ISSN 1307-8798

Official Publication of the Turkish Society of Anatomy and Clinical Anatomy

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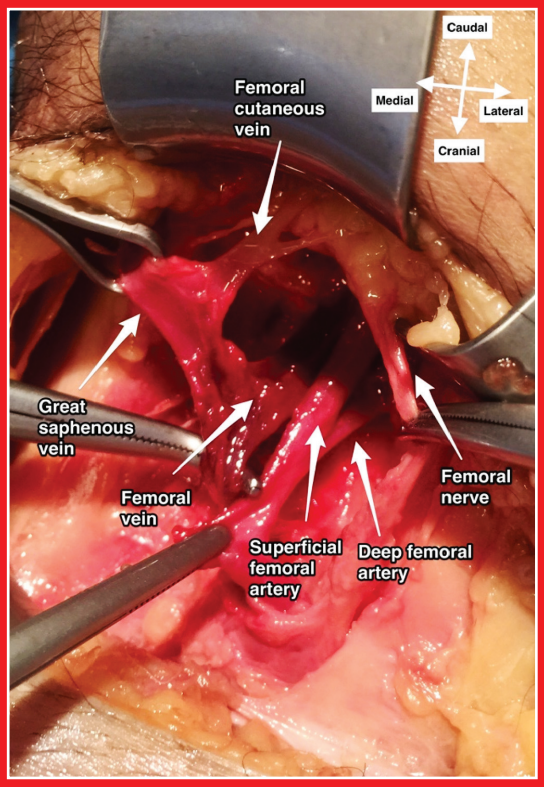
An International Journal of Experimental and Clinical Anatomy

Volume 13 / Issue 1 / April 2019

Published three times a year



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Güven Mah. Güvenlik Cad. Onlar Ap. 129/2 Aşağı Ayrancı, Ankara
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Publisher
Deomed Publishing
Gür Sok. No:7/B Kadıköy, İstanbul, Turkey
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Printing and Binding

Yek Press, İstanbul, Turkey, Phone: +90 212 430 50 00
Printed in Turkey on acid-free paper (April 2019).

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www.anatomy.org.tr
Received: July 17, 2018; Accepted: January 8, 2019
doi:10.2399/ana.18.045

anatomy
An International Journal of Experimental and Clinical Anatomy

Effects of alcohol and tramadol co-treatment on cognitive functions and neuro-inflammatory responses in the medial prefrontal cortex of juvenile male rats

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Abstract

Objectives: Misuse and abuse of drugs are on the increase amongst juvenile individuals across the world. This study was carried out to evaluate the cognitive and subcellular neuropathologic events in the medial prefrontal cortex (mPFC) of juvenile male rats following exposure to alcohol and tramadol hydrochloride.

Methods: The rats were assigned into four groups; vehicle group, alcohol group, tramadol group, and the alcohol+tramadol combined group. Twenty-four hours after the administration of the last dose, 5 rats from each group were sacrificed. The mPFCs were excised and were stained with either cresyl violet or glia fibrillary acidic protein immunoreactivity. The remaining rats in each of the groups were subjected to cognitive behavioural tests.

Results: The administration of alcohol, tramadol and the co-administration of alcohol+tramadol triggers astrogliosis, glial scars and inflammatory responses relative to the vehicle-treated with well-preserved profile. The distribution of Nissl substances suggested that the neurons are either undergoing neurodegeneration or neuronal metabolism impairment. The behavioural tests showed that the administration of the respective substances impaired cognition in the treated rats compared to the vehicle-treated rats.

Conclusion: This study concluded that alcohol, tramadol, and alcohol+tramadol misuse can impair the functional integrity of the medial prefrontal cortex.

Keywords: brain; cognition; drug misuse; learning; memory; neural degeneration

Anatomy 2019;13(1):1–12

Introduction

Tramadol is a synthetic centrally acting analgesic opioid which exerts its analgesic effect by blocking the re-uptake of norepinephrine and serotonin.[1-3] It is structurally related to codeine and morphine. Tramadol is a racaemic mixture of two enantiomers, the (1R, 2R)-(+)- and (1S, 2S)-(–)-stereoisomers, which have differing affinities for μ-receptors and monoamine re-uptake. (+)-Tramadol enantiomer preferentially blocks serotonin re-uptake and (–)-tramadol is a potent suppressor of noradrenaline re-uptake.[1,4–6] It is used for the treatment of moderate to severe pain.[7–9] Observations from published reports have shown that tramadol can be effective in relieving the symptoms of anxiety, depression, and phobias,[9,10] and is used in the treatment of opiate withdrawal,[11] as well as premature ejaculation.[12]

Tramadol is assumed to be a safe drug devoid of the large number of severe adverse effects associated with many of the traditional opioids. However, tramadol might

be additive and could be a substantial contributor to fatal intoxication when consumed in excess with other drugs depressing the central nervous system.[13–16]

Alcohol use and abuse are factors associated with tremendous burden of diseases, injury, and medico-economical costs worldwide. Repeated and excessive alcohol consumption is associated with more than 60 diseases. According to published findings by Rehm et al.,[17] it was estimated that approximately 4% of the total mortality and between 4% and 5% of the disability-adjusted life-years (DALYs) recorded worldwide are attributable to alcohol. Furthermore, while the greater share of the alcohol-associated disease burden occurs in advanced countries, the medical burden inflicted per unit consumption of alcohol is highest in developing and poorer countries, thereby setting a major barrier for additional developments in these countries.[17]

In a demographic study by The World Health Organization, it was reported that about 10–16% of individuals who consume episodic alcohol excessively and repeatedly are aged15 years or above are considered to be 'problem drinkers'.[17,18] Many from these age group have a mild to moderate form of alcohol use disorder (AUD) and despite the negative consequences, are abnormally and excessively preoccupied with alcohol craving, seeking and consumption.[17,19]

Over time, there has been considerable progression in the pattern of alcohol consumption among teenagers.[20] A substantial body of evidence in clinical- and laboratory-based studies have described the vulnerability of the central nervous system to the deleterious effects of alcohol and that exposure to alcohol during ontogenesis can confer morphological and functional abnormality on the brain and other structures.[21–23] It has been reported that many of the neurotoxic abnormalities associated with adolescent or juvenile exposure to abused substances occur at the same time with biological modifications in the functional integrity of bio-chemistry of the nervous system, which significantly determine the excellent transmission of information from one brain area to another via neural circuits.[24,25]

The prefrontal cortex (PFC) is the association cortex of the frontal lobe.[26] It receives inputs from many cortical areas of the brain, and functions in executing affective, cognitive, and social behaviour and many other complex functions. The PFC constitutes the highest level of the cause of ontogeny. It has an extended ontogenesis, which permits the accession of higher cognitive and executive functions via experience, but makes it susceptible to factors that can lead to aberrant and defective operational performance, manifested in neuropsychiatric disturbances.[27]

In the developing and advanced countries across the world, young people have the tendency of consuming alcohol in combination with other abused substances;[28] however, there is dearth of information on the effects of alcohol and its possible association with tramadol on the medial prefrontal cortex. Therefore, the main aim of this study was to examine the neuropathologic changes in the medial PFCs of juvenile male rats following exposure to alcohol and/or tramadol. Specifically, the objectives of the study were to determine the effects of treatments on the general cytoarchitecture, cell count of normal and degenerating neurons and astrocytes and expression of glial fibrillary acidic protein (GFAP) in the medial prefrontal cortex, and behavioural tests including MWM, passive avoidance, and novel object recognition.

