performed through the foramen ovale, which decreases patient morbidity and is cost-efficient.[14,16] The foramen ovale is also used to diagnose squamous cell carcinomas and meningiomas via computed tomography (CT) guided transfacial fine needle aspiration.[17]

It is through the foramen ovale that the trigeminal nerve is accessed. Therefore, the variations in the length and in the width of the foramen ovale are of utmost importance during the anesthesia of the trigeminal nerve.

Conclusions

The present study demonstrates the anthropometric significance of the foramen ovale, which may help radiologists and neurosurgeons. These variations in measurements of foramen ovale found in the present study may be useful for treating trigeminal neuralgia, abnormal tumors, and to perform biopsies from deeper parts of the brain.

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Effect of Diabetes on the Male Reproductive System—A Histomorphological Study

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Abstract

Introduction Type 1 diabetes is an autoimmune disorder characterized by lack of insulin production by the β cells of the pancreas. This lack of insulin causes a variety of systemic effects on the metabolism of the body, one of which is reproductive dysfunction. The present study investigates the effects of diabetes on the male reproductive system of streptozotocin (STZ)-induced diabetic rats.

Material and Methods A total of 18 adult male Wistar rats weighing ∼ between 250 and 300 g were included in the present study. The animals were divided into normal and diabetic groups. The diabetic group was further subdivided into 2 subgroups with durations of 24 and 48 days. A single dose of STZ (40 mg/kg body weight) was administrated intraperitoneally to the animals of the diabetic group. After the planned duration, the testes and epididymides were dissected, and their gross weight was measured. The tissues were then processed for histological study.

Results The gross weight of the testes and epididymides in diabetic rats at 24 and 48 days showed a decrease in comparison to the control. ($p < 0.01$ for testes and epididymides).

Diabetic animals presented a significant decrease in the diameter of the seminiferous tubules compared with the control group ($p < 0.01$). The epididymides in the diabetic groups showed a considerable reduction in the tubular surface area compared with the control group ($p < 0.01$). There was also a reduction in the mean diameter, which was measured using the maximum and minimum diameter of the tubules ($p < 0.01$).

Conclusion The present study is an insight into the adverse effects that diabetes can have on the tissue structure of the testes, of the epididymides, and ultimately on the process of spermatogenesis.

Keywords
- diabetes mellitus
- testis
- epididymis
- streptozotocin (STZ)
- male infertility

Introduction

Diabetes mellitus (DM) is characterized by an increased blood glucose level (hyperglycemia). The worldwide prevalence of DM was of ∼ 2.8% in 2000, and it has been projected to extend to 4.4% in 2030. The total figure of individuals with DM is estimated to escalate and double by 2030. Moreover, the occurrence of DM is higher in men than in women.[1]

Persistent hyperglycemia for long durations can lead to several problems, such as diabetic neuropathy, nephropathy, retinopathy, male impotence, and cardiovascular disease.[2]

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Although DM is responsible for the aforementioned problems, its effect on male sterility based on impotence, retrograde ejaculation, and hypogonadism is not widely understood.

The suggestion that DM has unfavorable effects on male fertility has long been contentious.[3] Nevertheless, current studies have discovered that DM can affect the development of sperm and the production of androgens, which finally causes male infertility.[4]

Morphometric studies have shown a significant difference in the diameter of the seminiferous tubules in diabetic individuals and although a constant (20–25%) total or subtotal block of spermatogenesis at spermatocytes stages 2 and 3 occur in small tubules of all animals.[5]

The streptozotocin (STZ)-induced diabetic rat model is one of the most extensively used models to study the effect of DM on fertility. Testicular dysfunction and degeneration were observed under conditions of experimentally induced DM in animal prototypes.[6,7]

The present study is, therefore, an attempt to investigate the effects of DM on the male reproductive system of STZ-induced diabetic rat models. It also aims to quantify, through histological analysis, the rate of degeneration observed in the testes and in the epididymides caused by DM.

Materials and Methods

Experimental Animals

A total of 18 healthy adult male Wistar albino rats of both genders, weighing between 150 and 200 g, housed under standard environmental conditions of temperature and humidity (25 ± 0.5°C) and 12 hour light/dark cycle were used for the present study. The animals were fed with standard pellet diet and water ad libitum. The experimental study was performed in the central animal house after obtaining the approval from the Institutional animal ethics committee (Ref. number: IAEC/KMC/49/2013).

The rats were randomly divided into 3 groups: control group ($n = 6$), 28-days diabetic group ($n = 6$), and 46-days diabetic group ($n = 6$).

Induction of Diabetes by Streptozotocin

After 7 days of acclimatization, Wistar rats were used for the induction of DM.[6] After overnight fasting, the rats were injected with a single intraperitoneal dose of streptozotocin (STZ) (40 mg/kg). Drinking water was added with glucose (5%) to overcome the STZ-induced hyperglycemia. Fasting blood glucose levels were measured using an Accu-Chek Active glucometer with glucose oxidase-peroxide reactive strips (Roche Diagnostics India Pvt. Ltd., Mumbai, India). Animals with fasting blood glucose levels > 250 mg/dl were included in the present study.

On the confirmation of DM, the animals were observed for the planned duration, that is, 24 and 48 days, at the end of which the animals were sacrificed. The testes and the epididymides were dissected, and their gross weight was measured. The tissues were then fixed in 10% formalin for further studies.

Microscopic Observations

The fixed tissues were processed using paraffin embedding, and sections of 5 µm thickness were acquired at 200 µm recesses. The sections were subjected to hematoxylin and eosin (H&E) staining to evaluate the testicular and epididymal morphology. The extent of tissue damage was analyzed, quantified and documented using microscopic images with the help of ImagePro Premier 9.1 (Media Cybernetics, Rockville, MD, USA).

Histological Analysis

The mean diameters of the seminiferous tubules in the testes and of the epididymal tubules were measured using an oculometer (Erma ocular micrometer, Japan) using a magnification of 10 x. It was performed in randomly selected 25 circular tubules in different slides of the same group and calculated with the following formula.[8]

$$\text{Mean} = \sqrt{\text{smallest diameter} \times \text{larger diameter} \times \text{magnification}}$$

Here, the smaller diameter was considered as the width of the tubules, and the large diameter as the length of the tubules.

Next, the histopathological changes in the testicular tissue were evaluated by the Johnsen testicular biopsy score system. It describes a new and rapid method for the registration of spermatogenesis in human testes. According to this system, each tubular section is given a score from 10 to 1 according to the incidence or to the nonappearance of the chief cell types organized in the order of development. Presence of spermatozoa scores 10 or 9; of spermatids scores 7 or 6; of spermatocytes scores 5 or 4; only spermatogonia scores 3; only Sertoli cells scores 2; and no cells scores 1. A total of 50 cross-sectioned tubules in each group were evaluated systematically, and a score between 1 (very poor) and 10 (excellent) was given to each tubule according to the Johnsen criteria.[9]

Statistical Analysis

The results were expressed as mean ± standard error of the mean (SEM). The data were analyzed using R software (R Foundation for Statistical Computing, Vienna, Austria). One-way analysis of variance (ANOVA) followed by the Dunnett post-hoc test was employed to compare the control and treated groups. The Tukey post hoc test was also used to compare between the different groups (control, 24-days, and 48-days). A p-value ≤ 0.05 was considered statistically significant.

Results

The Gross Size and Weight of the Testes and of the Epididymides

The size of both the testes and the epididymides continued to show a major reduction on the 24th and 48th days in the diabetic groups compared with the control group (normal) (►**Fig. 1**).

The gross weight of the testes and of the epididymides in diabetic rats continued to reduce at 24 and 48 days in

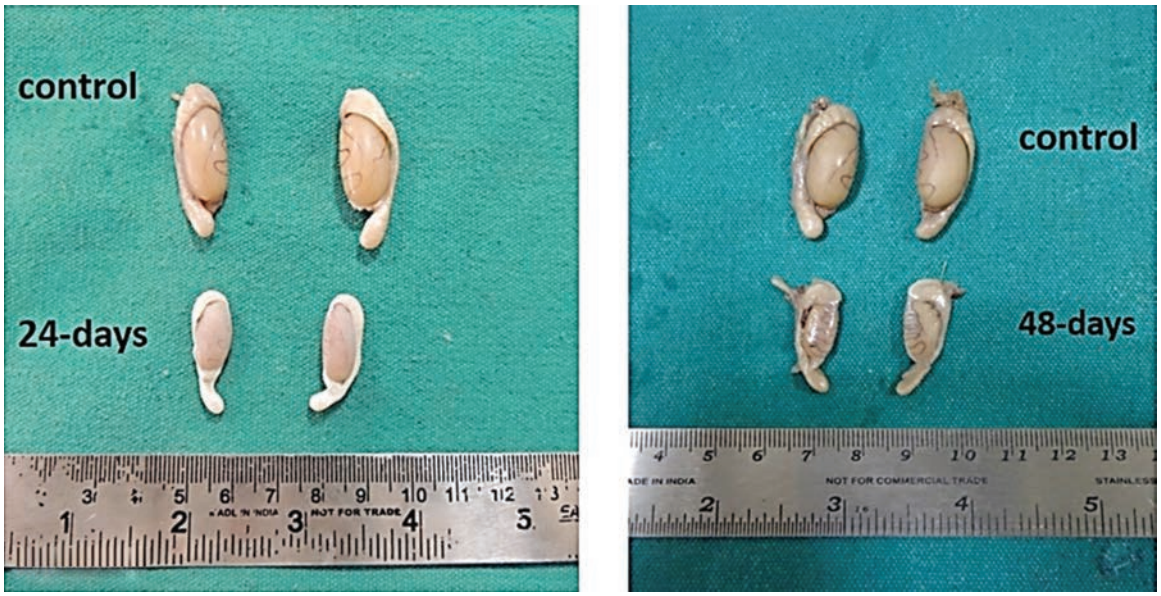

Fig. 1 Image showing the gross reduction in the size of the testes and of the epididymides in the 24- and 48-days diabetic groups in comparison with the control group.

comparison with the control group. The mean weight of the testes and of the epididymides was considerably lower in the rats of both 24- and 48-days groups compared with the control group (►**Fig. 2**), implying a significant weight reduction in the test groups ($p < 0.01$ for testes, $p < 0.001$ for epididymis). Among the treated groups, the 48-days diabetic rats showed further reduction in the weight compared with the 24–days group. The findings were statistically significant ($p < 0.01$ versus the 24-days diabetic group). Although the epididymis showed a reduction, the findings were not statistically significant.

Microscopic Observation

Histological Observation of the Testes

The seminiferous tubules of the testes of the control group were healthy, showing normal testicular morphology, as well as all levels of spermatogenic cells. The Sertoli cells and the Leydig cells were viewed as normal (►**Fig. 3a** and **b**).

The diabetic animals presented a significant decrease in the diameter of the seminiferous tubules compared with the control group ($p < 0.01$). The structure of the tubules was disrupted. There was a considerable reduction in the number of the spermatogenic cells, that is the primary and secondary spermatocytes. Reduction in the number of spermatids was also perceived. There was a decrease in the Sertoli cells, as well as in the Leydig cells. The increased thickness of the basement membrane of the tubules was observed. Further, the spaces between the seminiferous tubules showed very less/scanty connective tissue (►**Fig. 3c**, **d**, **e**, **f**).

The surface area of the most circular seminiferous tubules was reduced pointedly in the diabetic groups as opposed to the control group. The mean diameter that was calculated using the maximum length and breadth was decreased in the diabetic group. There was a steady decline in the testes biopsy score as calculated using the Johnsen criteria to quantify the nature of the tubules.[9] All of the values were considerably reduced in the 48-days diabetic group in

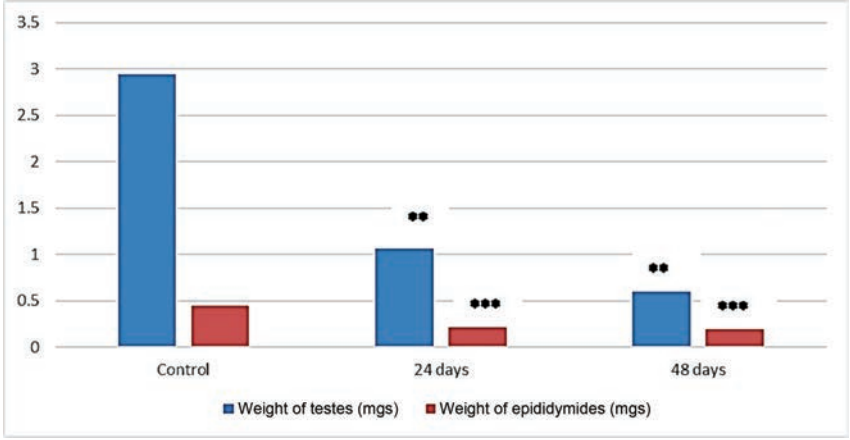

Fig. 2 Chart showing the comparison between the mean weight the testes and of the epididymides in the control and in the diabetic groups sacrificed at 24 and 48 days. ** p-value <0.01; *** p-value <0.001 versuss control.

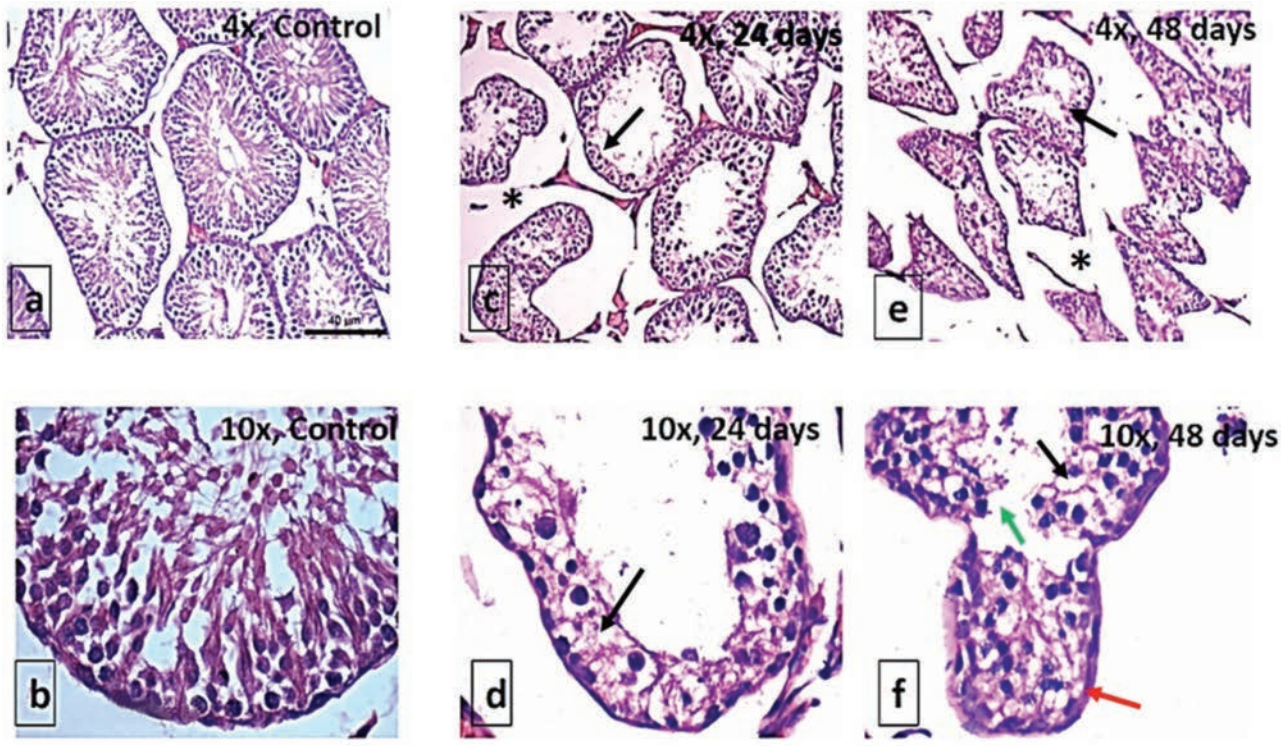

Fig. 3 Hematoxylin and eosin sections showing microscopic changes in the testicular tissue of the control and diabetic groups. (a) 4x magnification shows the normal morphology of the seminiferous tubules, of the connective tissues, and of the Leydig cells. (b) 10x magnification shows the normal morphology of single seminiferous tubules with normal Sertoli cells and all the layers of spermatogenic cells, from spermatogonia to spermatids. (c) 4x magnification shows the degenerated seminiferous tubules (indicated by black arrow) and reduced levels of connective tissue and of Leydig cells (indicated by *). (d) 10x magnification of a single tubule with decreased spermatogenic cells and Sertoli cells (indicated by black arrow). (e) 4x magnification showing the decreased diameter of the seminiferous tubules (indicated by black arrow) and the degeneration of the connective tissue in the spaces between them (indicated by *). (f) 10x magnification of a single seminiferous tubule showing a thickened basement membrane (indicated by red arrow), very few spermatocytes (indicated by black arrow), and a narrow lumen (indicated by green arrow).

comparison with both the control and the 24–days diabetic group (►**Table 1**).

Histological Observation of the Epididymis

The microscopic structure of the epididymides in the control group showed normal morphology, the typical structure of the epididymal tubule, lining epithelium which is stratified columnar was noted, and abundant spermatozoa within the lumen were present (►**Fig. 4a** and **b**).

The epididymides in the diabetic groups showed a considerable reduction in the tubular surface area compared with the control group ($p < 0.01$). There was also a reduction in the mean diameter, which was measured using the maximum and minimum diameter of the tubules ($p < 0.01$) (►**Table 2**). The lumen of the epididymis also showed a reduction in its dimensions, almost occluded lumen and completely devoid of spermatozoa (►**Fig. 4c**, **d**, **e**, **f**). Clamping of the nuclei was observed, which was due to tubular shrinkage. The connective tissue between the tubules was sparse. The clamping of the nuclei showed an increase in the 48-days group in comparison both with the control and with the 24–days groups (►**Table 2**).

Discussion

Chronic hyperglycemia is a metabolic disease that threatens the world with multiple organ and system imbalances, including reproductive system dysfunction. Several results

Table 1 Comparing diameter, surface area and Johnsen score of seminiferous tubules of testes, between control and diabetic groups

Parameter	Control	24 days	48 days
Diameter of the tubules (μm)	73.83 ± 12.95	34.48 ± 4.97***	20.82 ± 6.41***
Surface area of the tubules (μm^2)	449.22 ± 150.69	115.98 ± 22.41***	70.39 ± 40.41***
Johnsen score	9.32 ± 0.81	4.06 ± 0.91***	3.12 ± 0.6***

*** p-value < 0.001 versus control.

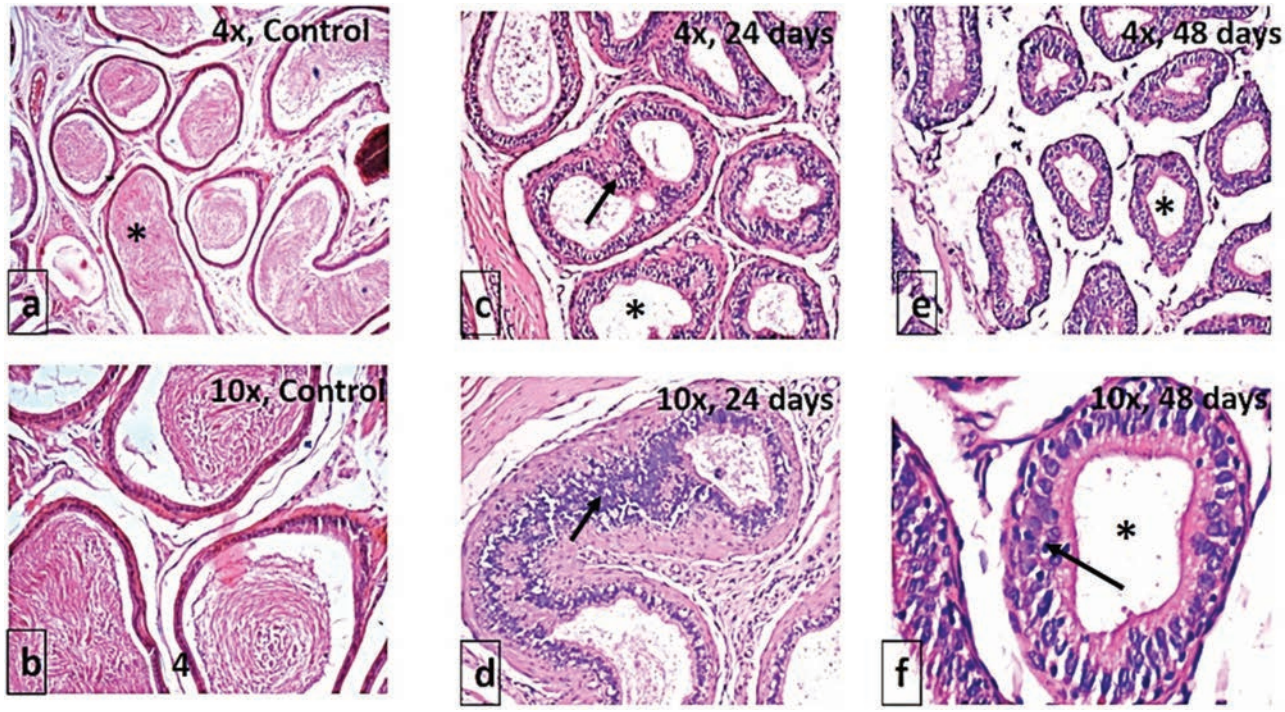

Fig. 4 Hematoxylin and eosin sections showing microscopic changes in the epididymides of the control and diabetic groups. (a) 4x magnification showing the normal morphology of the epididymides, and the lumen filled with abundant spermatozoa (indicated by *). (b) 10x magnification showing normal lining epithelium and smooth muscle layer surrounding it. (c) 4x magnification of the 24-days diabetic group, note the shrinkage of the epididymal tubule; clamping of nuclei in the lining epithelium (indicated by black arrow) and reduced spermatozoa in the lumen (indicated by *). (d) 10x magnification of the seminiferous tubule of 24-days diabetic group showing nuclei clamping (indicated by black arrow), which almost occludes the lumen. (e) 4x magnification of the 48-days diabetic group, showing the tubular diameter reduced, as well as the lumen reduced in size with no spermatozoa (indicated by *), connective tissue lost between the tubules, and the muscle layer diminished. (f) 40x magnification shows a single epididymal tubule of the 48-days diabetic group reduced in diameter with clamping of nuclei (indicated by black arrow), and the lumen completely devoid of spermatozoa (indicated by *).

showed that sexual behavior and reproductive tract functions are markedly affected by DM, which can lead to reduced fertility. Diabetic testicular damage may be brief or lasting, depending on the degree and on the duration of the disease.[10]

The present study showed that STZ-induced DM and increased blood glucose causes extensive histological changes in the testes and in the epididymides of rats.

In a study conducted by Guneli et al on STZ-induced diabetic rats, it was observed DM causes the reduction of spermatogenic cells, decreases the diameter of the seminiferous tubule, and increases the thickening of the basement membrane. It was perceived that the degeneration was caused by cell apoptosis as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive cells were suggestively more in the test group.[11] Ballester et al showed that the testicular sperm count, motility and testicular weight was pointedly reduced in diabetic rats. These changes were attributed to a significant reduction in the sex hormones (luteinizing hormone [LH] and follicle stimulating hormone [FSH]) secreted by the pituitary gland in diabetic rats, which led to a decrease in the testosterone levels.[5]

In the present study, the gross weight of the testes and of the epididymides was considerably reduced in the test group. The diameter of the seminiferous tubule and the number of spermatogenic cells also showed a marked reduction in the rats of the diabetic groups compared with the control group.

Similar studies were conducted in the past by authors who concluded that the effects of DM on spermatogenesis

Table 2 Comparing diameter and surface area of tubules of the epididymides between control and diabetic groups sacrificed at 24 and 48 days

Parameter	Control	24 days	48 days
Mean diameter (μm)	1002.08 ± 260.19	701.24 ± 132.57***	421.16 ± 69.41***
Mean surface area (μm^2)	1857.58 ± 291.120	831.47 ± 160.11***	474 ± 64.04***
Clamping of nuclei (μm)	215.75 ± 63.76	294.07 ± 83.91**	305.30 ± 180.24**

*** p-value < 0.001.
** p-value < 0.01 versus control.

could be identified as a lessening in the testicular diameter. Histological observation of calcification and sloughing of germ cells were also unquestionably seen.[12,13] In the present study, the degeneration of the testes as calculated using the Johnsen testicular degeneration method further confirmed infertility in the diabetic groups.

Diabetes mellitus increases the thickness of the basal lamina of the tubules, which supplements the reduction of the total size of the tubular diameter of the epididymal tubules.[2] Likewise, the histological studies performed in the present scenario discovered a substantial increase in the thickness of the basal lamina, a reduction in the size of the tubules and in the lumen of the epididymal segments. Due to tubular shrinkage, the principal cells were packed tightly, showing clamping of the nuclei on one edge of the tubule. We have also measured the thickness of the clamping of the nuclei, which was seen to almost completely occlude the lumen. This finding was not documented previously in the literature.

Based on the studies conducted in the past, oxidative stress mechanisms were understood to contribute entirely or partially to the direction of the development of gonadal degeneration, utterly bereft of spermatozoa in STZ-induced diabetes in animal prototypes.[14] Diabetes-induced weight loss of reproductive organs in males has also been reported to be caused by oxidative stress leading to the atrophy of the sex organs.[15] The present study also showed a consistently significant reduction in the weights of the testes and of the epididymides. Numerous epididymal tubules were noted to be completely lacking in spermatozoa.

Hyperglycemia in diabetes increases the level of reactive oxygen species (ROS), which leads to DNA damage in the testes and, therefore, to a major reduction in the sperm motility, count, and viability.[16] Diabetes mellitus also results in an increase in the levels of testicular malondialdehyde (MDA), a product of lipid peroxidation, and a reduction in the antioxidant levels, such as in the activity of superoxide dismutase (SOD), which leads to oxidative injury.[17] An obvious increase in the MDA levels in diabetic rats has also been reported experimentally.[17]

Diabetes mellitus is also accompanied by the overexpression of inducible nitric oxide synthase (iNOS) and of nuclear factor kappa-light-chain-enhancer of activated B cells p-65 (NF-κB-p65), with a simultaneous upsurge in the testicular nitric oxide (NO) levels. The NO further produces reproductive dysfunction by causing testicular injuries that result in testicular atrophy and apoptosis.[18,19]

Aromatase, a member of the cytochrome P450 family, plays a vital role in the process of development and reproduction.[20] It has been reported that the expression of aromatase markedly decreases in the testicular tissues of diabetic rats.[21] Therefore, the reduced aromatase levels might be one of the critical mechanisms responsible for male reproduction dysfunctions in DM.[16]

The present study also endorses these molecular mechanisms as a possible explanation for the degenerative changes encountered in the testes and in the epididymides of diabetic rats.

In a study, 4 weeks after the STZ treatment, a significant increase in degenerated germ cells at various stages of development was observed.[22] In the current scenario, degeneration was observed as beginning at 4 weeks, and it was more marked at 8 weeks. However, although STZ-induced DM in various animal studies has been demonstrated as a successful model for studying the manifestations of DM, it has been reported that high doses of STZ might induce damage in tissues besides the pancreas.[10] In the past, authors have opined that the morphologic alterations observed in the testes of STZ-induced diabetic rats are not caused by a direct effect of the drug, but rather by DM.[23] It remains uncertain if the damages are owing to DM or to STZ itself. Diabetic testicular dysfunction may be brief or lasting, depending on the degree and on the duration of the disease.[12] However, the present study suggests that the long-term follow-up of 24 days and of 48 days of STZ-induced diabetic rat models showed significant changes in the histology of male reproductive organs. Moreover, it also showed a decrease in all types of spermatogenic cells in the seminiferous tubules, as well as absence of spermatozoa in the lumen of the epididymal tubules with clamping of the nuclei, which occluded the lumen of the tubules.

Conclusion

The present study gives an insight to the microscopic changes occurring in male reproductive organs, such as testes and epididymides in DM. We have made an attempt to quantify the changes and to provide reference data for future studies in the field. The present study contributes to the existing literature regarding the degenerative effects of STZ-induced DM on the male reproductive system.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Participation of the Intercostal Nerves to the Innervation of the Diaphragm Muscle in *Cavia porcellus*

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Abstract

Introduction The diaphragm is the leading respiratory muscle. It is innervated mainly by the diaphragmatic nerve, and, in some species, by the delicate fibers of the intercostal nerves. In guinea pigs, there is no description of these anatomic structures that innerve this important muscle. This study aimed to analyze the participation of the intercostal nerves to the innervations of the diaphragm of guinea pigs of both sexes.

Materials and Methods We studied 40 guinea pigs (*Cavia porcellus*) of both sexes. We fixed and dissected the diaphragm of the specimens used in the experiment to assess the path of the intercostals nerves in both the body antimeres.

Results The diaphragm was innervated by the intercostal nerve pairs 6 through 12, and, less frequently, by the 8^{th} nerve (38/40 = 95%), followed by the 7^{th} (36/40 = 90%) and subsequently by the 9^{th} (32/40 = 80%). The 12^{th} nerve presented the lowest frequency (2/20 = 10%) in both genders. All nerve pairs displayed similar occurrence compared with the gender and the antimeric disposition. The only exception was the 9th nerve, which presented a significant variation of the occurrence, both in relation to gender and antimeric disposition. From a statistic point of view, all nerves were independent. We observed no correlation between the gender and their position.

Conclusions We shall conclude that the diaphragm of guinea pigs is innervated by the 6^{th} through 12^{th} pairs of intercostal nerves, with the 7^{th}, 8^{th}, and 9^{th} being the primary providers. There is no interference of the variables gender or antimeric disposition on the behavior of the intercostal nerves of guinea pigs as refers to their origin and participation to the innervations of the diaphragm.

Keywords
- guinea pig
- diaphragmatic muscle
- innervation

Introduction

Breathing is the only form to provide oxygen to human beings and animals. As it enters into the organism, the oxygen combines with most of the nutrients before entering the cells and producing the energy that is crucial for the performance of physiologic processes required for survival. The muscles enrolled in the respiratory process are the diaphragm, the scalene, and the intercostal muscles.[1] The diaphragm is deemed to be only one and essential respiratory muscle,[2] contributing from 60 to 80% of the respiratory process,[3] as it is the main muscle

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involved in the inspiration process. The diaphragm is not only enrolled in the act of breathing. It is also crucial to assist the abdominal muscles during vomiting, coughing, urination, sneezing, defecation, and labor.[4] Almeida et al (2008)[5] describe the intercostal nerves, mainly those from the 7th to the 9th, as primary efferent pathways for some of these actions or reflexes.

The diaphragm receives motor nerves mainly, or solely from the phrenic nerves.[6–13] Even the same, in some species, there is a participation also from branches derived from the intercostal nerves.[5,14–19] There are 12 pairs of intercostal nerves. They derive from the ventral branches of the thoracic nerves, do not form plexus and distribute to the walls of the thorax and the abdomen. Almost all nerves lie in the intercostal space (therefore they are denominated "intercostal nerves"). The 12th intercostal nerve is named *subcostal*, as it lies under (behind) the last rib.[20]

Guinea pig (*Cavia porcellus*) is a primary species for the science. It is frequently used for studies on the anatomy and physiology that shall be applied to the humans and other species.[21–23] Even with the diaphragm being responsible for such an essential function as breathing, there are no studies on the role of the intercostal nerves to its innervation in guinea pigs.

This study aimed to assess the participation of the intercostal nerves to the innervation of the diaphragm in guinea pigs *Cavia porcellus* of both genders and to describe the collateral innervation of this organ by the intercostal nerves.

Materials and Methods

In this study, 40 healthy adult male ($n = 20$) and female ($n = 20$) guinea pigs *Cavia porcellus* (Linnaeus, 1758) were used. The animals came from the central laboratory animal house of the Universidade Federal do Vale do São Francisco (UNIVASF, in the Portuguese acronym). This study was approved and certified by the ethical committee for the use of animals of the UNIVASF (CEUA, in the Portuguese acronym, protocol number 0012/160512).

The specimens were transported to the laboratory of domestic and wild animal anatomy of the UNIVASF (LAADS, , in the Portuguese acronym), where they were euthanized (40 mg/kg of 5% ketamine; 10 mg/kg of 2% xylazine intraperitoneally to induce anesthesia, followed by 5 ml of 10% potassium chloride, intraperitoneally). As a criterion for the enrollment, the animals should not present any anomaly or pathology in the thoracic or abdominal region.

After the euthanasia of the guinea pigs, the left common carotid artery was cannulated to perfuse a 10% water solution of formaldehyde using 20 mL syringes and 25 × 7 needles. After this, the anatomic pieces were dipped in 10% formaldehyde solution for 72 hours to fix the tissues. The costochondral articulations were incised to fold them to the exterior from the fifth rib on, in both antimeres. Every intercostal nerve was dissected. All the ramifications were conserved, especially those directed to the diaphragm. After their identification, the ramifications of the intercostal nerves were photographed and compared, to assess their position in the antimere and the sex of the animal.

Statistical analysis was performed using the Statistical Analysis System (SAS) software (SAS Institute Inc., Cary, NC, USA), considering the chi-square test and phi coefficient, and analyzing the independence between the gender (female, or male) and the position (left or right); the independence level between the position and the intercostal nerves and the occurrence of the participation of these nerves to the innervation of the diaphragm. A 5% significance level was adopted ($p < 0.05$).

Results

As described in ►**Table 1**, the ramifications derived from the intercostal nerve pairs 6 to 12 innerve the diaphragm of guinea pigs. According to the frequency of occurrence, the intercostal nerves were classified in four groups: A group (average) represented by the nerves 6 and 10; B group (high) represented by the nerves 7, 8, and 9; C group (intermediate) represented by the 11th nerve, and D group (low), represented by the nerve 12. We observed no significant difference among the nerves classified in the same group of occurrences.