Materials and Methods

Forty juvenile male Wistar rats (postnatal day (PND) 28, body weight: 78- 98 g) were used in this study. The rats were randomly divided into four groups. The rats were housed under standard conditions (n=10 per group; 12h light/dark cycle; 24±1°C room temperature; 53±12% relative humidity; rodent feed and clean drinking water *ad libitum*). All the experimental procedures documented in this experiment were performed in strict compliance with the ethical guidelines for the use of animals in laboratory research outlined by the Health Research Ethics Committee (HREC), College of Health Sciences, Osun State University (Osogbo, Nigeria) which is in conformity with the approved NIH Guidelines for the Care and Use of Laboratory Animals.[29]

The rats were randomly assigned into one of the following four groups; vehicle, alcohol-treated, tramadol hydrochloride-treated, and alcohol+tramadol hydrochloride co-treated. The rats in the vehicle group (n=10) were subcutaneously (s.c.) injected with double distilled water two times daily; alcohol-treated group (n=10) 1 ml of 15% v/v ethanol twice daily; tramadol-treated group (n=10) with 1 ml of 60 mg/kg/bw tramadol hydrochloride twice daily, and the alcohol-tramadol hydrochloride group (n=10) were with a combination of 1.0 ml of 15% v/v ethanol and 1 ml of 60 mg/kg/bw of tramadol hydrochloride two times daily. The dose of the drugs used in this study was adopted from our previous pilot study. The duration of treatments was 30 days. 24 h after the administrations of the last respective doses, 5 rats from each of the experimental groups were deeply anaesthetized and transcardially perfused for histochemical or immunohisto-

chemical staining procedures while the remaining five rats were exposed to MWM, passive avoidance, and novel object recognition behavioural tests. The summary of the experimental procedures is presented in **Table 1**.

Twenty-four hours after the administration of the last subcutaneous injection, 20 rats (n=5 from each of the experimental groups) were subjected to MWM, passive avoidance, and novel object recognition behavioural tests. All behavioural procedures were recorded with ANY-maze video tracking system (Stoelting; http://www.anymaze.co.uk). This test plays pivotal role in the validation of rodent models for cognitive evaluation and results are expressed by escape latencies to find the hidden platform in milky water. The modified method of Piermartiri[30] was used in this study. Briefly, the experimental apparatus consisted of white metallic circular white pool (diameter of 120 cm and a depth of 50 cm) containing non-toxic milky water (temperature of 25±1°C and height of 35 cm). An opaque Plexiglas escape platform (10×10 cm) was submerged 1.2 cm below the water surface at a constant position in the middle of the North-West (NW) quadrant. To reduce stress effects, the rats in each of the experimental groups were habituated to the MWM 24 hours prior to training sessions. The pool was placed in a test room containing various prominent visual cues. The animals were allowed to a spatial reference memory version of the water maze as previously described.[30] To reduce stress effects, the rats in each of the experimental groups were habituated to the MWM 24 hours prior to training sessions. The acquisition training sessions was performed on PND 59 and consisted of 10 consecutive trials, during which the animals were left in the pool facing the wall and allowed to swim freely to the escape platform. If an animal did not find the escape platform within a period of 60 s, it was gently guided to it. The animal was allowed to remain on the platform for 10 s after locating the escape platform, and it was then removed from the tank for 5 min before being placed at the next starting point in the pool. This procedure was repeated 10 times, with the starting points varying in a quasi-randomized manner. The test session was carried out on PND 60 after the training session. The test session consisted of a single probe trial where the escape platform was removed from the tank and each animal was allowed to swim for 60 s in the maze. The ANY-maze (Stoelting; www.anymaze.co.uk digital tracking system) was used in videotaping and recording the trials and probe tests. The tracks from trials and probe tests were statistically analyzed.

On PND 61, the rats in the respective experimental groups were trained on a one-trial step-through passive avoidance test. The passive avoidance chamber was compartmentalized into two (one illuminated and one dark; a door separated the illuminated section from the dark section) equal sections, fitted with a white plastic laminate grid floor. During the training trial, each rat was placed in the illuminated section; as soon as the rat entered the dark section, the door was automatically shut, and the rat received an inescapable foot shock (0.25 mA, 1 s).[31] In the testing trial, given on PND 62, the rat was once again placed in the illuminated section and the duration the rats entered the dark section again was measured and the step-through latency maximum testing limit duration was 180 seconds.[32]

Table 1
Synopsis of experimental protocols (n=40 rats).

Days	Procedure
0 – 27	Animal breeding (40 F1 generation)
28 – 58	Experimental treatments/drug exposure
59	Animal sacrifice (n=5 from each group) Morris water maze training (n=5 from each group)
60	Morris water maze testing (n=5 from each group)
61	Passive avoidance training (n=5 from each group)
62	Passive avoidance testing (n=5 from each group)
63	Habituation to the novel object recognition chamber (n=5 from each group)
64	Novel object recognition testing (n=5 from each group)

The novel object recognition (NOR) behavioural test was carried out in a wooden square chamber (60 × 60 × 45 cm) with non-glossy painted ply board walls and a white Formica floor divided by blue-black painted lines into 36 squares (10 × 10 cm). On PND 63, the rats were allowed 24 h habituation period to the NOR chamber, followed by the training and testing (PND 64) sessions as outlined by Marco et al.[33] with slight modification. During the habituation period, the rats were allowed to freely explore the chamber, under dim light conditions, for 300 s. The behaviour of the rat in the NOR chamber was video recorded for subsequent behavioural assessment. On PND 64, the procedure started with the training session. The rats were first exposed to two identical objects (two ceroplastic spherical balls) for them to explore the objects for 5 min. After a 5 h inter-trial interval, the test session began with the rats been exposed to one of the previously encountered objects (familiar object, F1 or F2) and to a novel, unfamiliar object (metallic red tinted spherical ball, N) for 5 min. The objects were placed in adjacent corners, at an approximate distance of 10 cm from the walls. At the commencement of each session, the rats were placed in the

middle of the chamber facing the objects. For each rat, the position of the objects was not changed between the training and test session. However, the objects' position was changed between the rats in order to prevent spatial preference. The NOR chamber and the objects were completely cleaned between tests on different rats with a 20% (v/v) ethanol solution. Both training and test sessions were video recorded (ANY-maze video tracking system; Stoelting; www.anymaze.co.uk) and the rats' behaviour was later evaluated by an experienced observer by means of event-recorder software (Observer®, Noldus, Netherlands). Exploration of an object was considered whenever the rats pointed their nose toward an object at a distance ≤1.0 cm, while turning around, climbing and/or biting the objects was not regarded as exploration. The time the rats' spent exploring the objects during the two sessions was recorded, and the discrimination index (DI) was calculated as the difference between the time spent exploring the novel object (N) and the familiar one (F1 or F2) in relation to the total time spent exploring the objects [(N-F) / (N+F)]. Rats that explored for less than 30 s during the training session and those only exploring just one of the objects during the test session were not included in the statistical analyses.

The rats then were deeply anesthetized with pentobarbital sodium and perfused transcardially with 50 ml normal saline and followed by 4% paraformaldehyde in tris buffer (pH 7.4) through the right cardiac ventricle and ascending aorta. The brains were then removed from the skulls of the rats, post-fixed in 10% phosphate-buffered formalin overnight, embedded in paraffin wax, and then sectioned at 5 µm thickness on a microtome.

Sections obtained were used for Nissl staining and glial fibrillary acidic protein (GFAP) immunohistochemical staining.

Nissl staining was done according to the reported method of Hamburg et al.[34] Briefly, every 10th sections of the right halves of the mPFCs were mounted on glass slides, air dried, and immersed in 70% ethanol for 12 h. Treatment with 0.1% cresyl violet solution (Sigma-Aldrich, St. Louis, MO, USA) for 20 min was followed by differentiation in 70% ethanol for 10 min. Subsequently, the sections were dehydrated in ascending grades of ethanol (70% for 10 min, 96% for 20 min and 100% for 30 min), treated with two changes of xylene (100%, 10 min each) and covered with coverslips using DPX mounting medium (Sigma-Aldrich, St. Louis, MO, USA). Five periodic Nissl-stained sections at 4.7 to 2.7 mm ventral and 4.7 to 2.7 mm dorsal to the bregma were used for cell counts and the average was calculated. In the mPFC, neurons were counted in two different regions delineated with a rectangle measuring 0.47×0.35 mm^2. The midpoints of both counting boxes were 5.0 mm apart from each other. The specimens were scanned at a magnification of x200 within the identical areas in the mPFC, using a Zeiss Axioscope A1 with a camera scope (AxioCamMRc, Carl Zeiss MicroImaging GmbH, Göttingen, Germany) attached to a computer interface. The intact neurons were outlined as non-basophilic neurons with both the pale nuclei and the discrete nuclei. Any neurons that had nucleolar fragments bigger than one-half of the average nucleolar diameter were included in the count as well as the neurons that had intact neuronal bodies.[35] The number of degenerating neurons was quantified as; (neuronal cell number in vehicle-treated rats) - (number of neuronal cells from the other respective-treated groups). All neuronal cell counting was performed by pathologist blinded to the grouping and treatment conditions.