Table 1 Frequency and percentage of occurrence of the intercostal nerves in the innervation of the diaphragm of guinea pigs (*Cavia porcellus*), according to the antimere and the gender—Petrolina (PE), 2018

Nerve	VI		VII		VIII		IX		X		XI		XII	
Gender	R	L	R	L	R	L	R	L	R	L	R	L	R	L
Females ($N = 20$)														
FR	12	13	16	18	18	18	17	14	10	11	4	5	1	2
%	24.5	26.5	22.9	25.7	24.3	24.3	27.0	22.2	24.4	26.8	26.7	33.3	14.3	28.6
Males ($N = 20$)														
FR	11	13	18	18	20	18	17	15	10	10	3	3	2	2
%	22.4	26.5	25.7	25.7	27	24.3	27.0	23.8	24.4	24.4	20	20	28.5	28.5
Total	23	26	34	36	38	36	34	29	20	21	7	8	3	4
%	46.9	53.1	48.6	51.4	47.4	48.6	54	46	48.8	51.2	46.7	53.3	42.9	57.1

Abbreviations: F, female; FR, frequency; L, left antimere; M, male; R, right antimere.
%= percentage

The nerve pairs of the A group, 6 and 10, displayed similar occurrence (p 0.05) according to both the gender and the antimeric disposition. Both nerves presented 65% of occurrence in males, and females.

Nerves pairs of the B group 7, 8, and 9 displayed similar occurrence according to both the gender and the antimeric disposition. The 7th nerve was present in 90% (18/20) of the specimens, both male, and female. The 8th nerve too was present in 90% (18/20) of the females, and 100% (20/20) of the males. The 9th nerve was visible in 85% (17/20) of the females and males. ►**Fig. 1** shows the innervation of the diaphragm by the 7th intercostal nerve.

The nerve of the C Group, 11th, showed a similar occurrence ($p > 0.05$) as concerning the gender and the antimeric disposition, in agreement with the other nerves. Even if the participation of this nerve in the innervation of the diaphragm was not relevant, it was present in 55% (11/20) of the female, and 50% of the male subjects.

The nerve of the D Group, 12th, showed a similar occurrence ($p > 0.05$) as concerning the gender and the antimeric disposition, in agreement with the other nerves. Its participation to the innervation of the diaphragm was the lowest. It was only present in 10% (2/20) of the female, and male subjects.

The Chi-square test over 0.05, with a value of 0.82, corresponding to 8%, and Phi-coefficient of -0.026 statistically confirm the independence and the absence of any correlation between the gender and the position among all the nerves that have been studied.

When analyzing the relationship between gender and the occurrence of the nerves either in the left, or in the right antimere, we observed that in females the occurrence was more frequent ($p > 0.05$) in the left antimere (51% = 81/159), than in the right (49% = 78/159). On the other hand, in males, the occurrence was more common in the right antimere (51% = 81/160) than in the left (49% = 79/160). Among the females, all intercostal nerves except the 8th and 9th were observed more frequently in the left antimere. The 8th nerve displayed the same occurrence in both antimeres and the 9th was more frequently observed in the right antimere. Among the males, the nerves 8 and 9 displayed more frequent occurrence in the right antimere. The nerves 7, 10, 11, and 12 displayed the same occurrence in both antimeres. The nerve 11 was more frequent in the left antimere than in the right one (►**Table 1**).

Discussion

The results of this study highlight the importance of the intercostal nerves as main efferent ways for the breathing in guinea pigs, not just as motor fibers. These findings disagree with the results of previous studies, which claimed that the phrenic nerve was the main, and probably the only source of innervation of the diaphragm in some mammalian species.[6–13] On the other hand, our findings are in agreement with other studies that pointed out the contribution of the intercostal nerves to the innervation of the diaphragm.[4,5,14–19]

We observed the innervation of the diaphragm by the intercostal nerves 6 to 12, mainly by the 8th nerve. Rosenblueth et al (1961),[4] even if they observed the participation of the intercostal nerves 4 to 10, observed that the major intercostal nerve acting in the innervation of the diaphragm was the 8th nerve. In dogs, MELO et al (1999)[24] observed the innervation of the diaphragm by the intercostal nerve pairs 8 to 12 in 93.54% of cats and dogs. Faria et al (2011)[16] observed the central innervation of the diaphragm by the intercostal nerves 9 through 11, but the possible participation of the nerve fibers from the intercostal nerves 7, 8, and 12. Oliveira et al (2001)[25] observed that the diaphragm of hybrids of bovines and zebus received the innervation of the intercostal nerves 7 through 12 in 50% of the animals; from the intercostal nerves 6 through 12 in 33.3%; from the left antimere of the 6th intercostal nerve in 13.3% of the animals, and from the right antimere of the 7th nerve in 3.3% of the animals. Almeida et al (2008)[5] observed in bovines from the Santa Inês breed, the contribution to the innervation of the diaphragm of the intercostal nerves 8 to 12

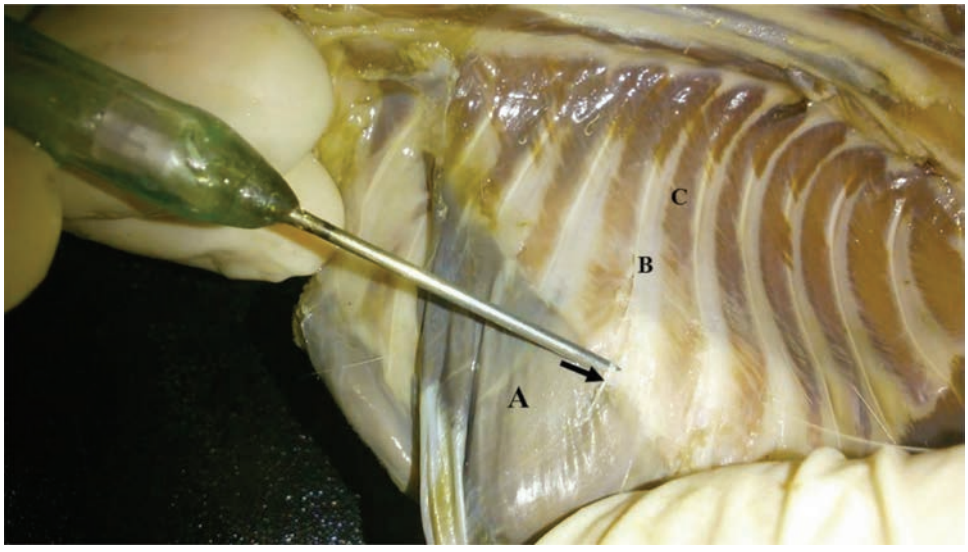

Fig. 1 Photograph highlighting the participation of the VII intercostal nerve (arrow) to the innervation of the diaphragm (A) of guinea pigs (*Cavia porcellus*). In (B), the seventh rib is visible and in (C), the intercostal muscle—Petrolina (PE), 2018.

in 63.33% of the animals, and of the pairs 9 to 12 in 26.68% of the animals, in both antimeres. In agreement with our results, these authors also did not observe any difference in the participation of the intercostal nerves to the innervation of the diaphragm neither according to the gender nor the antimeric disposition.

In this study, we observed little participation of the intercostal nerve 12 (10%) to the innervation of the diaphragm in both genders, in disagreement with the study of Melo et al (1999),[24] who observed that the 7th pair of nerves innerved the diaphragm in only 3.22% of the cases.

Conclusions

We shall conclude that the diaphragm of guinea pigs is innervated by the 6th through 12th pairs of intercostal nerves, with the 7th, 8th, and 9th being the primary providers. There is no interference of the variables gender or antimeric disposition on the behavior of the intercostal nerves of guinea pigs as refers to their origin and participation to the innervation of the diaphragm.

Conflicts of Interest

The authors have no conflicts of interest to report.

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Correlation between Second to Fourth Digit Ratio and Anthropometric Variables Indicative of Cardiovascular Disease

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Abstract

Second to fourth digit ratio (2D:4D) is a sexually dimorphic biometric marker. Regarding indirect evidence, there have been several studies that link the waist-to-hip ratio (WHR) with the 2D:4D ratio. If the 2D:4D ratio is associated with testosterone and estrogen levels, it may be correlated with a risk of myocardial infarction (MI). The aim of the present study is to find out the correlation between anthropometric risk factors for cardiovascular disease and the 2D:4D ratio in 250 young females of the state of Haryana in Northern India. The present study was conducted on 250 females of the Haryana population aged between 17 and 35 years old. A series of 8 anthropometric measurements was obtained from the participants: height, weight, 2D:4D ratio, body mass index (BMI), waist circumference (WC), hip circumference (HC), neck circumference (NC), and WHR. The data was collected, tabulated and subjected to statistical computation using SPSS Statistics for Windows, Version 13.0 (SPSS Inc., Chicago, IL, USA). Strong positive associations between the NC and the WHR confirm that both measures are indicative of body fat. Also, a positive correlation between the 2D:4D ratio and the WHR suggests that low androgen levels in women are associated with a greater risk of obesity. Moreover, this result, as well as the positive correlation between 2D:4D ratios and NC, suggest that the digit ratio is indicative for being overweight in women and suggest a predisposition toward cardiovascular disease – however, these correlations of body measurements with digit ratios are not significant.

Keywords
- 2D:4D ratio
- coronary heart disease
- correlation
- waist-to-hip ratio
- neck circumference

Introduction

Second to fourth digit ratio (2D:4D) is a sexually dimorphic biometric marker. It is influenced by prenatal estrogen and testosterone levels. High prenatal levels of androgens (high testosterone/estrogen) determine lower values of 2D:4D, and vice-versa. The *Hox A* and *Hox D* genes are responsible for both gonadal and digital differentiation.[1]

Relative finger lengths are determined before birth at ~ 13 weeks of gestation.[2] The gender difference is present in children,[2,3] and gender differences in the 2D:4D ratio are robust across several ethnic groups and races.[4–6]

Regarding indirect evidence, there have been several studies that link the waist-to-hip ratio (WHR) with the 2D:4D ratio.[5,7] In female subjects, the WHR appears to be directly linked to health and fertility, since it has been shown to be an accurate predictor of risk for various diseases.[8,9] In men, studies have shown that aging is accompanied by decreasing levels of testosterone, which in turn decrease

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lean body mass and increase the deposition of abdominal fat. Testosterone supplementation in elderly male subjects and in healthy eugonadal men decreases the WHR and increases lean body mass.[10,11] Manning et al found a negative relationship between the 2D:4D ratio and the age at the first myocardial infarction (MI), which means that men with a low 2D:4D ratio tended to have their first MI later in life than men with high 2D:4D ratios.[12] Consequently, they suggested that the formation and maintenance of the cardiovascular system is sensitive to testosterone and estrogen in men, and that the 2D:4D ratio is a marker for in utero and adult levels of these hormones. This has led to the assumption that if the 2D:4D ratio is associated with testosterone and estrogen levels, it may be correlated with a risk of MI. Manning et al[12,13] found a negative relationship between the 2D:4D ratio and the age at the first MI, which means that men with a low 2D:4D ratio tended to have their first MI later in life than men with high 2D:4D ratios.[12,13] Consequently, they suggested that the formation and maintenance of the cardiovascular system is sensitive to testosterone and estrogen in men,[14] and that the 2D:4D ratio is a marker for in utero and adult levels of these hormones.

Body obesity and metabolic syndrome, a cluster of conditions associated with an increased risk for type 2 diabetes and hypertension, are considered a major risk factor for coronary heart disease (CHD), associated with an elevated risk for stroke and early mortality. However, Ben-Noun et al[15] tested a method of identifying overweight or obese patients solely by measuring their neck circumference (NC). Their results indicated a significant association between the NC and body mass index (BMI), age, weight, waist circumference (WC), hip circumference (HC), and WHR. A follow-up study also demonstrated that a higher NC is positively correlated with the factors of the metabolic syndrome and, therefore, it is likely to increase the risk of CHD.[16]

Finally, Fink et al[17] studied the 2D:4D ratio in relation to measurements of body shape and body fat distribution and found some support for an early organizational effect of sex hormones through the association between indices of female body shape, male BMI, and human finger length.[17]

White et al[18] have shown the relationship between the 2D:4D ratio and elevated triglycerides, which supports the use of the 2D:4D ratio as a non-invasive screening tool to assess the risk of metabolic syndrome.

The aim of the present study was to assess the correlation of the 2D:4D ratio with known risk factors for developing cardiovascular disease (CVD) in young females of the state of Haryana in Northern India. Because it is reflective of fetal androgen exposure, we have hypothesized that the 2D:4D ratio would correlate with parameters associated with CVD. If our hypothesis is correct, measuring the 2D:4D ratio of a patient would be a non-invasive way to determine their risk of developing cardiovascular disease.

Materials and Methodology

The present study was conducted on 250 females of the state of Haryana in northern India, aged between 17 and 35 years old. Approval by the institutional ethical committee was obtained and the informed consent from all subjects was taken before conducting the study.

Inclusion Criteria

1. Young individuals between 18 and 35 years old.
2. Individuals of the state of Haryana.
3. Individuals with no morphologically identifiable physical anomalies or deformities.

Exclusion Criteria

1. Individuals who were not from the state of Haryana.
2. Pregnant females.
3. The subjects with any apparent physical hand anomalies, inflammation, trauma, or deformities, and those who had underwent a recent major surgery were excluded because of their unsuitability for the present investigation.
4. Subjects having any genetic, psychological, neurological or chronic diseases affecting the hand parameters.
5. Individuals with a history of any recent drug intake.
6. Females who were wheelchair bound or had difficulty in standing.
7. Diagnosed cases of heart disease, hypertension, diabetes mellitus, chronic diseases of major organs, and endocrine disorders.
8. Subjects $<$ 18 years old and $>$ 35 years old.

A series of eight anthropometric measurements were obtained with the participants:

1. Height
2. Body weight
3. 2D:4D ratio
4. Body mass index
5. Waist circumference
6. Hip circumference
7. Neck circumference
8. Waist-to-hip ratio
9. Waist-to-height ratio (WHtR)

1. **Height**: Height was measured (to the nearest 0.5 cm) with the subject standing in an erect position against a vertical scale of portable Seca 213 Portable stadiometer (Seca Deutschland,22089 Hamburg, Germany), with the head positioned so that the top of the external auditory meatus was in level with the inferior margin of the bony orbit.[19]
2. **Body weight**: Body weight was measured (to the nearest 0.5 kg) with the subject standing motionless on a bathroom weighing Venus Electronic Bathroom scale (Ace incorporation, Jaipur, Rajasthan, India).[19]
3. **2D:4D ratio**: It is defined as the ratio of the length of second digit (index finger) to the length of the fourth digit (ring finger). The measurement was taken from both hands with an electronic sliding Mitutoyo 6 AOC Digital Sliding Caliper (Mitutoyo, Japan) from the palmar side with the digits fully stretched and touching on a hard flat surface, with the second to fifth digits adducted and the thumb slightly extended.

4. **Body mass index:** The BMI was calculated as weight in kilograms divided by the squared height in meters (weight in kg/height in m^2).[19]
5. **Waist circumference:** The WC was measured by using bone landmarks as references with the help of a measuring tape. The World Health Organization (WHO) guidelines recommend the measurement of the WC at the midpoint between the lowest rib and the iliac crest (the highest point of the ilium).[19]
6. **Hip Circumference**: The HC was measured at the level of the greater trochanters with the help of a measuring tape.[19] It is defined as the maximum circumference in the gluteal area.
7. **Neck circumference:** The NC (cm) was measured at the level of the upper margin of the thyroid cartilage by using a measuring tape.[20]
8. **Waist-to-hip ratio:** The WHR was calculated using the following formula: WHR = WC (cm)/HC (cm)

Each measurement was taken three times by the same individual, and the mean of the three measurements was considered as the final reading.

Statistical analysis

The data was collected, tabulated and statistically analyzed using SPSS Statistics for Windows, Version 13.0 (SPSS Inc., Chicago, IL, USA).. Descriptive statistics of each risk factor and pairwise correlations with the 2D:4D ratio were conducted. The descriptive statistics, means and standard deviations (SDs) of the body measurements and of the 2D:4D ratios are shown in ►**Table 1**. The Pearson correlation (r) was used for assessing the relationships between the 2D:4D and the body measurements (►**Table 2**).

Results

Strong significant positive associations between NC and WHR confirm that both measures are indicative of body fat (►**Table 3**). The present findings of positive correlations between 2D:4D ratios and NC (►**Table 2**) in women of the state of Haryana suggest a possible predisposition toward CHD. Neck circumference is also significantly positively correlated with weight, WC, and HC in the present study (►**Table 3**).

Table 1 Descriptive statistics, means and standard deviations of body measures and 2D:4D ratios

Measurement	Mean	Standard deviation
Height (cm)	145.85	7.85
Weight (kg)	37.95	12.67
WC	67.93	7.85
HC	85.62	8.57
NC	28.60	2.70
BMI	21.24	3.15
WHR	0.72	0.20
RT 2D:4D	0.98	0.03
LT 2D:4D	0.97	0.01

Abbreviations: BMI, body mass index; HC, hip circumference; LT 2D:4D, second to fourth digit ratio of left hand; NC, neck circumference; RT 2D:4D, second to fourth digit ratio of right hand; WC, waist circumference; WHR, waist-to-hip ratio.

Table 2 Correlation coefficients for the relationships between 2D:4D ratios and body measures

	Rt 2D:4D ratio	*p-value*	Lt 2D:4D ratio	*p-value*
Age	0.034	0.66	0.044	0.57
Height	0.126	0.066	0.034	0.63
Weight	0.158	0.02	0.130	0.06
WC	− 0.004	0.96	0.022	0.75
HC	0.025	0.72	− 0.032	0.64
NC	0.031	0.66	0.023	0.74
BMI	0.034	0.66	0.044	0.57
WHR	0.127	0.06	0.114	0.09

Abbreviations: BMI, body mass index; HC, hip circumference; LT 2D:4D, second to fourth digit ratio of left hand; NC, neck circumference; RT 2D:4D, second to fourth digit ratio of right hand; WC, waist circumference; WHR, waist-to-hip ratio.

Discussion

No significant correlation between the 2D:4D ratio and the WHR for females was found in any of the studies shown in ►**Table 4**. The WC and HC are negatively correlated with the 2D:4D ratio, but this correlation is significant only in the study of Fink et al.[17] The WHR and the BMI were negatively correlated with the 2D:4D ratio in the studies by Fink et al[17] and by Fink et al,[20] but not in the present study.

Oyeyemi et al[21] have shown a positive significant correlation between the 2D:4D ratio, BMI, and the WHtR, suggesting a possible predisposition toward cardiovascular disease. The authors have also shown a significant correlation between the 2D:4D ratio and the NC, but this positive correlation was not significant in the present study.

Oyeyemi et al[22] have shown that digit ratios in both hands failed to show any significant correlations with the NC in female subjects. Metabolic syndrome markers (BMI, WC, NC) significantly correlate with both right and left 2D:4D ratios in males and females. Thus, the 2D:4D ratio could be used as a surrogate marker for the risk of metabolic syndrome and CVD in Ilorin, Northcentral Nigeria.

Danborno et al[23] showed a significant relationship between 2D and 4D lengths in the right and left hands with chest circumference, WC and HC only in males, but not in females, which is in line with the present study.

Manning[4] has proven in his study that mothers with high WHR, which is associated with high testosterone and low estrogen, tend to have children with low 2D:4D ratios. Mothers with low 2D:4D ratios tend to have children with low 2D:4D ratios, and their children have high concentrations of testosterone in their amniotic fluid.

Table 3 Pearson correlation coefficients of the body measures

	Height	Weight	WC	HC	NC	BMI	WHR
Height	1	0.418	0.511	0.536	0.652	− 0.032	0.368
Weight	0.418	1	0.376	0.311	0.373	0.046	0.250
WC	0.511	0.376	1	0.800	0.795	0.300	0.720
HC	0.536	0.311	0.800	1	0.698	0.268	0.563
NC	0.652	0.373	0.795**	0.698**	1	0.060	0.770**
BMI	− 0.032	0.046	0.306	0.268	0.060	1	0.009
WHR	0.368	0.250	0.720	0.563	0.770	0.009	1

Abbreviations: BMI, body mass index; HC, hip circumference; NC, neck circumference; WC, waist circumference; WHR, waist-to-hip ratio.
**Correlation is significant at 0.01 level

Table 4 Correlation between 2D:4D ratio and body measures in different studies

	Fink et al (2003)[17]		Fink et al (2006)[20]		Present study	
	Rt 2D:4D	Lt 2D:4D	Rt 2D:4D	Lt 2D:4D	Rt 2D:4D	Lt 2D:4D
WC	− 0.358	− 0.380	− 0.146	− 0.023	− 0.004	− 0.022
HC	− 0.307	− 0.296	− 0.029	− 0.014	0.025	− 0.032
BMI	− 0.193	− 0.130	− 0.006	− 0.070	0.034	0.044
WHR	− 0.082	− 0.140	− 0.152	− 0.012	0.127	0.114
NC	—	—	− 0.080	− 0.031	0.031	0.23

Abbreviations: BMI, body mass index; HC, hip circumference; LT 2D:4D, second to fourth digit ratio of left hand; NC, neck circumference; RT 2D:4D, second to fourth digit ratio of right hand; WC, waist circumference; WHR, waist-to-hip ratio.

White et al[18] have shown the relationship between the 2D:4D ratio and elevated triglycerides, but found no relationship between WC and the 2D:4D ratio of either hand, which is in line with the present study.

Conclusions

- There is a significant association between NC and height, weight, WC, HC and WHR. A higher NC is positively correlated with factors of metabolic syndrome and, therefore, is likely to increase the risk of CHD.
- The present findings of positive correlations between 2D:4D ratios and the NC in women of the state of Haryana suggest a possible predisposition toward cardiovascular disease, but the correlation is not significant.
- The present study did not show any significant correlation between the right and left 2D:4D ratios with WC, HC, BMI, NC, or WHR.
- Body mass index, WHR, and NC were positively correlated with the 2D:4D ratio, whereas WC and HC were negatively correlated with the 2D:4D ratio, but the correlation was not significant.

The present study is not without limitations. The first limitation is its small sample size. A large sample would provide power to limit the potential for bias in predictive models. Variations in the study design and in the mode of measurement of the digits may introduce variability that could result in differing outcomes when reporting data. A further investigation is required on a larger number of subjects in order to obtain a more detailed picture of possible associations.

Conflicts of Interests

The authors have no conflicts of interests to declare.

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Relationship of Handedness with Second to Fourth Digit (2D: 4D) Ratio and its Role in Sexual Dimorphism in Tibeto-Nepalese and Indo-Nepalese Adult Population of the Dharan Municipality, Sunsari District of Eastern Nepal: An Anthropometric Study

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Abstract

Introduction The importance of handedness lies in its relationship with the lateralization of the brain function, especially of language. The aim of the present study was to provide an authentic database on right and left second to fourth digit (2D:4D) ratios in 2 different ethnic groups of a particular age and gender, and to study its correlation with handedness and sexual dimorphism.

Materials and Methods The present study was conducted among 400 Tibeto-Nepalese (TN) subjects (200 of each gender) and 400 Indo-Nepalese (IN) subjects (200 of each gender) aged $\geq$ 18 years old. The values for the 2D:4D ratio were calculated for both hands. Hand preference was established according to the Edinburgh Handedness Inventory, and five hand preference determination groups were constituted after the calculation of the laterality score. The results were tabulated and subjected to statistical analysis. A *p*-value < 0.05 was considered as statistically significant.

Results The mean value of right sided 2D:4D ratio of TN female was higher as compared to IN female; and for left sided 2D:4D ratio, the value was lower in TN female as compared to IN female. When the values of the 2D:4D ratios were assessed by gender, the values were found to be statistically significant ($p < 0.001$), whereas when they were assessed by ethnicity, only the right hand 2D:4D ratio was found to be statistically significant ($p < 0.001$).

Conclusion When the relationships between the laterality score and the 2D:4D ratios were examined, the values were found to be statistically significant in both ethnic groups only on the left side. The 2D:4D ratio shows significant ethnic and population differences in the present study.

Keywords
- handedness
- lateralization
- tibeto-nepalese
- indo-nepalese

Introduction

The political scientists Joshi and Rose broadly classify the Nepalese population into three major ethnic groups in terms of their origin: Indo-Nepalese (IN), Tibeto-Nepalese (TN), and Indigenous (IND). The first group, comprising those of IN origin, inhabits the more fertile lower hills, the river valleys, and the Terai plains adjoining the borders of India. The second major group consists of communities of TN origin occupying the higher hills from the west to the east.[1] The distribution of

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hand preference is J-shaped, reflecting the predominant use of the right hand.[2] The importance of handedness lies in its relationship with the lateralization of the brain function, especially of language.[3] The Geschwind-Behan-Galaburda theory states that the influence on early cell loss and prenatal levels of testosterone are related to cerebral lateralization and handedness.[4] The sexual dimorphism in the 2D:4D ratio is established as early in as the 9th week of fetal life. It is found in children, is little affected by puberty, and appears to be universal in human populations.[5–8]

The main objectives of the present study were:

- To calculate the 2D:4D ratios of both hands of adult individuals from 2 different Nepalese ethnic groups (TN and IN) of both genders.
- To study the correlation of the 2D:4D ratios with handedness or hand preference.

Materials and Methods

A population-based cross-sectional study was conducted among TN and IN subjects aged ≥ 18 years old of both genders from the Dharan municipality, in the Sunsari District of Eastern Nepal, using the Edinburgh Handedness Inventory[9] hand preference determination questionnaire and a separate pre-designed questionnaire for recording socio-demographic variables and length of the second and fourth digits of both hands. The sample size was calculated with an allowable error of 20% (95% confidence interval [CI]) by using the following formula:

Sample size (n) = $4pq/L^2$

In which:

p = population proportion of positive character,

q = 1-p and

L = allowable error.

Hence:

Sample size (n) = $4pq/L^2$ = 780 individuals

Thus, a total of 800 adults (≥ 18 years old) were recruited for the study of both ethnic groups, TN and IN (400 each), including both genders (200 males and 200 females in each ethnic group) by a systematic random sampling technique. The cutoff point for the age of the subjects was the end of January, so all of the participants had to be of a certain age on February 1st.

Inclusion Criteria

- Residence in the Dharan Municipality.

Exclusion Criteria

- Subjects with any apparent physical hand anomalies, inflammation, trauma, deformities and hand surgery
- Subjects with any genetic, psychological, neurological or chronic diseases affecting hand parameters
- Individuals who disagreed to take part in the study

Digit lengths (►**Figs. 1**, **2**) were measured on the ventral surface of the hand from the basal crease of the digit to the tip of the finger using a vernier caliper (Cescorf Equipamentos para Esportes, Porto Alegre, RS, Brazil) with a precision of 0.01 mm. Then, they were reported on the questionnaire and the 2D:4D ratio was calculated. This technique of measuring digit length has been reported to have a high degree of repeatability.[10,11] Hand preference or handedness was determined according to the Edinburgh Handedness Inventory, which evaluates the direction and the degree of hand preference.[9]

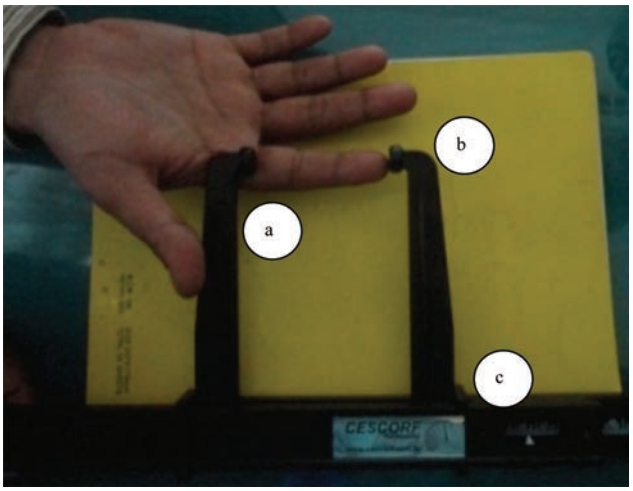

Fig. 1 **Measurement of the right 2nd digit length** (a= basal crease; b= the tip of the finger and c= vernier caliper (Cescorf Equipamentos para Esportes, Porto Alegre, RS, Brazil)

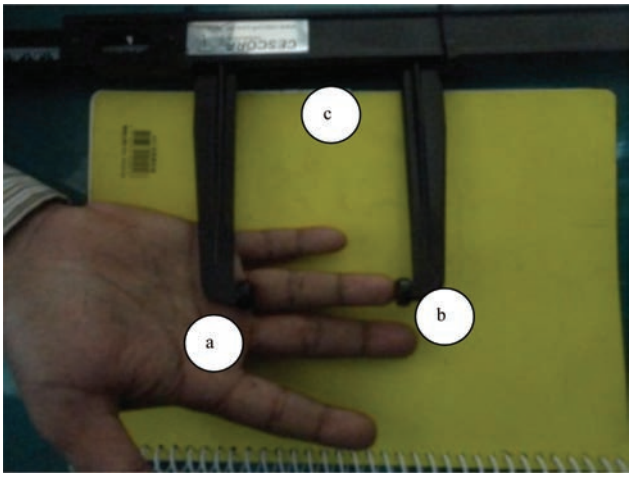

Fig. 2 **Measurement of the right 4th digit length**(a= basal crease; b= the tip of the finger and c= vernier caliper (Cescorf Equipamentos para Esportes, Porto Alegre, RS, Brazil)

Preinformed written consents were obtained from each respondent, and we have also assured them that their anonymity would be preserved during and after the present study. Ethical clearance for the performance of the present study was taken from the institutional ethical and review board. Participation in the present study was voluntary and the purpose of the study was explained to the participants prior to the distribution of the questionnaires. Permission from the Office of the Dharan municipality was also obtained.

Questions regarding the hand preferences of the participants in: 1) writing; 2) drawing; 3) throwing balls; 4) using scissors; 5) using toothbrush; 6) knife without fork; 7) using

Table 1 Hand preference were evaluated in 5 groups depending on the value of the Geschwind laterality score

Hand Preference*	Geschwind Score**	
	Minimum value	Maximum value
Right hand		
Strong	+80	+100
Weak	+20	+75
Ambhidextrous	−15	+15
Left hand		
Weak	−75	−20
Strong	−100	−80

*Hand preference was determined according to the Edinburgh Inventory, which evaluates the direction and the degree of hand preference (Oldfield Rc,1971).
**The resultant sum of these points were used to determine the Geschwind (laterality) score, an indicator of the direction and of the degree of hand preference.

a spoon 8); using a broom 9); striking matches; and 10) opening boxes were asked to the subjects by providing the questionnaire. Subjects had to put a " + " in the column associated with the hand that they was used to carry out each activity. They had to put a " + +" in the associated column if their preference for one hand was very strong; and to put a " + " in both columns if they used both hand equally. A " + +" in the right column was assigned 10 points, a " + " in the right column 5 points, a " + +" in the left column - 10 points, and, a " + " in the left column - 5 points. The resultant sum of these points was used to determine the Geschwind (laterality) score, an indicator of the direction and of the degree of hand preference. The $-100 \leq$ Geschwind score $\leq +100$ (right hand preference decreases and left hand preference is quantified by Geschwind score, value of which ranges from − 100 (strong left hand preference) to + 100 (strong right hand preference). Hand preference was evaluated in 5 groups depending on the value of the Geschwind laterality score, as shown in ►**Table 1**.

The results were expressed as mean ± standard deviation (SD). The gender differences in the 2D:4D ratios and in hand preference in each ethnic group were evaluated by an unpaired *t*-test. The right-left differences in 2D:4D ratios (2D:4D right hand – 2D:4D left hand) in each gender and ethnic group were evaluated by an unpaired *t*-test. The differences of laterality in the 2D:4D ratios, taking into account the hand preference, were evaluated by the paired student *t*-test. A $p < 0.05$ was considered statistically significant. The correlation between the hand preference and the 2D:4D ratios were evaluated by the Pearson correlation coefficient (r) analysis. Statistical analyses were performed using SPSS Statistics for Windows, Version 11.4 (SPSS Inc., Chicago, IL, USA).

Table 3 Interpretation of 2D:4D ratio by gender, hand and ethnic group

Ethnicity	Hand	Female	Male	*p-value*
TN	**Right**	1.007 ± 0.039	1.005 ± 0.042	<**0.001**
	Left	0.999 ± 0.040	1.003 ± 0.045	<**0.001**
IN	**Right**	1.005 ± 0.024	1.005 ± 0.044	0.686
	Left	1.001 ± 0.031	1.003 ± 0.045	**0.04**

Abbreviations: 2D:4D, second to fourth digit ration; IN, Indo-Nepalese; TN,Tibeto-Nepalese.
Results are shown as mean ± standard deviation, with *p-value*.

Results

In the present study, 82% of the TN males were found to have a strong right hand (SRH) whereas 82.5% of the TN females were found to have a SRH, followed by 10% and 12.5% strong left hand (SLH) in males and females, respectively, whereas in IN males and females, SRH were 86%, followed by 10% and 12.5% SLH in males and females, respectively (►**Table 2**).The 2D:4D ratio of the TN subjects in both hands of both genders were found be statistically significant ($p < 0.001$). However, in the IN subjects of both genders, only the left side 2D:4D ratio was found be statistically significant ($p < 0.001$) (►**Table 3**).The right-left difference by gender in each group were found be statistically significant ($p < 0.001$) (►**Table 4**). The right-left 2D:4D ratios in the 5 groups evaluated by hand preference in the TN population were found to be not statistically significant ($p > 0.05$) (►**Table 5**). The right-left 2D:4Ds ratios in SRH and SLH TN males were found be

Table 2 Distribution of subjects by hand preference and gender

Hand preference	TN male *n* (%)	TN female *n* (%)	IN Male *n* (%)	IN female *n* (%)	Total *n* (%)
Strong right hand	164 (82)	165 (82.5)	172 (86)	172 (86)	673 (84)
Weak right hand	13 (6.5)	9 (4.5)	8 (4)	2 (1)	32 (4)
Ambidextrous hand	0	1 (0.5)	0	0	1 (0.1)
Weak left hand	3 (1.5)	0	0	1 (0.5)	4 (0.5)
Strong left hand	20 (10)	25 (12.5)	20 (10)	25 (12.5)	90 (11.2)
Total	200 (25)	200 (25)	200 (25)	200 (25)	800 (100)

Abbreviations: IN, Indo-Nepalese; IND, Indigenous; TN,Tibeto-Nepalese.
Results are shown as number and percentage of frequency.