The IHC of glial fibrillary acidic protein (GFAP) was performed according to the method of Adekomi.[32] Briefly, immunohistochemical analysis of GFAP was carried out on every 10th section of the left halves of the mPFC from the rats in the respective treatment groups. 5 µm-thick sections of the mPFC were fixed with 10% phosphate-buffered formalin for 6 hours. After blocking endogenous peroxidase, the sections were incubated with the primary antibodies; polyclonal anti-GFAP (1:100, Dako, Glostrup, Denmark). The peroxi-dase reaction was visualized using 0.03% DAB and 0.005% hydrogen peroxide. The immunostained sections were slightly counterstained with cresyl violet, dehydrated, cleared, and mounted in DPX (Dako, Glostrup, Denmark). The control sections for the IHC of GFAP were performed by excluding primary antibody and substituting it with a non-immune serum. The count of GFAP immunoreactive cells in the mPFC of the treated rats were quantified in 2.5 mm^2 fields of mPFC sections of five rats each from all the experimental groups using a X40 objective with a calibrated ocular micrometer system (Olympus Corporation, Tokyo, Japan). Counting of the GFAP immunoreactive cells in the mPFC were done by blinded microscopic observation by three pathologists who had no inkling of the study. Similar levels of mPFC (4.7 to 2.7 mm ventral and 4.7 to 2.7 mm dorsal to bregma) sections were maintained across the experimental groups according to the stereotaxic mouse brain atlas by Paxinos and Franklin.[36]

Data obtained are expressed as mean±SEM. The studied parameters were analysed using one-way analysis of variance. The statistical evaluation of the results was carried out using one- or two-way ANOVA with treatment

and number of trials (repeated measures) as the independent variables. Following significant ANOVAs, multiple post hoc comparisons were carried out using the Duncan test. After subjecting the data obtained from the cell count to one-way ANOVA, the data were further analysed using Kruskal–Wallis test. If any significance was observed, independent comparisons were made using Mann–Whitney's U test. A p-value <0.05 compared to vehicle values was considered statistically significant.

Results

The general cytoarchitectural profile of the neurons in the cresyl violet stained sections of the mPFCs of the rats in the vehicle-treated group was well preserved. The mPFC of the rats in this group showed neurons with normal appearance, prominent basophilic cytoplasm, and small-sized neuroglia cells uniformly dispersed within the neuropil (**Figure 1a**). On the other hand, the cellular profile of the mPFC of the rats in the alcohol-treated group displayed intense deviations from the normal cytoarchitecture. These changes were characterized by heterogeneous pattern, including increased neuronal vacuolization and less cytoplasm, chromatolysis, loss of nissl substances/deposition of nissl substances at the perinuclear membranes of the neurons, and neuronal shrinkage with small pyknotic or karyorrhectic nuclei (**Figure 1b**). The cytoarchitectural profile of the mPFC in the tramadol-treated group had similar features, but the neuropil had varying sizes of vacuolations and a couple of perinuclear spaces were present around the monomorphic neurons (**Figure 1c**). Marked neuropathological characteristics were observed in the mPFCs of the rats treated with alcohol+tramadol combination. These characteristics include chromatolysis, accumulation of neurofibrils at the perinuclear rim of the neurons, heterogenous neuronal apoptotic appearance, fragmented cytoplasmic contents with deposition of nissl substances at the perinuclear membranes of the neurons, hypertrophy of the neurons (**Figure 1d**). There was a significant loss in the number of normal neurons obtained from the cresyl violet stained sections of the mPFCs of the rats in the alcohol-treated, tramadol-treated and alcohol+tramadol combined groups compared to those of control group ($p<0.05$). There was no significant difference in the number of normal neurons in the alcohol treated compared to the tramadol treated groups. Significant difference ($p<0.05$) was observed in the number of normal neuron among groups. Furthermore, the number of normal neurons in both alcohol-treated and tramadol-treated groups were significantly higher ($p<0.05$) than the number of normal neurons in the alcohol+tramadol co-treated group (**Figure 1e**). The number of degenerating neurons in the vehicle group was significantly less ($p<0.05$) than the alcohol-treated, tramadol-treated, and alcohol+tramadol co-treated group, respectively (**Figure 1f**).

The activation of astrocytic cells following alcohol, tramadol and alcohol+tramadol-induced neuronal damage was demonstrated by using anti-GFAP antibody as a marker of astrocytic reaction in neuroinflammation (**Figures 2a–d**). In the vehicle treated group, immunohistochemical staining of the mPFC for GFAP displayed sparsely distributed GFAP-immunoreactive astrocytes with normal spatial arrangement, size, and dark brown cytoplasmic fibres which formed an organized array of network in the neuropil (**Figure 2a**). On the other hand, the astrocytes in the alcohol-treated group showed disruptive features, including astrogliosis with numerous small-sized astroglia ebbing around the pyknotic neurons (**Figure 2b**). The immunohistochemistry of GFAP in the tramadol-treated group had comparable cellular morphology with the alcohol-treated group. The mPFC was likewise characterized by reactive astrocytes traversing the entire neuropils (**Figure 2c**). GFAP-immunoreactive astrocytes increased across the mPFC section of the rats in the alcohol+tramadol co-treated group. The astrocytes were hypertrophied, and the neuropils had numerous glia scar around the degenerating neurons (**Figure 2d**).

Quantitative astrocytic cell count was used to confirm the GFAP immunohistochemical staining in the mPFCs of the rats in the treatment groups. In the mPFC, quantification of the number of GFAP-immunoreactive cells showed that the mean number of astrocytes in mPFC of the rats in the alcohol-treated, tramadol-treated, and alcohol+tramadol combined groups was significantly increased ($p<0.05$) relative to the vehicle group (**Figure 2e**). Significant difference ($p<0.05$) was observed in the number of GFAP-positive cells between alcohol-treated, tramadol-treated and alcohol+tramadol co-treated groups. There was no significant difference ($p>0.05$) in the number of GFAP- immunoreactive cells in the alcohol treated compared with the tramadol treated groups. The alcohol-treated group had significant lower number of astrocytes than the alcohol+tramadol co-treated group ($p<0.05$). Similarly, the number of GFAP-positive cells in the tramadol-treated group was significantly reduced ($p<0.05$) than the number of GFAP-positive cells in the alcohol+tramadol co-treated group (**Figure 2e**).

In this study, we examined the ability of the rats to acquire (training session) and retrieve (test session), spatial information suggestive of learning and memory capabili-