Table 4 Right-Left difference by gender in each group

Ethnicity	Male	Female	*p*-value
TN	-0.0021 ± 0.044	0.0012 ± 0.053	<**0.001**
IN	0.0021 ± 0.036	0.0012 ± 0.029	<**0.001**

Abbreviations: 2D:4D, second to fourth digit ratio; IN, Indo-Nepalese; TN,Tibeto-Nepalese.
Results are shown as mean ± standard deviation, with *p-value*.

Table 5 Right-Left 2D:4D ratio in five groups evaluated by hand preference in Tibeto-Nepalese

Handedness	Right	Left	*p*-value
SRH	1.0032 ± 0.041	1.0033 ± 0.042	> 0.05
WRH	0.979 ± 0.52	0.997 ± 0.48	> 0.05
AMBH	0.984	0.857	––
WLH	0.998 ± 0.097	0.967 ± 0.41	> 0.05
SLH	1.0049 ± 0.043	1.0047 ± 0.033	> 0.05

Abbreviations: 2D:4D, second to fourth digit ratio; AMBH, ambidextrous; SLH, strong left hand; SRH,strong right hand; WLH, weak left hand; WRH, weak right hand.
Results are shown as mean ± standard deviation, with *p-value*.

Table 6 Right-Left 2D:4D ratio in 5 groups evaluated by hand preference in Tibeto-Nepalese by gender

Gender	Handedness	Right	Left	*p*-value
Male	SRH	1.0033 ± 0.0421	1.0056 ± 0.0416	< **0.001**
	WRH	0.9914 ± 0.054	1.0014 ± 0.052	0.581
	WLH	0.9882 ± 0.097	0.9677 ± 0.041	0.71
	SLH	1.0121 ± 0.061	1.0132 ± 0.044	<**0.001**
Female	SRH	1.0032 ± 0.041	1.0013 ± 0.041	0.151
	WRH	0.9624 ± 0.046	0.9920 ± 0.043	0.601
	AMBH	0.9848	0.8571	––
	SLH	0.9992 ± 0.0228	0.9979 ± 0.0214	0.623

Abbreviations: 2D:4D, second to fourth digit ratio; AMBH, ambidextrous; SLH, strong left hand; SRH,strong right hand; WLH, weak left hand; WRH, weak right hand.
Results are shown as mean ± standard deviation, with *p-value*.

statistically significant ($p < 0.001$) (►**Table 6**). The right-left 2D:4D ratios in SRH and in weak right hand (WRH) of IN subjects were found be statistically significant ($p < 0.001$) (►**Table 7**). The right-left 2D:4D ratios in SRH IN males and in SRH, WRH, and SLH in IN females were found be statistically significant ($p < 0.001$) (►**Table 8**). The right 2D:4D ratio in WRH ($p = 0.007$) and in SLH ($p = 0.048$) groups in males in both ethnic groups were found be statistically significant (►**Table 9**). The right 2D:4D ratio in SRH ($p < 0.001$) and the

Table 7 Right-Left 2D:4D ratio in 5 groups evaluated by hand preference in Indo-Nepalese

Handedness	Right	Left	*p*-value
SRH	1.0034 ± 0.036	1.0023 ± 0.038	0.04
WRH	0.998 ± 0.023	1.027 ± 0.077	< 0.001
AMBH	0	0	––
WLH	1.109	0.939	––
SLH	0.9976 ± 0.031	0.998 ± 0.022	> 0.05

Abbreviations: 2D:4D, second to fourth digit ratio; AMBH, ambidextrous; SLH, strong left hand; SRH,strong right hand; WLH, weak left hand; WRH, weak right hand.
Results are shown as mean ± standard deviation, with *p-value*.

Table 8 Right-Left 2D:4D ratio in 5 groups evaluated by hand preference in Indo-Nepalese by gender

Gender	Handedness	Right	Left	*p*-value
Male	SRH	1.0069 ± 0.046	1.0034 ± 0.047	< **0.001**
	WRH	1.0027 ± 0.021	1.0076 ± 0.047	0.875
	SLH	0.9909 ± 0.037	0.9977 ± 0.025	0.287
Female	SRH	0.9998 ± 0.023	1.0011 ± 0.028	< **0.001**
	WRH	0.9797 ± 0.028	1.107 ± 0.151	< **0.001**
	WLH	1.1093	0.9393	––
	SLH	1.002 ± 0.0255	0.999 ± 0.199	< **0.001**

Abbreviations: 2D:4D, second to fourth digit ratio; SLH, strong left hand; SRH,strong right hand; WLH, weak left hand; WRH, weak right hand.
Results are shown as mean ± standard deviation, with *p-value*.

right 2D:4D ratio in WRH ($p = 0.011$) groups in females in both ethnic groups were found be statistically significant (►**Table 10**). A positive correlation between the Geschwind score and the 2D:4D ratio was found in both ethnic groups, but this correlation was significant only in the right 2D:4D ratio of both ethnic groups (< 0.001 level; 2-tailed) (►**Table 11**).

Discussion

The differences in hand preferences may be linked to the prenatal production of testosterone and estradiol and, in the case of testosterone, to interactions with the homeobox genes *Hoxa* and *Hoxd*, which control the differentiation of the urogenital system and the development of the digits.[12] It is generally agreed in previous studies that 2D: 4D ratios tend to be greater in females (closer to 1.0), and that gender differences tend to be larger for the right hand than for the left. However, in the present study, only IN females had a greater ratio than males.[13,14] ►**Table 12** shows the comparative evaluation of the 2D:4D ratios between the present study and previous studies.

Table 9 Right-Left 2D:4D ratio in 5 groups evaluated by hand preference in males in both ethnic groups

Handedness	Right/ Left	TN	IN	*p*-value
SRH	Right	1.0033 ± 0.0421	1.0069 ± 0.046	0.831
	Left	1.0056 ± 0.0416	1.0034 ± 0.047	0.821
WRH	Right	0.9914 ± 0.054	1.0027 ± 0.021	**0.007**
	Left	1.0014 ± 0.052	1.0076 ± 0.047	0.466
WLH	Right	0.9882 ± 0.097	.....	.....
	Left	0.9677 ± 0.041	.....	.....
SLH	Right	1.0121 ± 0.061	0.9909 ± 0.037	**0.048**
	Left	1.0132 ± 0.044	0.9977 ± 0.025	0.064

Abbreviations: 2D:4D, second to fourth digit ratio; IN, Indo-Nepalese; SLH, strong left hand; SRH,strong right hand; TN, Tibeto-Nepalese; WLH, weak left hand; WRH, weak right hand.
Results are shown as mean ± standard deviation, with *p-value*.

Table 10 Right-Left 2D:4D ratio in 5 groups evaluated by hand preference in females in both ethnic groups

Handedness	Right/ Left	TN	IN	*p*-value
SRH	Right	1.0032 ± 0.041	0.9998 ± 0.023	< **0.001**
	Left	1.0013 ± 0.041	1.0011 ± 0.028	0.06
WRH	Right	0.9624 ± 0.046	0.9797 ± 0.028	0.282
AMBHI	Left Right Left	0.9920 ± 0.043 0.9848 0.8571	1.107 ± 0.151	**0.011** ....
WLH	Right	.....	1.1093	.....
	Left	.....	0.9393	.....
SLH	Right	0.9992 ± 0.0228	1.002 ± 0.0255	0.373
	Left	0.9979 ± 0.0214	0.999 ± 0.199	0.972

Abbreviations: 2D:4D, second to fourth digit ratio; IN, Indo-Nepalese; SLH, strong left hand; SRH,strong right hand; TN, Tibeto-Nepalese; WLH, weak left hand; WRH, weak right hand.
Results are shown as mean ± standard deviation, with *p-value*.

Conclusion

In the present study, 82% of TN males were found to be SRH, whereas 82.5% of TN females were found be SRH, followed, by 10% and 12.5% SLH in males and females, respectively. The 2D:4D ratio showed significant ethnic and population differences.

Table 11 Relationship between G- score and 2D:4D ratio

Ethnic group	2D:4D ratio	Coefficent of correlation#
TN	**Right**	0.573**
	Left	0.093 (NS)
IN	**Right**	0.348**
	Left	0.084 (NS)

Abbreviations: 2D:4D, second to fourth digit ratio; IN, Indo-Nepalese; NS, Not significant; TN, Tibeto-Nepalese.
#Correlation is significant at a level < 0.001 (2-tailed).

Table 12 Comparative evaluation of 2D:4D ratios in males and in females in different studies

Author	Population	*n*	Gender	2D:4D
Manning et al (1998)[10]	English	400	M F	0.98 1.00
William et al (2000)[15]	American	108 146	M F	0.96 0.97
Swami et al (2013)[16]	Harayani Brahmins Kashmiris pandits	150 150	M F M F	0.95 0.98 0.99 1.001
Present study	Tibeto-Nepalese Indo-Nepalese	200 200	M F M F	1.004 0.99 1.003 1.004

Abbreviations: 2D:4D, second to fourth digit ratio; F, female; M, male.

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Conflicts of Interest
The authors have no conflicts of interest to declare.

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Hepatoprotective Activities of Ethanolic Roots Extract of *Ageratum Conyzoides* on Alloxan-Induced Hepatic Damage in Diabetic Wistar Rats

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Abstract

Introduction The aim of the present study was to evaluate the hepatoprotective activities of the ethanolic roots extract of *Ageratum conyzoides* (AC) in alloxan-induced hepatic damage in diabetic rats.

Materials and Methods Diabetes was induced in Wistar rats by the administration of alloxan (150 mg/kg, intraperitoneal). The ethanolic roots extract of AC, at doses of 250 and 500 mg/kg of body weight, was administered to diabetes-induced rats at a single dose per day for a period of 28 days.

Results The effect of the ethanolic roots extract of AC on blood glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and hepatic oxidative stress markers was measured in the diabetic rats. The ethanolic roots extract of AC exhibited significant reduction of blood glucose ($p < 0.05$) at the dose of 500 mg/kg when compared with the standard drug glibenclamide (600 µg/kg). The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels increased significantly ($p < 0.05$) in the diabetic group without treatment when compared with the control group. In addition, the levels of oxidative stress markers, such as superoxide dismutase (SOD), catalase (CT), glutathione peroxidase (GPx), and glutathione (GSH), were significantly decreased in the diabetic rats compared with the normal rats, while the lipid peroxidation significantly increased in the diabetic group without treatment compared with the control (normal) group. The results demonstrated that the morphological, functional and oxidative stress changes in the liver caused by the ingestion of alloxan were attenuated in diabetic rats treated with the ethanolic roots extract of AC.

Conclusion We concluded that the ethanolic roots extract of AC possesses significant antidiabetic, antioxidant and hepatoprotective effects on alloxan-induced diabetic rats.

Keywords
- hepatoprotective activity
- antioxidants
- alloxan
- rats
- ageratum conyzoides

Introduction

Diabetes mellitus (DM) is a chronic carbohydrate, lipid and protein metabolic disorder that contributes to several kinds of complications, including diabetic hepatopathy. Diabetes mellitus has now become an epidemic, with a worldwide incidence of 5% in the general population.[1]

Some reports have shown that antioxidant treatment reduces diabetic complications and protects the cellular components from oxidative damage.[2] It has been reported

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that diabetic complications are associated with the pathogenesis of many serious systemic diseases, such as the overproduction of reactive oxygen species (ROS), and the accumulation of lipid peroxidation byproducts.[3]

Dietary fibers supplementation with powerful antioxidants, such as terpenoids, flavonoids, vitamins and medicinal plants, has been used to prevent the occurrence of DM and its complications. [4]

Plants have been the major source of drugs for the management of DM in Nigerian herbal medicine and in other ancient systems in the world, and for a long time DM has been managed with the use of orally administered herbal medicines or their extracts,[5] because plant products are frequently considered to be less toxic and to cause less side effects than synthetic ones.[6]

Ageratum conyzoides (AC) belongs to the Asteraceae family, which includes other medicinal plants like *Vernonia amygdalina* and *Tridax procumbens*, which have a proven antidiabetic potency.[7,8] *A. conyzoides* is an annual herbaceous plant which has a wide tropical distribution and is widely used in Southern Nigeria; it is a tree of up to 30 m high with a wide crown, fissured bark and fragrant white flowers.[9] The plant is commonly found in West Africa and in abundance particularly in the Southern part of Nigeria. It is found in the savannah regions and in the swampy areas of Nigeria. Its stems are covered with fine white hairs. It is commonly called goat weed in English, Imi esu and rerinkomi in Yoruba, igwulube in Igbo, alkama tuturuwa in Hausa, otiti in Efik, and nnyano in Ibibio. The root of AC is used in India for the treatment of fever and gastrointestinal diseases such as diarrhea, dysentery and intestinal colic with flatulence.[10]

In Brazil, the roots and leaves are of value in the treatment of malaria, ovarian inflammation, amenorrhea, dysmenorrhea, rheumatism and dysentery.[11,12] In Cameroon and in the Congo, it is used traditionally to treat fever, rheumatism, headache, and colic.[13,14] The leaves are also used in dressing wounds and burns, and they have been shown to present antibacterial activities.[15,16] In Nigeria, the leaves and roots are useful in the treatment of boils, leprosy, skin diseases, eye diseases, and inflammations.[17] It has been shown to possesses antidysentric,[18] fertility,[19] antispasmodic and muscle relaxation properties.[20] The leaves have been found to be a potential source of antidiabetic agents.[21]

The aim of the present study was to investigate the potentials of the ethanolic root extract of AC as an antioxidant and hepatoprotective agent in rats with alloxan-induced DM.

Materials and Method

Collection of the Plant Material

The AC roots were collected from a cultivated farmland at Ijebu Ilugun, northeast of Ijebu, Ogun State, Nigeria, in the month of June 2016. The plant was identified and authenticated at the Forestry Research Institute of Nigeria (FRIN), where a voucher specimen has been deposited in the herbarium (FHI 107873).

Preparation of the Plant Extract

The roots of the plant were shade-dried at room temperature for 7 days and then powdered using mortar and a pestle. The root powder (2,500 g) was soaked in a solution of 96% ethanol containing 4% water in 3 cycles using a Soxhlet extractor. The crude extract was filtered with Whatman filter paper 4 (Whatman, Maidstone, Kent, UK), and the filtrate was concentrated and dried in a rotary vacuum evaporator under reduced pressure in vacuole at 30° C to obtain 287.2 g of dry residue (11.5% volume) to yield a viscous brownish-colored extract that was stored in an airtight bottle kept in a refrigerator at 4° C until used.

Preliminary Phytochemical Analysis

The preliminary phytochemical screening of the powdered roots of AC for carbohydrates, glycosides, flavonoids, terpenoids, tannins, saponins, steroids and alkaloids was performed according to standard laboratory procedures.[22,23]

Laboratory Animals

Twenty-five healthy Wistar rats weighing between 180 and 200 g were obtained from the Laboratory Animal Center of the College of Medicine, University of Lagos, Idi-Araba, Lagos, Nigeria. The rats were housed in clean metallic cages and kept in a well-ventilated room at 24 ± 2°C at the Animal House of the Faculty of Basic Medical Sciences, Obafemi Awolowo College of Health Sciences, Olabisi Onabanjo University, Ikenne, Ogun, Nigeria, with a 12 h light/dark cycle throughout the experimental period and were allowed to acclimatize to the laboratory condition for 1 week before being used. They were fed with standard animal pellet (Pfizer Feeds Plc., Ikoyi, Lagos, Nigeria) and had free access to water *ad libitum*

Acute Toxicity Study

The toxicity study was performed using a group of 5 rats weighing an average of 200 g, after depriving them of food overnight with access to water only. The rats fasted for 14 hours before being administered a single dose of 5,000 mg/kg of the ethanolic roots extract of AC by gavages. They were closely monitored in the first 4 hours and then hourly for the next 12 hours, followed by hourly intervals for the next 48 hours after the drug administration to observe any deaths or changes in general behavior and in other physiological activities. The animals did not show any mortality at the dose administered, since 1/10th of the regular dose (500 mg/kg of body weight [bwt]) was chosen as the highest extract dose. The dose used is consistent with previous finding on the plant.[24]

Alloxan-induced Diabetic Experiment

Alloxan was purchased from the representative of the Sigma Company (Zayo-Sigma Chemicals Ltd, Jos, Nigeria) and was prepared in freshly normal saline solution. Diabetes was induced by intraperitoneal (IP) injection of alloxan monohydrate (150 mg/kg bwt) in a volume of 3 mL.[25] After 72 hours, blood was drawn for blood glucose estimation monitored with an Infopia Finetest glucometer (Infopia Co Ltd., Anyang, Gyeonggi, Korea). The animals with blood

glucose level $\geq$ 250 mg/dl were considered diabetic and included in the experiment.[26]

The diabetic animals were randomly distributed into four groups of five animals each while the last group, the positive control, had five normal rats.

The treatments for each group were as follows:

Group I: Normal rats (positive control).
Group II: Alloxan diabetic untreated (control negative).
Group III: Alloxan diabetic treated with glibenclamide at a dose of 600 µg /kg bwt [26]
Group IV: Alloxan diabetic treated with AC at a dose of 250 mg/kg bwt.
Group V: Alloxan diabetic treated with AC at a dose of 500 mg/kg bwt.

Weight Variation: The animals were initially weighed and then weighed every 7 days from the beginning of the treatment until the 28th day.

Animal Sacrifice

After 28 days of treatments (24 hours after the last dose), the animals were anaesthetized with diethyl ether and the blood was collected through cardiac puncture into sample bottles devoid of anticoagulant. The samples were centrifuged at 4,000 rpm for 5 minutes to obtain the sera. The abdominal cavity of each rat was opened through a midline abdominal incision to expose the liver. The liver was excised and then weighed with an electronic analytical and precision balance. The liver of each animal was fixed in Bouin fluid for histological procedures.

Histological Analysis

The liver tissues were carefully dissected, and the organs were cut on slabs $\sim$ 0.5 cm thick and fixed in Bouin fluid (10%) for 1 day, after which they were transferred to 70% alcohol for dehydration. The tissues were passed through 90% alcohol and chloroform for different durations before they were transferred into two changes of molten paraffin wax for 20 minutes each in an oven at 57° C. Serial sections 5 µm thick were obtained from a solid block of tissue and were stained with hematoxylin and eosin stains, after which they were passed through a mixture of equal concentration of xylene and alcohol. Following the clearance in xylene, the tissues were oven-dried. Photomicrographs were taken.

Serum Alanine Aminotransferase and Aspartate Aminotransferase Parameters

The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured spectrophotometrically as described by the method of Reitman et al (1957),[27] as modified by Rajan et al (2012).[5]

Hepatic Antioxidant Enzymes Assay

Assay of catalase (CT) activity: The catalase activity was evaluated according to the method described by Chance et al (1955).[28] The catalase activity was assayed spectrophotometrically in the supernatants by measuring the decrease in absorbance of H_2O_2, and it was expressed as μmg^{-1} protein.

Superoxide Dismutase Activity Assay

The superoxide dismutase (SOD) activity was evaluated according to the method described by Marklund et al (1974).[29] It was expressed as μmg^{-1} protein.

Glutathione Peroxidase Activity Assay

The glutathione peroxidase (GPx) activity was determined by the method described by Pagila et al (1967).[30] The absorbance of the product was read at 430 nm and it was expressed as $nmol^{-1}$ protein.

Liver Reduced Glutathione Concentration Assay

The reduced glutathione (GSH) was measured according to the method described by Rukkumani et al (2004).[31] The absorbance was read at 412 nm, it was expressed as $nmol^{-1}$ protein.

Lipid Peroxidation (Malondialdehyde) Assay

The lipid peroxidation in the liver tissue was measured colorimetrically by the thiobarbituric acid reactive substance (TBARS) method described by Park et al (2002).[32] The concentration was estimated using the molar absorptive of malondialdehyde, which is $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and it was expressed as nmol mg^{-1} protein.

Statistical Analysis

The Student t-test was used, and differences were considered significant at $p < 0.05$. All data were expressed as mean $\pm$ standard error of the mean.

Results

The phytochemical analysis revealed the presence of carbohydrates, glycosides, flavonoids, terpenoids, steroids, saponins, tannins, and alkaloids (►**Table 1**).

Effect on the Body Weight of Male Rats

The control group (I) gained weight over the 4 weeks of the experimental period, with the mean body weight increasing by 22.2 g after 4 weeks (►**Table 2**). In contrast, the untreated

Table 1 Phytochemical composition of the extract of the roots of *Ageratum conyzoides*

Phytochemical	Availability
Carbohydrates	+
Glycosides	+
Cardiac glycosides	+
Saponins	+
Steroids	+
Flavonoids	+
Terpenoids	+
Anthracene derivatives	−
Alkaloids	+
Tannins	+

Abbreviations: +, present; -, absent.

Table 2 Body weight changes

Groups	Initial body wt (g)	Final body wt (g)	Difference in body wt (g)	Liver wt (g)
I	185.4 ± 0.7	197.6 ± 1.1	22.2	9.2 ± 1.0
II	192.8 ± 2.0	168.7 ± 2.2	-24.11	2.8 ± 0.8
III	195.6 ± 1.4	222.9 ± 1.7	27.3	8.5 ± 0.7
IV	189.2 ± 2.5	199.6 ± 1.5	10.4	7.4 ± 0.4
V	185.6 ± 1.8	198.3 ± 1.8	12.7	7.9 ± 1.0

Abbreviations: g, grams; wt, weight.

diabetic group (II) lost an average of 24.1 g after 4 weeks ($p < 0.05$). The treatments with glibenclamide and AC resulted in significant weight gain to levels approaching the control group (groups III, IV and V, versus group I). The mean liver weight in the diabetic untreated group decreased significantly compared with that of the control group, while the mean liver weight of the diabetic group treated with AC and glibenclamide decreased by improving the restoring activity of the extract of AC to the weight lost due to the administration of alloxan.

Effect on Blood Glucose Level

The blood glucose level in the diabetic group was significantly higher ($p < 0.05$) than that of the control group (►**Fig. 1**). On the other hand, the administration of the ethanolic roots extract of AC for 28 days was found to lower significantly the blood glucose levels in a dose dependent manner in the treated diabetic groups ($p < 0.05$) when compared with those of the diabetic untreated (negative) group. The antihyperglycemic effect of the ethanolic extract of AC (250 /500 mg/kg) was found slightly effective than the reference standard glibenclamide produced a significant reduction in blood glucose compare with diabetic control.

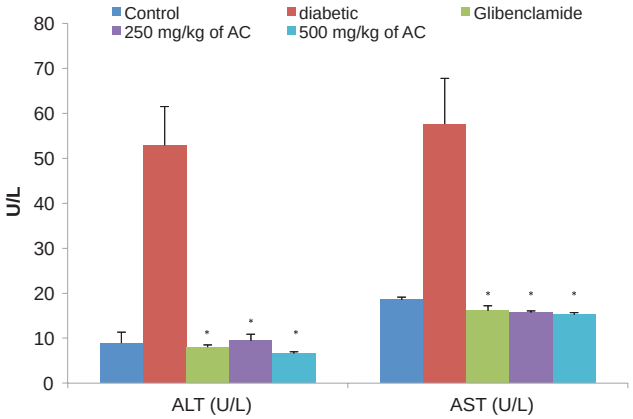

Fig. 2 Biochemical profiles of untreated diabetic rats; diabetic but treated with glibenclamide and extract respectively; and the normal rats untreated for 4 weeks. Mean ± SD, ($n = 8$) *p < 0.05 vs control group. Abbreviations: AC, *Ageratum conyzoides*; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Effect on Some Biochemical Parameters

The study also indicates that ALT and AST increased significantly ($p < 0.05$) in the diabetic group compared with the control group, as shown in ►**Fig. 2**. However, the administration of the ethanolic roots extract of AC for 28 days was capable of slightly lowering the ALT and AST levels in the diabetic groups.

Effect on Hepatic Antioxidant Enzymatic and Non-enzymatic Markers

The present study indicated that the diabetic group showed a statistically significant decrease ($p < 0.05$) in SOD, CT and GPx

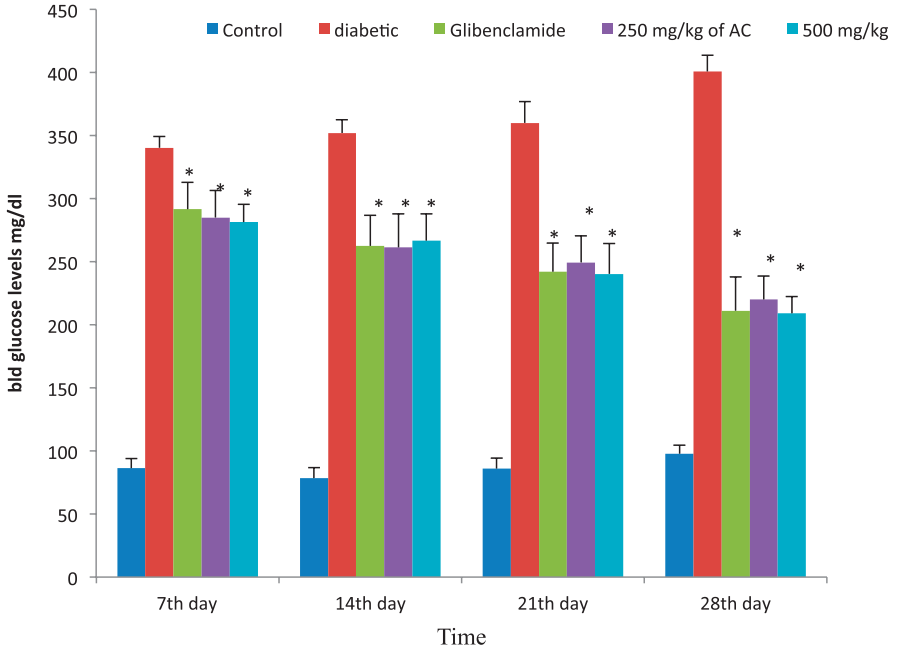

Fig. 1 Blood glucose level of Wistar rats treated with glibenclamide and ethanolic roots extract of *Ageratum conyzoides* for 4 weeks. Values represent Mean ± SEM ($n = 8$). $P < 0.05$ vs control group*: Statistically significant when compared with control group at $p < 0.05$.

activities compared with normal rats without treatment. Diabetic rats treated with AC significantly increased ($p < 0.05$) the liver SOD, CT and GPx activities compared with diabetic untreated animals, however. There was no significant ($p < 0.05$) change in the liver content of GSH and MDA on the diabetic group following the treatment with AC when compared with the normal rats without treatment. However, a significant reduction ($p < 0.05$) in the GSH content as well as a significant increase in the MDA content were observed in the diabetic untreated group when compared with the normal animals. The diabetic group treated with AC, however, presented a significantly elevated liver content of GSH, but also a significantly reduced liver content of MDA compared with the diabetic untreated group.

Hepatocytoarchitectural Findings

The cytoarchitecture of the normal hepatic tissues (►**Fig. 3a**) showed hepatocytes radially arranged from the lobular margins toward the center vein with each column interspaced by sinusoids.

The hepatic tissues of the diabetic group (►**Fig. 3b**) showed a marked distortion of the liver cytoarchitecture resulting from the degeneration of the hepatic parenchyma.

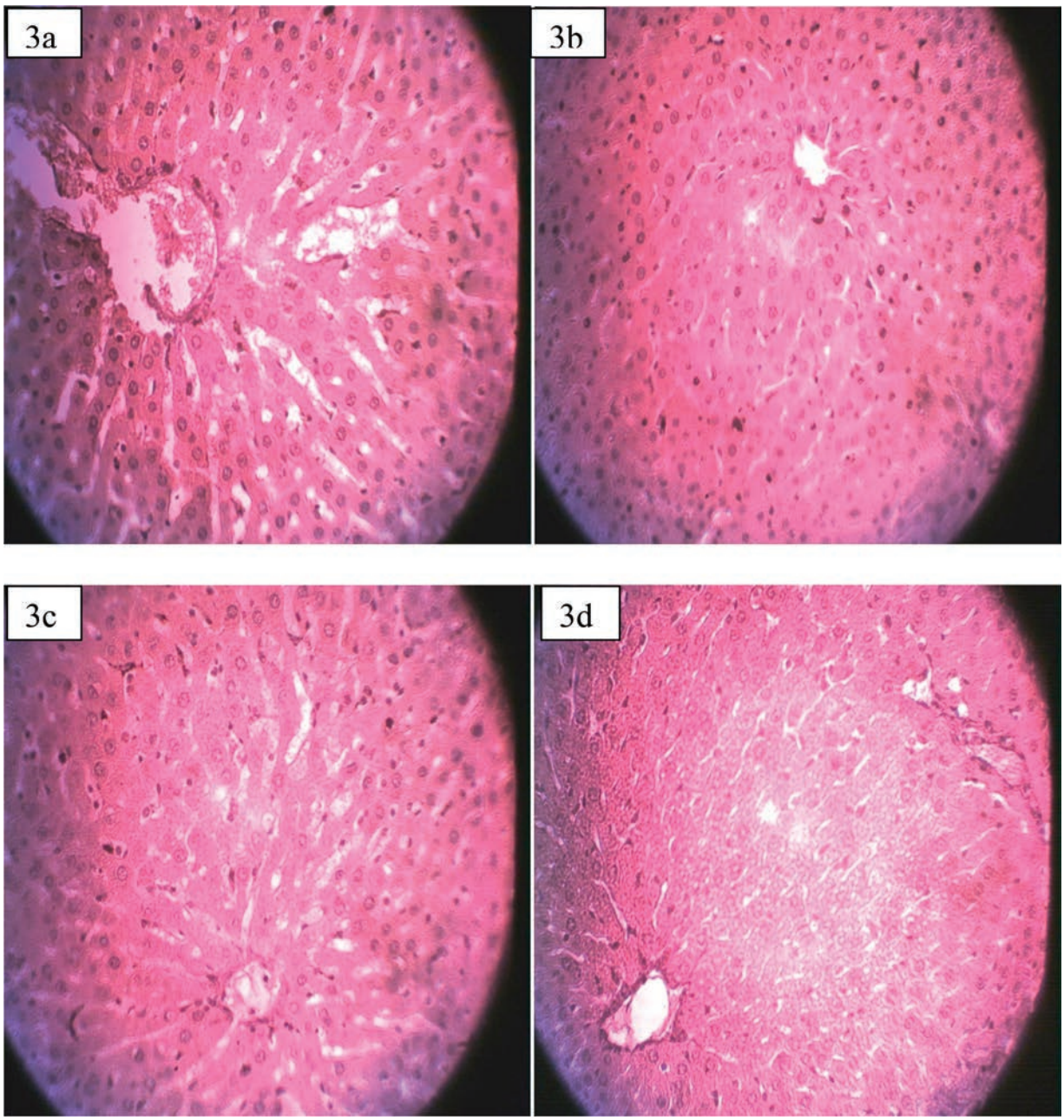

Fig. 3 (a) Cross section of (Group I) normal rat (positive control) stained with H and E × 400. (b) Cross section of (Group II) diabetic rat (negative control) stained with H and E × 400, Figure (c) Cross section of (Group III) diabetic rat treated with glibenclamide stained with H and E × 400, (d): Cross section of (Group IV) diabetic rat treated with *Ageratum conyzoides* stained with H and E × 400.

Table 3 Effect of oral administration of *Ageratum conyzoides* extract after 4 weeks on hepatic antioxidant enzymatic in alloxan-induced diabetic male rats

	Groups				
Parameters	I	II	III	IV	V
CT (μmg^{-1} protein)	17.2 ± 1.6	5.2 ± 2.4*	15.8 ± 2.8**	13.9 ± 2.6**	14.7 ± 1.4**
SOD (μmg^{-1} protein)	44.3 ± 4.2	12.4 ± 1.7*	38.1 ± 3.1**	29.3 ± 2.8**	38.5 ± 2.5**
GPx ($nmolmg^{-1}$ protein)	0.85 ± 0.2	0.25 ± 0.1*	0.72 ± 0.3**	0.57 ± 0.2**	0.69 ± 0.3**
GSH ($nmolmg^{-1}$ protein)	2.7 ± 0.2	0.57 ± 0.1*	2.5 ± 0.1**	1.4 ± 1.1**	2.2 ± 0.2**
MDA ($nmolmg^{-1}$ protein)	0.7 ± 0.1	4.5 ± 0.4*	1.45 ± 0.2**	1.5 ± 0.2**	1.1 ± 0.1**

Values are the mean values ± standard error of the mean of 8 rats. *: Statistically significant when compared with control group (I) at $p < 0.05$. **: Statistically significant when compared with untreated diabetic group (II) at $p < 0.05$.
Abbreviations: CT, catalase; GPx, glutathione peroxidase; GSH, reduced glutathione; MDA, malondialdehyde; SOD, superoxide dismutase.

The hepatic tissue of the glibenclamide treated group (►**Fig. 3c**) showed mild pyknotic changes coupled with early periportal inflammation. The hepatic tissue of the AC treated groups (►**Fig. 3d**) showed regeneration of the liver parenchyma and portal trait dilatation. (►**Table 3**)

Discussion

Diabetes mellitus has been identified as one of the leading metabolic disorders worldwide. It is characterized by hyperglycemia associated with impairment in insulin secretion as well as with an alteration in the intermediary metabolism of carbohydrates, proteins and lipids. Reactive oxygen species play a major role in the etiology and in the pathogenesis of DM and of its complications. Lipid peroxidase-mediated tissue damage has been demonstrated in insulin-dependent and non-insulin-dependent DM.[33]

It could be concluded from the result that the median acute toxicity (LD_{50}) value of the extract was 5,000 g/kg bwt. According to Locke (1983),[34] the extract can be classified as being non-toxic, since the LD_{50} by oral route was found to be much higher than the toxicity index of 2 g/kg of the World Health Organization (WHO).

The significant slight weight gain observed in the diabetic animals treated with the extract clearly suggested that the extracts might not have had the obesity forming tendency compared with glibenclamide treated improved weight gain which is one of the undesirable side effects encountered when treating diabetes with sulphonylureas. There were also no changes observed in the microscopic examinations of the organs of the diabetic animals treated with the extract or with glibenclamide.

The present study reveals that the administration of the extract of AC presented a significant antihyperglycemic activity by improving the blood glucose level, and that it was considerably effective compared with glibenclamide.

Glibenclamide, being a second-generation sulfonylurea class of the oral hypoglycemic agents, is known to mediate its hypoglycemic effect by stimulating insulin release from the pancreatic β cells, causing a reduction in the hepatic insulin clearance by stimulating the release of somatostatin and suppressing the secretion of glucagon.[35]

Sulfonylureas have also been shown to induce hepatic gluconeogenesis.[36]

Comparing the results of glibenclamide with those obtained for the extract of AC in the present study, it appears that the AC may have its antihyperglycemic effect through the induction of hyperinsulinemia by the utilization of peripheral glucose.