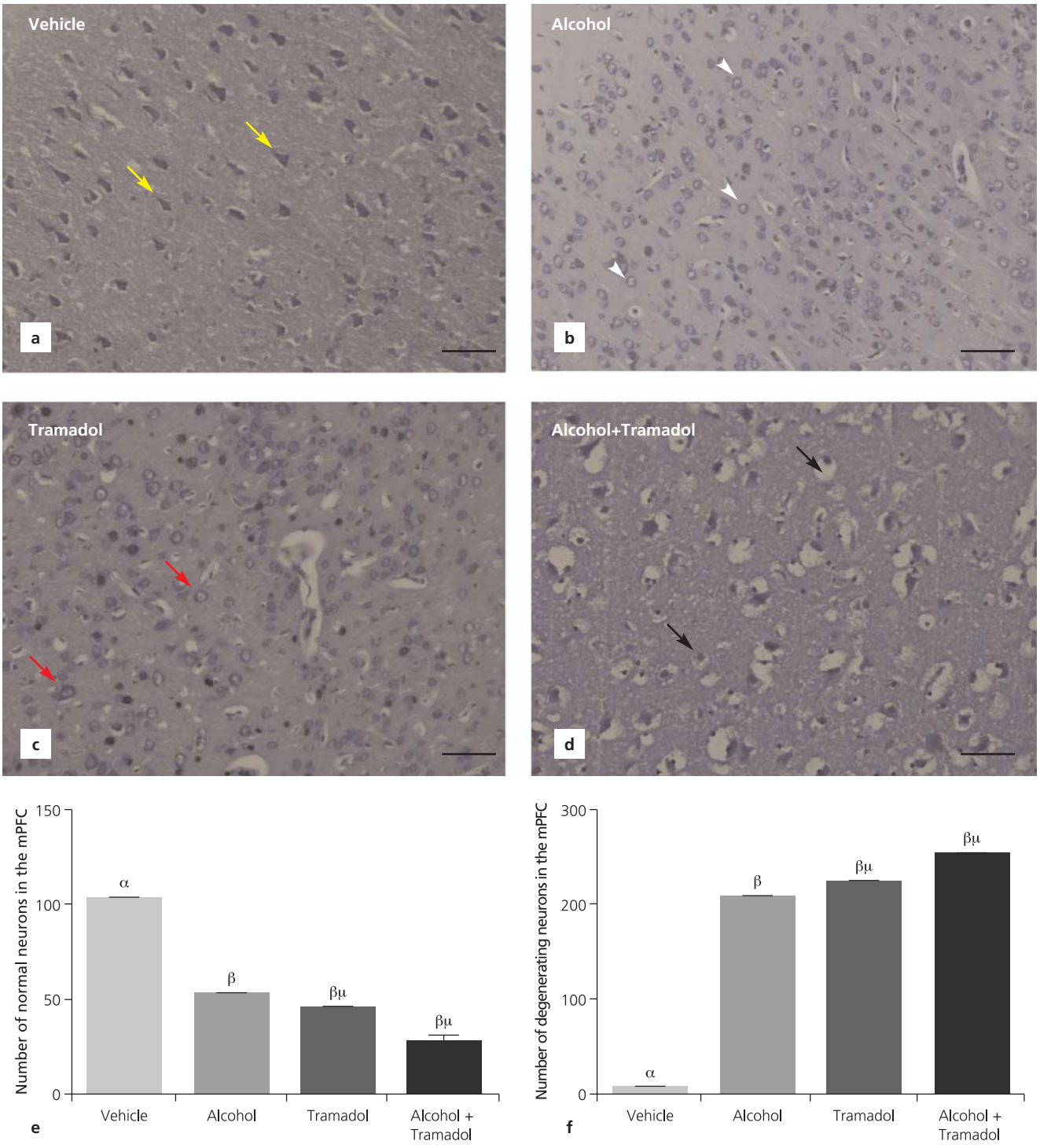

Figure 1. Histochemical demonstration of Nissl's substances using cresyl violet stain. In the vehicle group (**a**), CV-stained neurons are with normal appearance and prominent basophilic cytoplasm (**yellow arrows**). Observable changes were prominent in the distribution of the Nissl substances in the neurons (**arrowheads**) in the mPFC of the alcohol treated rats (**b**). These includes; chromatolysis, tred cytoplasm and peri-nuclear Nissl deposits, pyknotic neurons, and neurons with ruptured membrane (**white arrowhead**). In the tramadol treated group (**c**), there were patho-anatomical features such as pyknosis, chromatolysis, and accumulation of Nissl's substances at the perinuclear surface of the neurons, and heterogenous neuronal apoptotic appearances (**red arrows**). In the alcohol+tramadol treated group (**d**), there was marked hypertrophied neuronal cell degeneration as well as increased apoptotic cells. Scale bar=100 µm. The mean number of normal (**e**) and degenerating (**f**) neurons respectively per group (n=5 per group; "**α**" p<0.05, significant difference from the vehicle group and the other groups; "**β**" p<0.05, significant difference between alcohol treated, tramadol and alcohol+tramadol groups while; "**µ**" p<0.05, significant difference between tramadol and tramadol groups). [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

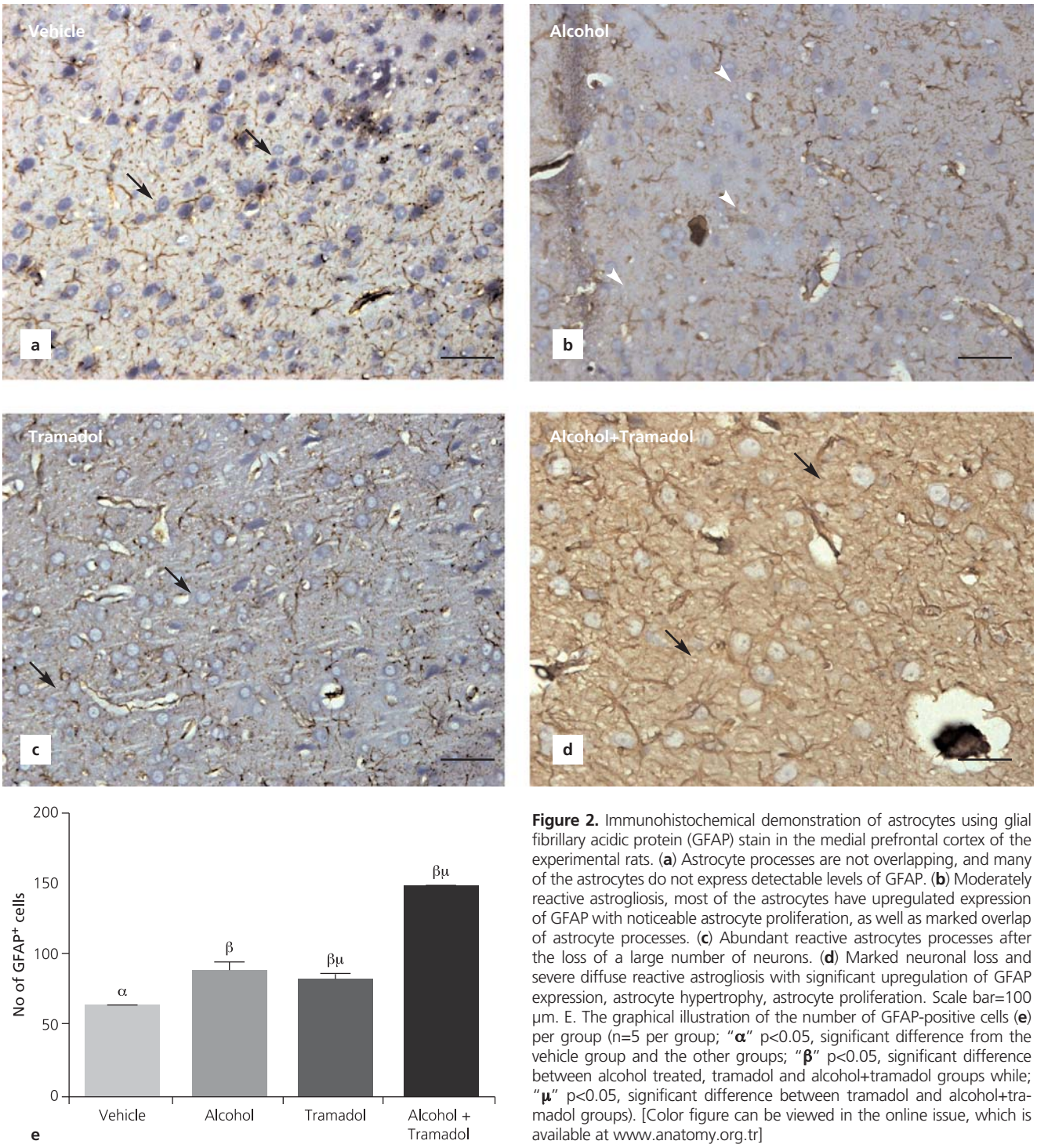

Figure 2. Immunohistochemical demonstration of astrocytes using glial fibrillary acidic protein (GFAP) stain in the medial prefrontal cortex of the experimental rats. (**a**) Astrocyte processes are not overlapping, and many of the astrocytes do not express detectable levels of GFAP. (**b**) Moderately reactive astrogliosis, most of the astrocytes have upregulated expression of GFAP with noticeable astrocyte proliferation, as well as marked overlap of astrocyte processes. (**c**) Abundant reactive astrocytes processes after the loss of a large number of neurons. (**d**) Marked neuronal loss and severe diffuse reactive astrogliosis with significant upregulation of GFAP expression, astrocyte hypertrophy, astrocyte proliferation. Scale bar=100 μm. E. The graphical illustration of the number of GFAP-positive cells (**e**) per group (n=5 per group; "**α**" $p<0.05$, significant difference from the vehicle group and the other groups; "**β**" $p<0.05$, significant difference between alcohol treated, tramadol and alcohol+tramadol groups while; "**μ**" $p<0.05$, significant difference between tramadol and alcohol+tramadol groups). [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