The present results show that the injection of alloxan induces hepatocellular damage, as evidenced by the high levels of ALT and AST in the untreated diabetic groups. These increases may be due to the leakage of these enzymes from the liver cytosol into the blood stream, which causes a change in the permeability of the membranes of the liver cells. On the other hand, the oral administration of the extract of AC lowered the ALT and AST levels slightly effective than the standard drug, glibenclamide.

Furthermore, AC has an ability to restore the protein breakdown and enhance the glycogenesis process in the liver of diabetic rats. The ALT and AST activities are known as cytosolic marker enzymes reflecting hepatocellular necrosis as they are released into the blood after cell membrane damage.[37,38]

In the present study, ►**Fig. 2** shows the activities of AST and ALT in experimental rats compared with normal rats. The diabetic rats showed more activities of serum AST and ALT. Therefore, both enzyme activities were used as indicators of hepatic damage.

Therefore, it is possible to suggest that the AC extract is safe and might confer protection against ALX-induced hepatocellular damage as evidenced by the normal serum levels of ALT and AST in the diabetic group treated with AC. The hepatoprotective activity of AC might be due to the presence of antioxidant compounds such as flavonoids, terpenoids, liminoids and vitamins. It has been reported that AC contains terpenoids, which shows that the results found in the present study are in conformity with the previous report by Ekundayo et al (1988).[18]

In addition, hepatotoxicity and oxidative stress mediated by diabetic are exhibited by a significant increase in the activities of antioxidant enzymes, SOD, CT, GPx and the liver content of MDA, and a significant decrease of GSH. By contrast, the diabetic group treated with the ethanolic roots extract of AC remarkably modulated the oxidative stress caused by alloxan induction because it has been reported

that the oxidative stress associated with the increased generation of reactive oxygen species (ROS) and the defective antioxidant defense mechanism system in the body are the main contributors of DM and its complications.[39]

It is possible to suggest that this extract might directly improve the structural and functional integrities of the liver.

Conclusion

The present study indicates that AC has the potential to manage DM and prevent DM-associated hepatic damage.

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The Effect of *Zingiber officinale* on the Spleen Tissue and Antibody Titer of Broiler Chickens

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Abstract

Introduction Increasing the immune system's function of fighting infectious diseases is very important in the poultry industry. Ginger, scientifically known as *Zingiber officinale*, belongs to the *Zingiberaceae* family. The use of ginger in the diet of poultry increases serum levels of superoxide dismutase enzymes and glutathione peroxidase, which are considered to be important antioxidant enzymes. The main objective of the present study is to evaluate the effect of ginger on the spleen tissue of broiler chickens.

Material and Methods The specimens comprised 2 groups of 20 Ross breed broiler chicks, for 42 days and were then, examined and tested. The diet was supplemented with 1 g/kg of ginger powder from the beginning of the rearing period. Blood samples of the chicks were randomly collected to measure the levels of hemagglutination (HI). The removed spleens were fixed with 10% formalin buffer. The specimens were cut in 5-micron diameters and stained with hematoxylin and eosin.

Results and Conclusion There was a statistically significant difference in the mean of HI blood titers between the chicks in the growth period and final period groups ($p < 0.05$). The white-pulp tissue samples were more clearly seen in the treatment group than in the control group, and also, it was observed that the wall of the central artery of the white pulp was thicker in the ginger-treated group as compared with the control group. The nutritional value of ginger may vary. Thus, it is necessary to investigate the effect of this plant final on weight gain; the serum factors associated with the metabolic chart, and the response of the immune system to this plant.

Keywords
- antibody titer
- chicken
- hemagglutination
- spleen
- *zingiber officinale*

Introduction

Increasing the immune system's function of fighting infectious diseases is very important in the poultry industry. Various factors, such as failure of vaccinations, diseases that suppress the immune system, and unusual use of antibiotics reduce immune responses. Due to high mortality rates in domestic animals and poultry, infectious diseases are a source of concern across the globe. The use of immune stimulants can be one way of improving immunity and reducing the risk of infectious diseases in animals. Ginger, scientifically known as *Zingiber officinale*, belongs to the *Zingiberaceae* family. This plant has a variety of species, some of which are edible and often used as medicinal and spice herbs[1] due to its antioxidant,[2] antiviral[3] and antifungal[4] properties, as well as its enhancing effect on the immune system.[5] Ginger is used to treat a wide range of diseases.[6] The use of ginger in the diet of poultry increases serum levels of superoxide dismutase enzymes, catalase, and glutathione peroxidase, which are considered to be important antioxidant enzymes.[7] Based on research results of Ueki et al[8] and

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Weidner and sigwart,[9] the antiinflammatory, antioxidant, antibacterial, antiviral and immunosuppressive properties of this plant make it quite effective in healing headaches, menstrual pains, arthritis, and fever caused by a variety of colds and influenza. As the largest lymph node, the spleen is involved in many activities, including the production of blood cells (lymphocytes), hemoglobin and iron metabolism, red blood cells destruction, blood purification, blood storage, phagocytosis, and immune responses. The most important function in the spleen is the immunological treatment of blood. Therefore, results obtained from this study can be of great value in the economic cycle of the poultry industry, including Jihad agriculture, veterinary offices, universities, chicken coops, and meat products processing. The main objective of the present study is to evaluate the effect of ginger on the spleen tissue of broiler chickens.

Materials and Methods

In this study, 40 day-old Ross breed broiler chicks were produced in a private sector broiler farm coop in the city of Tabriz. They were completely homogenous in terms of geographical and environmental conditions. The coop was disinfected after being washed with high pressure water. Thereafter, all the chambers, doors, and windows were covered. On the first day, the temperature of the coop was $\sim$ 32. It decreased by 1 degree, every 3 days, until it reached 21 at the 27th breeding season. This temperature remained constant for all groups until the end of the breeding season. In the course of the study, a mixture of natural light during the day and artificial lighting during the night was used to provide the required lighting. All chicks were fed in the same way in terms of diet components and the amount of each component. To provide practical results that can be applied to other studies, the seeds fed to the chicks followed the same current diet. Changes required by the geographical area of breeding parks were implemented according to notification protocol of the country's veterinary administration. The chicks were vaccinated against Newcastle disease, Gambaro, bronchitis, and influenza.

Treatment Implementation Process

The specimens were considered to be two groups of 20. The diet was supplemented with 1 g/kg of ginger powder from the beginning of the rearing period. A total number of 40 day-old Ross breed broiler chicks were grown in a bed system for 42 days, and were examined and tested. Blood samples of chicks were randomly collected on days 10, 24, and 42 to measure the levels of antibody. Blood samples (100 cc) were taken from each chick, and tests of hemagglutination (HI) were referred to a poultry laboratory.

After abdominal autopsy, the weight of spleen specimens was calculated by using digital scales. The spleen was completely removed from the body and fixed with 10% formalin buffer. Thereafter, histological specimens were sent to the histological laboratory. The specimens were cut in 5-micron diameters and stained with hematoxylin and eosin. Then, they were analyzed under an Eclipse E200-LED microscope (Nikon, Minato, Tokyo, Japan).

Table 1 Comparison of mean values obtained from the antibody titer against Newcastle virus (HI test) between units of control and ginger groups[a]

Parameters	Normal Group $n = 20$	Treatment Group $n = 20$
The initial period (1–10 days)	4.14 $\pm$ 0.253	4.17 $\pm$ 0.282
The growth period (11–24 days)	3.44 $\pm$ 0.274	4.36 $\pm$ 0.245*
The final period (24–42 days)	3.66 $\pm$ 0.233	4.64 $\pm$ 0.257*
Spleen weight (gm)	11.30 $\pm$ 0.3	12.6 $\pm$ 0.4*

Abbreviation: HI, hemagglutination test.
[a]Values are means $\pm$ standard deviation (SD).
*$p < 0.05$.

Statistical Analysis of Antibody Titer

This experiment was conducted with a completely randomized design. The collected data were analyzed using the General Linear Model (GLM) and the Statistical Analysis Software (SAS) (SAS Institute, Cary, NC, USA), and the means were tested using the Duncan multi-domain test (SAS, 2001), with a significance level of $p < 0.05$.[10]

Results

Results of HI Tests Variance Analyses

The Initial Period (1–10 Days)

As shown in ►**Table 1**, according to the results of this study, there was no statistically significant difference in the mean of HI blood titers between the normal and treatment groups at the beginning of the study ($p > 0.05$; 4.14 $\pm$ 0.253, 4.17 $\pm$ 0.282).

The Growth Period (11–24 Days)

The results in ►**Table 1** show a statistically significant difference in the mean of HI blood titers between the normal and treatment groups during the growth period ($p < 0.05$; 3.44 $\pm$ 0.24, 4.36 $\pm$ 0.245).

The Final Period (24–42 Days)

The results in ►**Table 1** show a statistically significant difference in the mean of HI blood titers between the normal and treatment groups during the final period ($p < 0.05$; 3.66 $\pm$ 0.223, 4.64 $\pm$ 0.257).

The Result of Mean Spleen Weight in Broiler Chicks at the End of the Period

The results in ►**Table 1** show a statistically significant difference in the mean of spleen weight between the normal and treatment groups at the end of the period ($p < 0.05$; 11.3 $\pm$ 0.03, 6.12 $\pm$ 0.40).

Result of Microscopy

The analysis of the spleen specimen showed that the spleen was surrounded by a hard connective capsule that had penetrated into the tissue through subtle branches. The

spleen tissue is formed from two red and white connectors. The white pulp contains the structure of the lymph nodes and central arteries, which was clearly evident in samples prepared in the ginger-treated group, at 42 days; areas covered by this pulp are generally unclear in birds and uncommon in mammals. In this study, white pulp tissue samples were more clearly seen in the treatment group than in the control group at the end of the period (►**Fig. 1A-B**).

Collagen conjugation and fibrocystic connective cells were observed while analyzing the spleen tissues. However, smooth muscle cells in the mammalian capsule were not seen in any of the two groups. It was observed that the central artery of the white pulp was thicker in the ginger-treated group as compared with the control group (►**Fig. 1C-D**). Differently from mammals, in which this area is clearly identifiable, the marginal area of the border between the white pulp and the red pulp was not observed in any of the groups. Although the compression and density of lymphocytic cells of the white pulp turned out to be higher in the treatment group, the number of these cells was higher in the control group. High compression and density of lymphocyte cells in the treatment group caused the white pulp border to be more recognizable in compare to normal group (►**Fig. 1E-F**). No smooth-cell membranes were observed in the analyzed spleen tissues of participating groups, and the trabecular penetration into the parenchyma tissue was rarely seen, which led to blood vessels passing into the spleen parenchyma tissue. The range of the red pulp in the studied samples turned out to be more limited than in mammals. The compression and density of red pulp cells turned out to be higher among the treatment group, and the nuclei of the RBC cells were clearly visible.

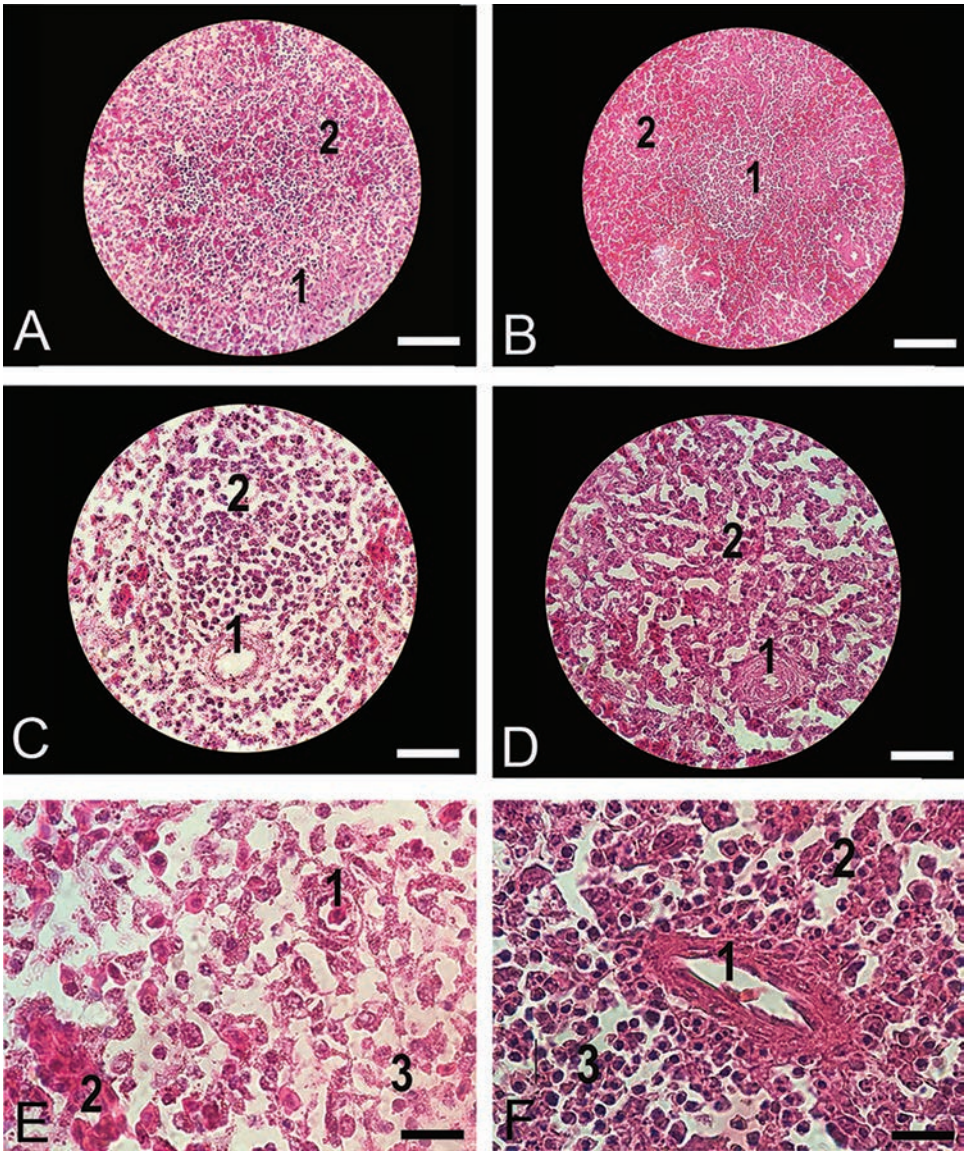

Fig. 1 Microscopic view of the spleen tissue of a broiler chicken. (A) Normal group (B) Treatment group. 1 - white pulp 2 - red pulp (hematoxylin & eosin [H&E] sainting, scale bar = 30 µm). (C) Normal group (D) Treatment group. 1 - central artery 2 - white pulp (H&E sainting, scale bar = 20 µm). (E) Normal group (F) Treatment group. 1 - central artery 2 - red pulp 3 - white pulp (H&E sainting, scale bar = 10 µm).

Discussion and Conclusion

Ginger reduces lipid peroxidation and increases antioxidant capacity of the plasma.[11] The use of ginger in the diet of poultry increases serum activity of superoxide dismutase enzymes, catalase, and glutathione peroxidase, which are considered to be important antioxidant enzymes.[7] In their histology book, Bloom and Fawcett[12] state that the spleen has been characterized by the accumulation of white pulp in tissues of central arteries and red pulp fills the spaces between the vascular sinus. The distribution and relative value of white and red pulp in different species of animals differ significantly so that during their immune responses or in adverse conditions, the formation and destruction of blood cells are altered. Animal species that have high blood volume (like horse, cattle, and dog) have low white pulp, solid structure, and smooth muscle connective tissue capsules. On the other hand, species that have low blood volume (like rabbits, rodents, and birds) have high white pulp, subtle structure, and poorly developed flat material. Based on the results of this study and consistent with Bloom and Fawcett,[12] a predominant white pulp to red pulp ratio was found in the spleen tissue of broiler chickens. The outer surface of the spleen is surrounded by a hard connective capsule, and the cylindrical trabeculae extend to the spleen parenchyma. Their cellular elements are fibroblasts. In rabbits, rodents, and birds, smooth muscle cells in the capsule and trabecula are rarely seen, and any changes in spleen volume are attributed to changes in blood flow to this organ. Smooth muscles are much more frequent in the capsules and trabeculae of equine, ruminant and carnivores, and regular contraction of the spleen is smooth due to contraction of muscle cells.[12] Hodges[13] also pointed out that the spleen tissue of poultry contains unclear red and white pulp, and differently from mammals, there is no trabeculae split. The results of a study conducted by Pourhaji and Abbaszadeh[14] indicated the presence of unclear pulps and the absence of splits in the spleen tissue of birds. However, Nasu et al[15] reported delicate connective trabeculae in the spleen tissue of pigeons. During observations conducted in this study, no trabecular structure or smooth muscle cells in the parenchyma of the spleen tissue were found. Additionally, areas covered with red and white pulp tuned out to be more identifiable in the treatment group as compared with the control group. In this study, it was observed that the central pulmonary artery of the white pulp was thicker in the ginger-treated group as compared with the control group, indicating the effectiveness of ginger in increasing blood flow to the tissue. The lymphocytic cells of the white pulp in the treatment group showed high compression and density as compared with the control group; whereas in the control group, there was a larger space between lymphocyte cells, showing a difference in the density of lymphocytic cells in the treatment and control groups. The analysis results of the immune response of broiler chicks showed that ginger can have an immune booster effect in a short period of time. Hence, there is a statistically significant difference between the control and treatment groups from the time of growth to the end of the final period. Results obtained from analyzing the weight of the spleen (considered to be the main indicator in the immune system) signified the fact that supplementing the diet of chicks with ginger caused weight gain at the end of the study. As reported by Katanbaf et al,[16] the relative weight gain of lymphoid organs is a sign of progression of the immune system. Al Khalifa et al[17] have shown that increasing the use of fish oil (as antioxidant) in the diet increases the weight of the spleen. Wang et al[18] have shown that diets of laying hens contain omega-3 fatty acids (as antioxidants), which improve the growth of lymphatic organs (like the spleen) within four weeks of feeding. In this study, consistent with the results of Wang's study, ginger functioned as an antioxidant, and increased the relative weight of the spleen and the hemorrhagic immune system. Considering that ginger contains the same antioxidants, it can be claimed that it protects fatty acids of the humoral immune system; hence, it can be noted that antioxidant agents like ginger prevent the formation of free radicals due to the oxidation of fatty acids, causing final improvement in the function of the humoral system. It can be concluded that ginger could have antiviral properties against the Newcastle virus by enhancing the efficiency of the immune system. According to the findings of this study, ginger powder could have a positive effect on growth performance and immune system in broiler chicks due to the presence of antibacterial properties of ginger oil and other compounds. Based on studies by Ueki et al[8] and Weidner and Sigwart[9] conducted to determine the effect of ginger on healing sore throat, arthritis, and fever caused by a variety of colds and influenza, it can be said that the antiinflammatory, antioxidant, antibacterial, and antiviral properties of ginger can function as an immune system booster through enhancing blood supply and strengthening the immune function of the body. The results of this study showed that using 1% ginger in the diet can improve immune functions and the immune system.