ties. In the training session (**Figure 3a**) carried out on PND 59, two-way ANOVA (treatment against repeated measures) showed that a significant effect for the main factors. There was a significant difference ($p<0.001$) between vehicle and the alcohol group in all the trials except for trial 1 and 3. Also, there was significant difference ($p<0.001$) between vehicle and tramadol in all trials except during the 3rd trial. Meanwhile, there was significant difference ($p<0.001$) in all the trials between vehicle and alcohol+tramadol. In the test session, performed 24 h after the training session, one-way ANOVA showed a significant effect for the treatments. Subsequent post-hoc compar-

isons showed that treatment with alcohol, tramadol and the combination of alcohol+tramadol showed a significant decline in both learning and memory as revealed by longer latencies (**Figure 3a**) compared to the vehicle, and reduced target quadrant preference during the probe trial (**Figure 3b**).

In the passive avoidance behavioural test, exposure to alcohol, tramadol and the combination of alcohol+tramadol altered the latency time when compared with the vehicle group. In the alcohol-treated group, the latency time in the passive avoidance test was significantly reduced ($p<0.05$) compared with the vehicle group. In addition, there was also a significant reduction ($p<0.05$) in the latency time between alcohol-treated group and the alcohol+tramadol co-treated group. However, the latency time was reversed in the tramadol-treated group compared with the alcohol-treated group. There was also significant difference ($p<0.05$) in latency time between the tramadol-treated group and the alcohol+tramadol co-treated group (**Figure 3c**).

Novel-object recognition test was done to evaluate non-spatial working memory in the experimental rats. Exposure to alcohol, tramadol, and alcohol+tramadol treatments triggers significant alterations in memory function relative to the vehicle-treated. Treatment with alcohol resulted in significant decrease ($p<0.05$) in memory function relative to the control. The tramadol-treated group displayed a less significant decrease in memory function

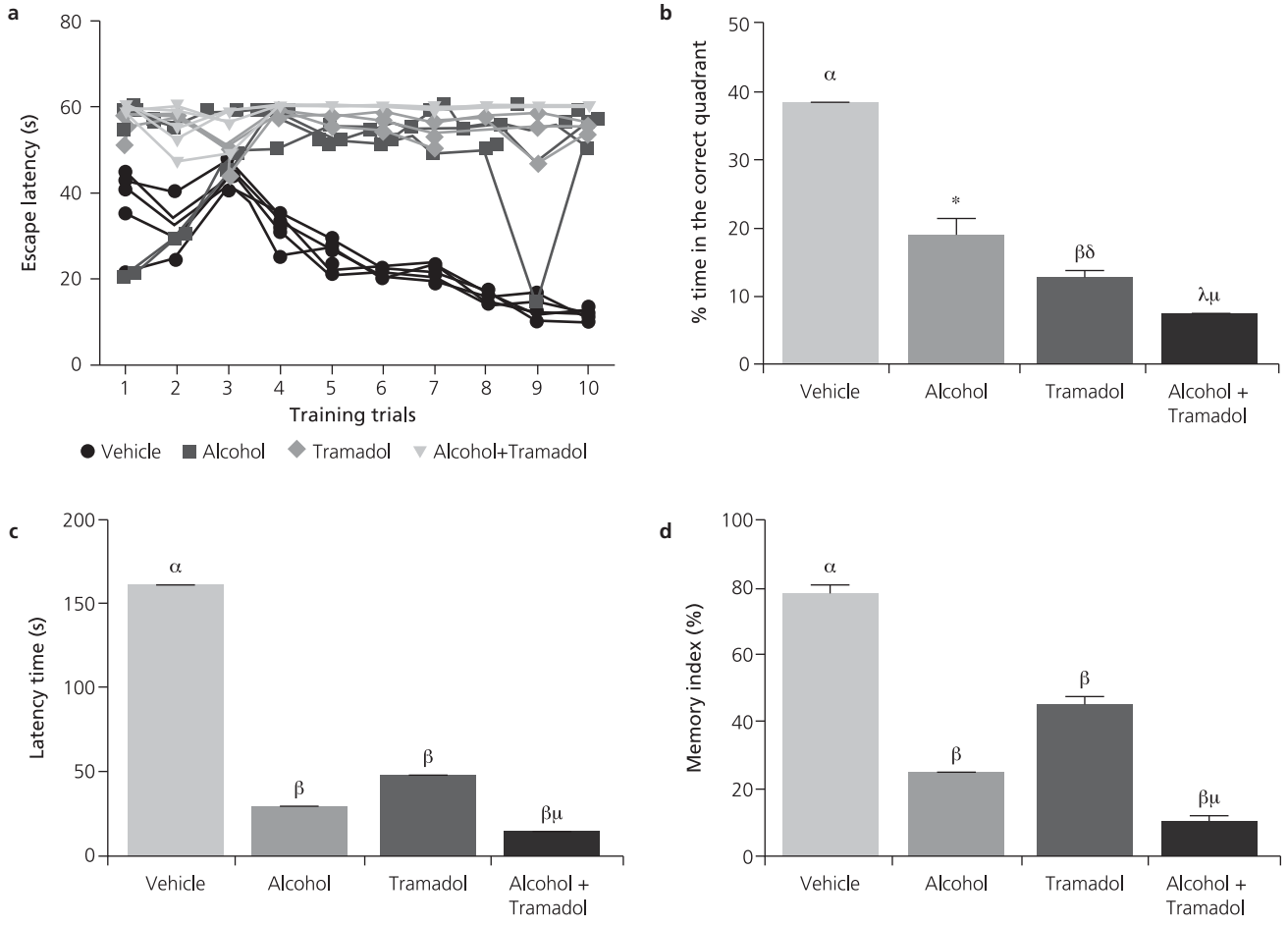

Figure 3. Exposure to alcohol, tramadol and the combination of alcohol+tramadol triggered cognitive deficits in the experimental rats. (**a**) Training trials were performed on PND 59. Values are expressed as mean ± SEM latency in seconds for escape to a hidden platform (n=5 for each group). (**b**) The probe test session was carried out 24 h after the training trials. Values are expressed as mean ± SEM of the time spent in the in the correct quadrant. (**c**) The latency of time of passive avoidance test across the groups. (n=5 per group at $p<0.001$). "**α**" represents significant difference between control and the other groups, **β** represent significant difference between alcohol, tramadol and alcohol+tramadol groups while "**μ**" represents significance difference between tramadol and alcohol+tramadol groups. (**d**) The memory index of novel object recognition test across the experimental groups. (n=5 per group at p-value <0.001). "**μ**" represents significant difference between vehicle and the other groups, **β** represent significant difference between alcohol, tramadol and alcohol+tramadol groups while "**μ**" represents significance difference between tramadol and alcohol+tramadol groups.

against the vehicle-treated ($p<0.05$), when considered with alcohol against the vehicle ($p<0.05$) and alcohol+tramadol against the vehicle ($p<0.05$). Furthermore, exposure to alcohol+tramadol co-treatment induced memory consolidation defect compared with the vehicle-treated (**Figure 3d**).

Discussion

In this study, we examined the effects of alcohol, tramadol, and the combination of alcohol and tramadol on some cognitive behavioural parameters using MWM, passive avoidance test, and novel object recognition test and subcellular neuropathological procedures using histological and immunohistochemical protocol in the mPFCs of juvenile male rats.

Data obtained in this study demonstrated that alcohol+tramadol co-treatment triggers shortfalls in the MWM test. Compared to the vehicle-treated group, alcohol-, tramadol-, alcohol+tramadol-treated groups spent significantly more time locating the escape platform in the swimming chamber. Observations from experiments in which animals and humans were exposed to alcohol and opioids suggest that stimulants and opioids are capable of conferring 'memory consolidation defect through alteration of the functional integrity of the opioid-neurotransmitter combination pathway(s), thereby triggering alcohol-opioid-related brain damage.[32,37–39]

In the MWM test, the vehicle-treated group easily found the location of the platform. No significant difference was observed when statistical comparison was made on the latency time to locate the escape platform between the alcohol and tramadol-treated groups. In addition, in the passive avoidance test, treatment with alcohol, tramadol, and, alcohol+tramadol impaired the latency time spent in the passive avoidance test (PAT) compared with the vehicle. These behavioral deviations are suggestive of the deleterious effects of alcohol, tramadol, and, alcohol+tramadol on cognition. There was a slight attenuation in the latency time in the PAT in the tramadol-treated group compared with the alcohol treated and the alcohol+tramadol groups, respectively. Also, there was a slight attenuation in the latency time in the PAT between alcohol and alcohol+tramadol treated groups. Therefore, based on these results, it could be suggested that the combined use of alcohol+tramadol could impair learning and memory in juvenile male rats. This observation is similar to a published report from our laboratory in which we observed that exposure to morphine-alcohol combination confers deleterious effects on cognition in juvenile male rats.[32] These impairments could also be linked to the deleterious effects of the substances administered on motivational and sensorimotor centers in the brain.

A large number of authors studied the singular effects of alcohol, and tramadol on neurobehavioral paradigm; however, to our knowledge, this is the first study to examine the effects of co-administration of alcohol+tramadol on cognitive behaviours in juvenile male rats.

Evidence from the histochemical and immunohistochemical data showed that the administration of alcohol, tramadol and the combination of alcohol+tramadol alters the cytoarchitectural profile of the mPFC of the treated juvenile male rats. Undoubtedly, adolescent and juvenile in the developing and developed world are significantly abusing drugs on daily basis. These drugs are either abused alone or in addition with other substances.

Studies have shown that tramadol abuse confers deleterious effects on the functional integrities of the CNS.[9,40,41]

In rats, tramadol preferentially gains access to the brain tissues compared to its active metabolite.[42] It was postulated that tramadol potentially induced neurotoxicity in rabbits by decreasing membrane fluidity of the blood brain barrier secondary to loss of unsaturation and fundamental changes in the structural concentrations and number of fatty acids.[43]

Evidences of neuronal degeneration and inflammation were observed under light microscopy in the mPFC of the rats receiving alcohol, tramadol, and the combination of alcohol+tramadol. In the mPFC, the degenerating neurons were characterized by fragmented cytoplasm, nuclear pyknosis, vacuolated neuropil, astrogliosis and glial scar surrounding the degenerating neurons. These features were further confirmed by the count of the normal and degenerating neurons, as well the number of astrocytes observed following the administration of the respective substances. Furthermore, the invasion of GFAP-immunoreactive cells in the mPFC of the rats in the alcohol, tramadol, and alcohol+tramadol treated groups was presumably the result of the chemotactic factors generated by the degenerating neuronal cells, and this further suggests that astrocytes can serve as facultative phagocytes in drug related neurotoxicity. In this context, the juvenile model of tramadol and alcohol-induced neuronal degeneration observed in this study provides a good system to study the neuron-glia interactions in response neuronal injury following exposure to alcohol and opioid combination. These observations are in consonant with the findings of Kofke et al.[44,45] and Miao et al.[46]

The significant difference in the numerical density of degenerating neurons in the mPFC of the experimental

rats could be an indication of severe damage to the neuronal cytoplasmic contents which contain the cellular machinery required for the functional biology of the cells.[47]

Numerous studies have identified that dysfunction of specific PFC subregions including the medial, anterior cingulate, and orbitofrontal cortices can promote drug- or ethanol-seeking behaviour.[48,49] Additional experiments in rodent models of ethanol consumption also further highlight the importance of the medial PFC. For example, lower numbers of GFAP-immunoreactive astrocytes have been observed in the prelimbic cortex of rats genetically bred for their high preference for ethanol over water and infusion of gliotoxins or a gap junction blocker into the rat prelimbic cortex increased preference for ethanol.[50]

Despite the changes in the number of GFAP-immunoreactive astrocytes within the mPFC of the alcohol treated rats, no significant changes were detected in the number of GFAP- immunoreactive astrocytes within the mPFC of the alcohol and tramadol treated rats. The nissl stain showed some changes that may have occurred within neuronal cell populations. It is known that neuronal loss occurs in the orbitofrontal cortex of rats following repeated ethanol consumption.[51] Regardless, the observed alterations in GFAP- immunoreactive cells could have profound impact on neural plasticity since astrocytes can actively modulate neuronal activity.[52]

Degenerating neuronal cells seem to be drained by the sustained augmented activity in response to the administration of alcohol+tramadol. It was shown that rats exposed to stress factors, including chemical substance, developed disturbance in the function of serotonin receptors in nerve cells and other tissues with subsequent occurrence of uncontrolled cholinergic action causing vasoconstriction and ischemia.[53] Moreover, stress-induced modulation of dopamine D1 and serotonin receptors functions through hyperactivation of cyclic adenosine 3-5-monophosphate which triggered neuronal degeneration as suggested by Tsukada et al.[54] Evidences from the cresyl violet stain further suggest that opioids may be involved in neurodegeneration. Although there are conflicting data in the scientific literature concerning the effects of opioids on programme cell death, *in vitro* experiments using specific cell lines revealed that opioids are capable of stimulating apoptosis.[9,55]

Alcohol-opioid combination may be involved in programmed cell death. There are conflicting results in the literature concerning the effects of opioids on apoptosis. *In vitro* studies using specific cell lines showed that opioids might induce or enhance apoptosis.[55] Another likely and/or possible mechanism of alcohol+tramadol induced brain damage is the decrement in the rat brain activities of Na^+/K^+-, Mg^{2+}- and Ca^{2+}-dependent ATPases with subsequent decrease in ATP turnover and energy metabolism, as well as loss of mitochondrial membrane transport functions.[56] In addition, tramadol and/or its active metabolite may react with alcohol to trigger the release of excessive ROS leading to DNA breakage.[57] Quantitative and pathological observations from this study is in support of the claims of Hauser et al.[58] and Eisch et al.[59] which suggested that opiate exposure can decrease the proliferation and survival of new neurons in the mature adult brain by acting directly on the neurocytes progenitor population, so decrease their proliferation and DNA synthesis via an opioid action at the μ-opioid receptor.

Conclusion

Exposure to alcohol and/or tramadol impaired cognition, learning and memory, and astrocytic activation became more prominent in the mPFC of the rats compared with the vehicle-treated rats. Furthermore, administration of these substances also conferred deleterious and toxic effects on the cellular profile of the mPFC of juvenile male rats.

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Conflict of interest statement: No conflicts declared.

 Please cite this article as: Adekomi DA, Adegoke AA, Olaniyan OO, Ogunrinde AE, Ijomone OK. Effects of alcohol and tramadol co-treatment on cognitive functions and neuro-inflammatory responses in the medial prefrontal cortex of juvenile male rats. Anatomy 2019;13(1):1–12.

Original Article

www.anatomy.org.tr
Received: January 22, 2019; Accepted: March 2, 2019
doi:10.2399/ana.19.018

anatomy
An International Journal of Experimental and Clinical Anatomy

Radiological hip indices correlate with GMFCS level I and GMFM-66 scores in cerebral pasy

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Abstract

Objectives: The aim of this study was to evaluate the certain radiological hip parameters and the effects of these parameters on the functional capacity of cerebral palsy (CP) children, to compare the Gross Motor Function Measure (GMFM-66 scores) of hemiparetic and diparetic children with spastic CP in Gross Motor Function Classification System (GMFCS) level 1, and to define possible differences or similarities with the control group.

Methods: The radiographic parameters measured for CP and control groups were caput-collum-diaphyseal angle (CCD), migration index (MI), center edge angle (CEA), acetabular index (AI) and pelvic obliquity. The functional capacity of the CP group was assessed by GMFM-66.

Results: No significant differences were found in terms of sides of the same individual in each group. Significant differences were found between groups for left CCD, right MI, right and left AI, and right and left CEA. Correlation analyses revealed significant relationships between radiological parameters. Hemiparetics had statistically higher GMFM-66 dimension E score than diparetics.