Promotion of growth indices in the poultry farming industry is of particular importance and different studies are being performed on nutritional and management principles to produce of the highest quality. Since ginger may have different nutritional values, it is necessary to investigate the effect of this plant on final weight gain, the serum factors associated with the metabolic chart, and the response of the immune system to this plant. Therefore, it is highly recommended that further research is conducted on the promotion of poultry immunity against Newcastle disease using different amounts of ginger in the broiler chicken diet.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Third Head of the Biceps Brachii Muscle and a Communication between the Musculocutaneous and Median Nerves: A Case Report

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Abstract

Many authors have reported and classified several anatomical variations between the musculocutaneous (Mc) and median (Me) nerves, regarding their origin, number, and proximity with the coracobrachialis muscle. There also are, in the scientific community, records classifying the origin of supernumerary heads of the biceps brachii muscle. However, the occurrence of both aforementioned variations in the same arm is very uncommon. During a routine dissection of the right upper limb of a male cadaver, a third head of the biceps brachii was found originating from the fibers of the brachialis muscle, as well as a communicating branch between the Mc and the Me nerves, in the same limb. The objective of the present case report is to describe these multiple variations found, relating them and discussing their relevant clinical implications.

Keywords

- anatomic variations
- clinical anatomy
- supernumerary head of biceps brachii muscle

Introduction

The brachial plexus (C5–T1 vertebrae) is a cervical swelling of the spinal cord that originates the nerves that supply the upper limbs. The communications between these 5 spinal nerve roots (C5–T1 vertebrae) originates 3 nerve fasciculus, which differ in terminal branches, including the musculocutaneous nerve (Mc) (C5–C7 vertebrae) and the median nerve (Me) (C5-T1 vertebrae).

The Mc is formed by the terminal portion of the lateral fasciculus and is responsible for the motor innervation of the muscles of the anterior compartment of the arm and for the sensory innervation of the lateral skin of the forearm.[1]

The Me is formed by a root from the lateral fasciculus and by a root from the medial fasciculus and is responsible for the motor innervation of a great part of the flexor muscles of the forearm, of half of the intrinsic muscles of the thenar palm, and of the skin of the palm.[1]

Therefore, the Mc and the Me nerves do not usually communicate (►**Fig. 1**).

The biceps brachii muscle has two origins, one in the supraglenoid tubercle, and another in the coracoid process, both located in the scapula, and their bellies unite to form a common tendon that inserts into the radial tuberosity and into the forearm fascia, through the aponeurosis of the biceps brachii.[1]

However, anatomical variations have been reported in the literature, with the occurrence of communications between the Mc and the Me nerves. Supernumerary heads of the biceps brachii muscle have also been reported.[2]

However, it is quite unusual for both of these anatomical variations to occur in the same cadaver and in the same limb.

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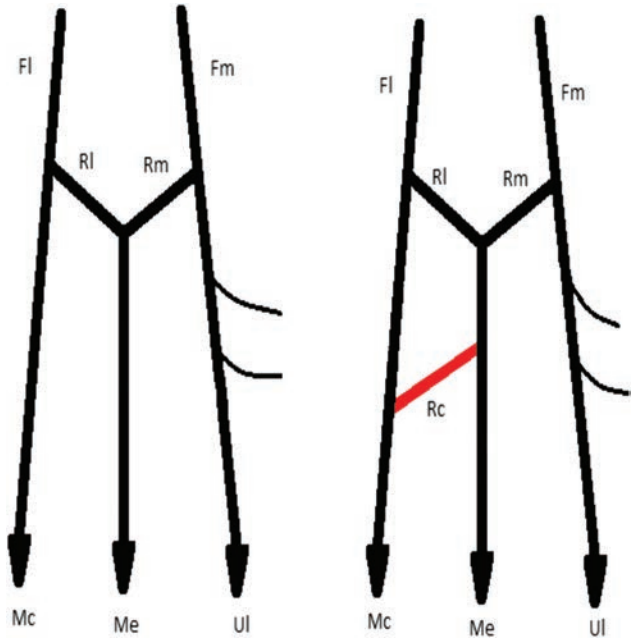

Fig. 1 Left drawing: normal arrangement of nerve fibers. Right drawing: variation presented in the present case report. **Fl**: lateral fasciculus; **Fm**: medial fasciculus; **Mc:** musculocutaneous nerve; **Me:** median nerve; **Rc:** communicating branch; **Rl**: lateral root; **Rm:** medial root.

The aim of the present study is to describe the relationship between multiple anatomical variations found in the right arm of a male cadaver, which were: a communication between the Mc and the Me nerves associated with a third head of the biceps brachii. The occurrence of a third head of the biceps brachii associated with this communication has clinical and surgical importance, and its knowledge can prevent iatrogenic mistakes.

Case Report

During a routine dissection, the right arm of a male cadaver presented a third head of the biceps brachii muscle. This variation has an incidence of 14.5% in the Brazilian population[3] (►**Table 1**).

The third heads of the biceps were sorted by Rodríguez-Niedenführ et al (1992),[2] according to the location of their origins in the humerus. There are three types of humeral heads: superior, inferomedial or inferolateral. It was also reported supernumerary heads arising from the pectoralis major muscle and from the coracoid process. The variation found in this cadaver, however, has its origin in the anterior third of the arm, from fibers coming from the brachial muscle, 18.0 cm away of the coracoid process of the scapula. It crosses the arm, from medial to lateral, joining its body with the biceps brachii to form a common tendon with this muscle. This third head has 11.0 cm in length, 1.8 cm wide, and continues inferiorly deep to the biceps brachii and superficially to the brachialis muscle (►**Fig. 2**).

Table 1 Incidence of the Third Head of the Biceps Brachii in Different Populations

Population	Incidence (%)
Chinese[a]	8
European[a]	10
African[a]	12
Japanese[a]	18
Brazilian[b]	14.5

[a]Data acquired from Jayanthi et al[11].
[b]Data acquired from Santo Neto et al[3].

Besides, this same arm had a communicating branch between the Mc and the Me nerves. The communication between these nerve was classified by Le Minor (1992)[4] and by Venieratos et al (1998).[5] The classification by Le Minor takes into account the height of this communication in the arm and how the fibers are arranged. There are five types:

Type 1: There is no communication between the Mc and the Me nerves.
Type 2: The fibers of the lateral root of the Me nerve go along with the Mc and the head to encounter and fuse with the Me nerve after a certain distance.
Type 3: The fibers of the lateral root of the Me nerve go along with the Mc nerve and, after a certain distance, leave it to finally form the lateral root of the Me nerve.
Type 4: Some of the fibers of the Mc nerve run through the lateral root of the Me nerve before merging, finally, in the Mc nerve.
Type 5: There is no development of the Mc nerve and every fiber that should be separated in this nerve is in the Me nerve, and they supply the muscles and the part of the skin that should be innervated by the Mc nerve.

In addition to the classification by Le Minor, there is also the classification by Venierators et al, from 1998, defined according to the relationship between the communicating

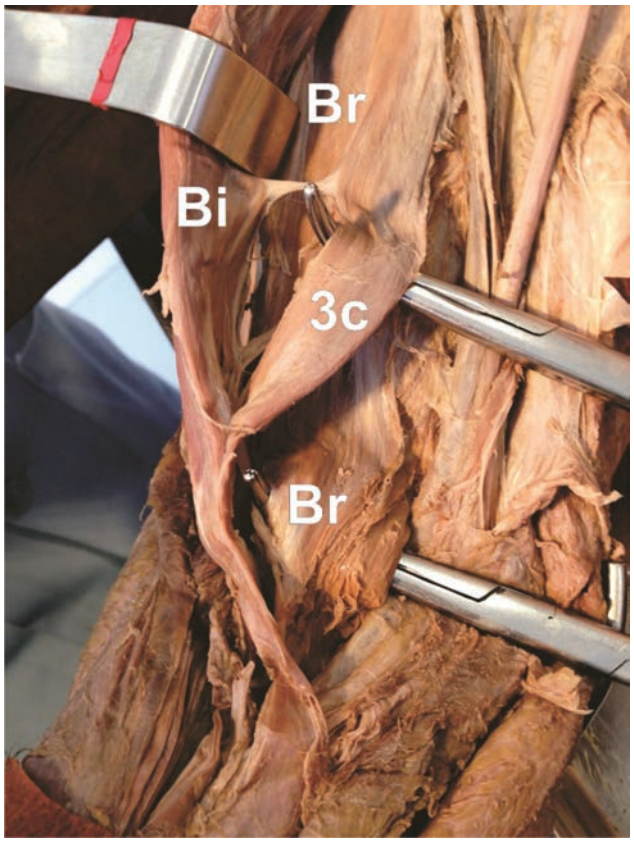

Fig. 2 **Bi**: biceps brachii; **Br**: brachial; **Me:** median nerve; **3c,** third head of the biceps brachii.

branch and the coracobrachialis muscle.[4,5] It is divided into three types:

Type I: The communicating branch is proximal to the drilling of the coracobrachialis muscle by the Mc nerve.
Type II: The communication occurs distal to the perforation of the coracobrachialis muscle by the Mc nerve.
Type III: There is no drilling of the coracobrachialis muscle by the Mc nerve or by the communicating branch.

The measurement of the third head of the biceps and of the communicating branch between the Mc and the Me nerves took as reference the distance from the coracoid process of the scapula. Based on these classification standards in the literature, the variation found in this body is type 2, according to Le Minor, and type II, according to Venieratators et al.

The communicating branch (Rc) was found 16.0 cm away from the coracoid process of the scapula drilling in coracobrachialis muscle (the branch's length was 8.0 cm). The length of the Mc was 17.5 cm (from its origin as a terminal branch of the lateral fasciculus, until the fibers that was provied for the formation of Rc). The Me in this cadaver was formed by a lateral root (derived from the lateral fasciculus), with 4.5 cm, and by a medial root (derived from the medial fasciculus), with 3.0 cm. From the location where the Me is formed (from the medial and lateral roots) to where it receives the Rc, the length of the Me was 20.0 cm (►**Fig. 3**).

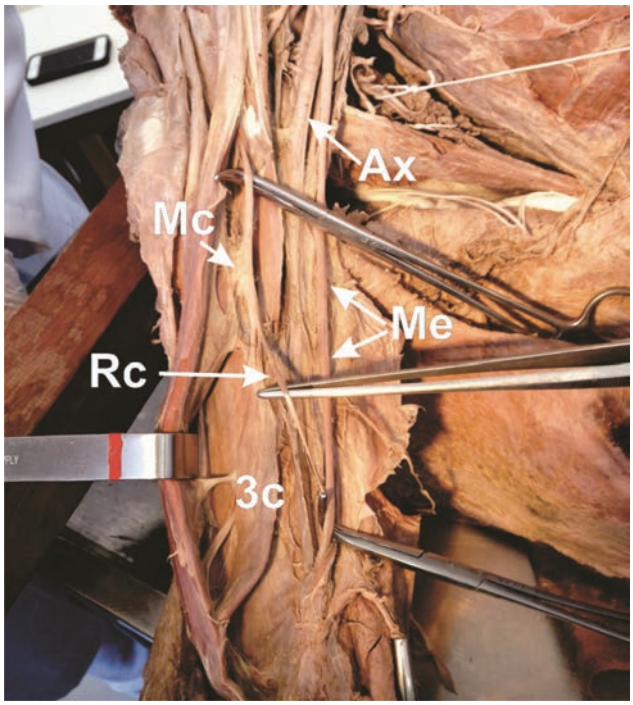

Fig. 3 **Ax:** axilar Artery; **Mc**: musculocutaneous nerve; **Me:** median nerve; **Rc:** communicating branch; **3c:** third head of the biceps brachii.

Discussion

The presence of a third head of the biceps associated with a variation of the Mc nerve may result from embryonic alterations during the development of the upper limb.[2,4,6,7,9] The mesenchymal forming muscles of the upper limb are pierced by primary ventral spinal nerve branches, whose contact is required for the mesenchymal condensation to the muscles.

As the development goes on, the somatic mesoderm invades the anlage, producing two condensations, a dorsal one and a ventral one, which will origin, respectively, the supinator-extensor muscle group and the flexor-pronator group. The nerves that invade the anlage of the developing limb avoid or do not penetrate the dense mesenchymal region or tissues with high rates of glycosaminoglycans. The places where a nerve can penetrate will collaborate with the development of muscles by signaling produced by the muscle itself. This may help to explain the mechanism of neuromuscular anatomical variations.[6]

The development of a third head of the biceps brachii can influence these nerve branching patterns, due to the close relationship of the mesenchyme with the primary ventral branches of the spinal nerves.[7–12] It is speculated that changes in muscle growth regulatory genes, such as *Pax 3* and *Myf 5*, and transcription factors, such as Myo D, may be involved in this kind of variation.[11]

In most cases, the communication between the Mc and the Me nerves, as well as the third head of the biceps brachii, are asymptomatic variations and incidental findings during surgeries or imaging studies.[7] The third head of the biceps brachii can, however, simulate a soft tissue tumor when it is

unilateral.[6] It can also complicate surgical procedures and confuse surgeons; as well as promote vascular and/or nerve compression in the arm, causing ischemia of the irrigation of the territory of the brachial artery and of its branches, or even pinching the Mc, causing paresis and paresthesia in its territory of innervations.[7,8,10,11,13]

The third head may still be responsible for the compression of the Me nerve, which runs through the arm and penetrates the region of the elbow in close relation with the brachial muscle and with the tendon of the biceps, with common symptoms when associated with doubling of the lacertus fibrosus. On physical examination, the compression of the Me can be evidenced by elbow flexion against resistance, with the supinated forearm, which will cause tension in the lacertus fibrosus associated with accessory aponeurosis and a third head of the biceps brachii, triggering neurological symptoms in the nerve territory, similar to pronator syndrome.[14]

The simultaneous presence of the communication between the Mc and the Me nerves and of the third head of the biceps brachii could also increase the incidence of compressive phenomena due to the proximity of these two variations. An hypertrophy of the third head of the biceps brachii that compresses the communicating branch between the Mc and the Me nerves could even simulate carpal tunnel syndrome. It could be difficult to diagnose and it could not be corrected by conventional surgical methods if the possibility of these variations is not borne in mind.[7] Humeral fractures in patients with a third head of the biceps brachii can be moved depending on the type and location of the fracture and on the presence of fragments.[9,11,15] Nerve damage from communicating branches between the Mc and the Me nerves may cause weakness in the compartment of the anterior arm, compromising the flexors of the forearm, as well as loss of skin sensitivity on the lateral side of the forearm and on the palm, depending on the fibers that pass through the injured communicating branch.

The third head of the biceps brachii can also generate extra flexion and supination force of the forearm, as well as elbow flexion independent of the position of the shoulder joint.[4–6] Some authors suggest that, depending on the position of the third head, it can contribute to the pronation of the forearm.[16] There have been also suggested that acessory heads with an accompanying artery or nerve may be useful in reconstructive surgery with flap removal.[11]

Conclusion

Due to the frequency of diagnoses and the number of surgical procedures performed in the upper limbs, it is extremely important for orthopedists, surgeons, neurologists, and general physicians to know the anatomical variations described in the present study. The information in the present report, therefore, helps in understanding these variations so that iatrogenic complications can be avoided.[7,15]

Conflicts of Interest

The authors have no conflicts of interest to declare.

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