Conclusion: The threshold values for hip parameters were determined with CP in GMFCS level 1. The hemiparetic and diparetic children with CP, who were at the GMFCS level I and age group, had similar hip morphology. Development of femoral head and acetabulum in these children were not different from control group. Evaluating the functional levels of patients according to GMFM-66 scores with radiographic parameters is believed to contribute to the monitoring CP children.

Keywords: cerebral palsy; functional capacity; gross motor functional classification system; gross motor function measure; radiographic hip parameters

Anatomy 2019;13(1):13–20

Introduction

Cerebral palsy (CP) comprises a group of permanent disorders of the development of movement and posture, which causes activity limitations.[1] Children with unilateral lesions are termed hemiparetic and children with bilateral lesions are termed diparetic. Hemiparetic group compared with the diparetic group with within the same Gross Motor Function Classification System (GMFCS) level would have better gait and lower extremity function, but worse upper extremity function. Children with hemiparetic CP tend to walk at an earlier age than those with diparetic.[2,3]

The hip joint plays a key role for the lower limb alignment, and deformity of this joint gives rise to function impairments in the lower limb.[4,5] Because of the muscle impairment during the growth phase, 15-20% of overall children population affected by CP have the risk of developing hip dislocation. A high correlation of hip dislocation

with the level of GMFCS has been reported.[5] Therefore, the functional capacity of the CP patients is affected and the quality of daily living activities decreases.[6]

The GMFCS was standardized as a method of classifying CP children by their level of functional mobility.[7] The GMFCS is a simple, valid, and objective classification method that consists of five levels. Level I, children with minimal or no disability with respect to community mobility; level II, children with limitations walking indoors and outdoors but not using devices; level III, children with limitations walking indoors and outdoors and using assistive devices; level IV, children using methods of mobility that require physical assistance or powered mobility in most settings; level V, children who are totally dependent on external assistance for mobility.[2,3,7,8]

Hip diseases are followed by surveillance programs in CP patients. This program encompasses the processes of the early identification and intervention of hip pathologies.[4,9,10] Data obtained by clinical examination and radiological imaging are the vital components of the program.[4,5,9–14] The Gross Motor Function Measure (GMFM-66) is used to measure changes in gross motor function over time or to evaluate interventions in CP patients.[15,16] The GMFM is a criterian-referenced observational measure to assess children with CP. It measures the the items of gross motor activitiea in five dimensions; A: lying and rolling, B: sitting, C: crawling and kneeling, D: standing, and E: walking, running and jumping.[16]

Hip dislocation/subluxation causes significant morbidity in CP patients with advanced GMFCS. It is rarely a problem in ambulatory and mild forms of CP (GMFCS level 1).[15,16] Although these types of CP children have been followed up, we have not encountered the data regarding the radiological hip indices. The aim of this study was to evaluate the certain radiological hip parameters and the effects of these parameters on the functional capacity of CP children, and to compare the GMFM-66 scores of hemiparetic and diparetic children with spastic CP in GMFCS level I. Also, control group with healthy hips were used to define possible differences or similarities with the CP group.

Materials and Methods

The antero-posterior radiographs (Hofmann DMT GmBH Selector D2 and Siemens Multix Tap equipments, Siemens Healthcare GmbH, Elangen, Eermany) of hips of the CP group in a standard technique in prone position[17] obtained at our institution were reviewed retrospectively. The CP group, followed at the Department of Child Neurology, was consisted of 34 children with spastic CP (24 right-sided hemiparetic and 10 diparetic) with GMFCS level I. Descriptive values of those with relevant to gender and age were shown in **Table 1**. Evaluation of the motor function capacity of the CP group according to the crawling and kneeling (C), standing (D) and walking (E) dimensions of the GMFM-66[12] was taken at their routine examination visits. Children with history of spine or hip operation, botulinum toxin injections, GMFCS level II-V, hypotonic/dyskinetic CP, uncoordinated children and low quality images were excluded. The control group consisted of 26 children submitted to the emergency department because of trauma. Descriptive values of this group were given in **Table 1**. The graphics excluding dislocation and broken hips, legs and spine were chosen carefully from the archive. The appropriate dosed and standardized positioned ones were taken into account for the study.[17] The graphics were digitalized by a CR device then evaluated at the KODAK workstation by a radiologist. The study was carried out according to the principles of the Declaration of Helsinki. The ethical approval was taken from the Clinical Research Ethics Committee of Mersin University. The following radiographic parameters for each hip measured for both groups were (**Figures 1** and **2**): The caput-collum-diaphyseal (CCD) angle: The angle formed between the femoral neck and shaft in the frontal plane (**Figure 1a**);[6,18] Migration index (MI): The percentage of the distance between the lateral cortex of the femoral head and the lateral margin of acetabulum to the distance between the lateral and medial cortex of the femoral head (**Figure 1b**);[5,10,12,13,19] Center edge angle (CEA): The angle between a line drawn from the center of the femoral head to the outer edge of the acetabular roof and a vertical line drawn through the center of the femoral head (**Figure 1c**);[12,19–21] Acetabular index (AI): The angle formed between the Hilgenreiner line and a line extending along the acetabular roof (**Figure 1d**);[5,12,19–21] Pelvic obliquity (PO): The angle formed between the line connecting both inferior margins of the pubis and the horizontal line extending from the inferior pubis (**Figure 1d**).[12,22]

Table 1
Descriptive value of the hemiparetic, diparetic and control groups.

	Gender		
	Female (n)	Male (n)	Years of age
Hemiparetic	10	14	7.5±2.4
Diparetic	4	6	8.4±3.1
Control	16	10	10.±2

The Shapiro Wilk test was used to control normality of the continuous measurements. The distribution was normal in terms of hip parameters and one-way-ANOVA test was used to compare group differences. Levene test was used to check homogeneity of variances. Differences between groups were tested by the Bonferroni post-hoc test. The paired sample t-test was used for differences between the left and right measurements in each group. Correlation between variables was investigated by calculating the Pearson correlation coefficient. Mean and standard deviation values were given as descriptive statistics, except PO. For PO, the median and quartile values were given and the Kruskal-Wallis test was employed for comparison between the groups. Also, because of not normally distributed scores, the median and quartile values are given and, Mann-Whitney U test was used to compare the CP groups in terms of GMFM-66 dimensions. SPSS Trial version was used for all statistical analysis. For the statistical significance $p<0.05$ was adopted.

Results

The descriptive values of radiological parameters except PO, regarding hemiparetic and diparetic CP children, and the control group were given in **Table 2**. The comparisons of these parameters within and between groups were analyzed in **Table 2**. No significant differences were found in terms of sides of the same individual in each group ($p>0.05$). Significant differences were found between groups for left CCD, right MI, right and left AI, and right and left CEA ($p<0.05$). When pair-wise comparisons were examined, it was observed that there was a significant difference for left CCD between hemiparetic (146.42±9.17) and diparetic (154.30±7.32) cases ($p=0.022$). There was a significant difference for right MI between diparetic (0.19±0.12) and the control groups (0.09±0.05), ($p=0.002$). There were significant differences for right AI between hemiparetic (11.95±3.05) and control groups (9.50±3.13), and diparetic (12.67±4.56) and control (9.5±3.13) groups ($p=0.02$ and $p=0.019$, respectively).

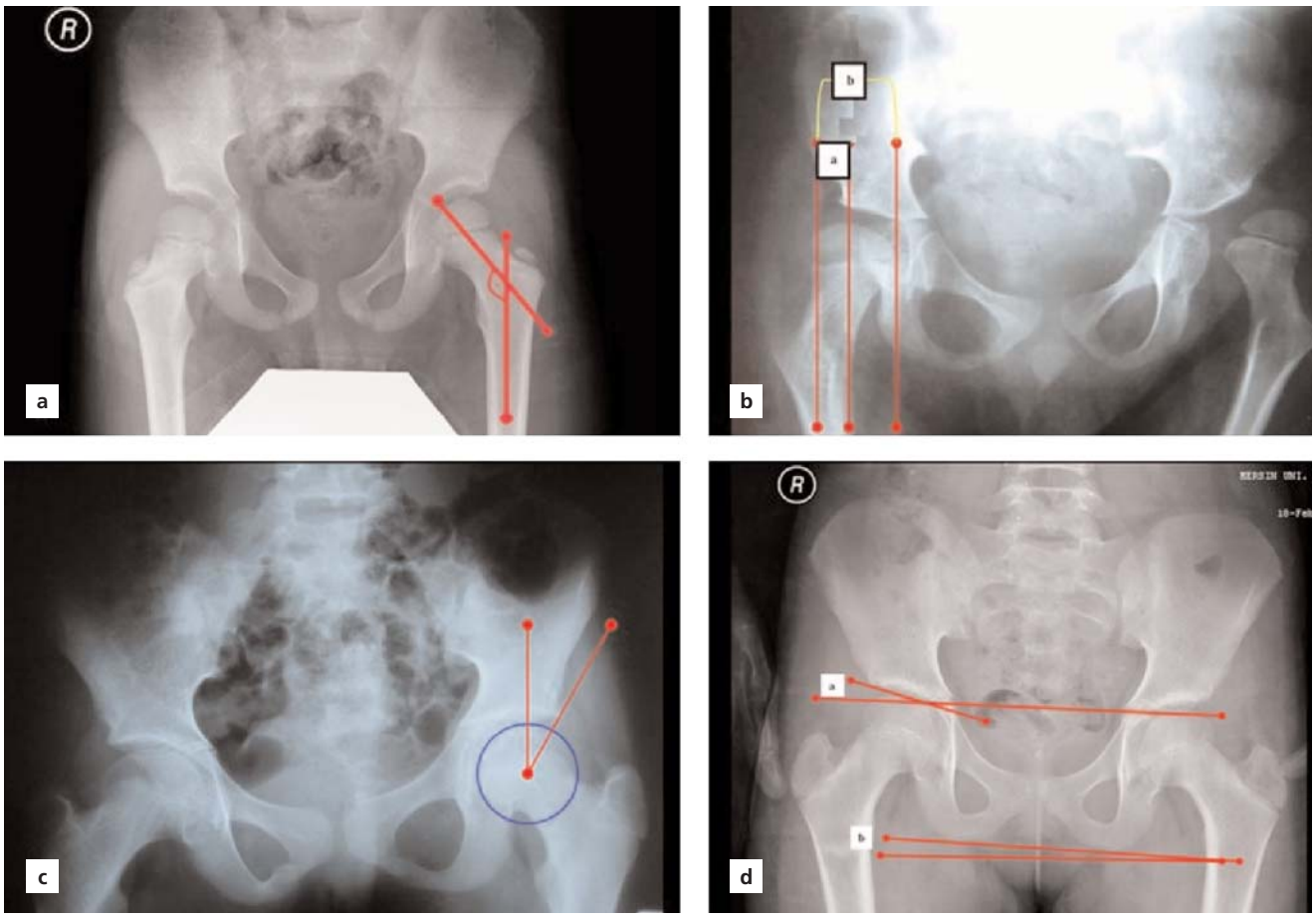

Figure 1. (**a**-**d**) The antero-posterior radiographics showing certain hip parameters. (**a**) Acetabular index diaphyseal angle (CCD). (**b**) Migration index (MI)=(a/b)x100. (**c**) Center edge angle (CEA). (**d**) Acetabular index (AI). **a:** acetabular index; **b:** pelvic obliquity (PO).

Table 2
Comparisons of certain radiological parameters within and between groups.

		Control n=26	Right hemiparetic n=24	Diparetic n =10	p[a]
CCD	R	147.69±6.84	149.52±9.34	152.90±6.2	0.199
	L	148.85±5.41	146.42±9.17	154.30±7.32†	**0.027**
	p[b]	0.259	0.058	0.285	
MI %	R	0.09±0.05	0.13±0.07	0.19±0.12*	**0.003**
	L	0.09±0.05	0.11±0.07	0.14±0.08	0.154
	p[b]	0.565	0.351	0.335	
AI	R	9.50±3.13	11.95±3.05*	12.67±4.56*	**0.017**
	L	8.81±2.71	12.11±4.48*	11.40±3.4	**0.008**
	p[b]	0.098	0.848	0.370	
CEA	R	34.85±5.18	28.31±5.18*	28.13±3.87*	**<0.001**
	L	34.88±5.57	30.58±5.75*	31.70±6.2	**0.044**
	p[b]	0.961	0.101	0.395	

p[a]: p-value of one-way ANOVA test; p[b]: p-value of paired t test; *indicator of difference with control group; †indicator of difference with hemiparetic group; **AI:** acetabular index; **CCD:** caput-collum-diaphyseal angle; **CEA:** center edge angle; **MI:** migration index; **L:** left side; **R:** right side.

There was a significant difference for left AI between hemiparetic (12.11±4.48) and the control (8.81±2.71) groups (p=0.003). There were significant differences for right CEA between hemiparetic (28.31± 5.18) and the control (34.85±5.18) groups, and diparetic (28.13±3.87) and control (34.85±5.18) groups (p=0.001 and p=0.005, respectively). There was a significant difference for left CEA between hemiparetic (30.58±5.75) and the control (34.88±5.57) groups (p=0.049).

For the PO, the median and 25-75% quartile values were: 1° and 0–3° for hemiparetics, 4 and 1-5 for diparetics, 10 and 0–30 for the control group. No significant difference was found between the hemiparetic, diparetic and control groups (p=0.163). Correlation analyses unveiled significant relationships between radiological parameters (**Table 3**). The scores of functional capacity of CP children measured with GMFM-66 were given in **Table 4**.

Discussion

The GMFCS have the advantages of reliability, validity and simple applicability. However, a significant ambiguity was reported in distinguishing level I from level II.[23] For further reliable classifications, we analyzed the radiological hip parameters of the children with GMFCS level I spastic type CP and evaluated their functional capacity according to these outcomes.

Although CCD angle was reported to have a high variance, it is normally measured approximately 150° in newborns, 135° at six years of age, 120–130° in adults, and 147° to 154° in CP children.[6,18,24] In the present study, the age distribution of children, both in CP and control groups, was between 5 to 12 years of age. Although, a certain CCD angle has not been described for these ages, the mean CCD angle of our cases has found to be over the average that was reported for the 6 years old group. When we compared the CCD angles of CP and control groups, the CCD angles in left hips of diparetics were found to be statistically significantly higher than left hips of hemiparetics (p=0.027). In the literature, where the CCD angle is reported to be high in CP patients, none of them have reported the specific angle values according to the CP subtype.[6,13] Particularly, no difference related to the CCD angle was observed in hemiparetic CP children in our study. Additionally, the CCD angle was found to have a positive correlation between sides in each group (**Table 3**).

The CCD angle and MI is reported to be significantly higher in GMFCS level IV-V quadriparetic patients than in the GMFCS level I-III diparetic patients. As well as, when there is a positive correlation between MI and CCD angles, a tendency to develop coxa valga is observed in the hips with higher MI values.[25] Our study demonstrated higher MI values in the right hips of diparetic CP cases than in the control group, while a powerful positive correlation was determined between CCD angle and MI (**Table 3**). According to the findings of the study, due to the mild increase of CCD in CP group, there need to be monitored closely in terms of coxa valga.

Table 3
Correlations between radiological parameters of the hemiparetic and diparetic CP groups.

			CCD-L	MI-R	MI-L	AI-R	AI-L	CEA-R	CEA-L
Right hemiparetic	CCD-R	r	0.738*	-0.092	-0.404	-0.464†	-0.531*	-0.193	0.140
		p	**<0.001**	0.708	0.086	**0.046**	**0.019**	0.428	0.568
	CCD-L	r	1	-0.055	-0.310	-0.337	-0.220	-0.148	0.077
		p		0.822	0.197	0.158	0.366	0.545	0.753
	MI-R	r		1	0.417	0.373	0.146	-0.533	0.037
		p			0.076	0.116	0.550	**0.019†**	0.879
	MI-L	r			1	0.502†	0.576*	-0.132	-0.519†
		p				**0.028**	**0.010**	0.590	**0.023**
	AI-R	r				1	0.619*	-0.461†	-0.423
		p					**0.005**	**0.047**	0.071
	AI-L	r					1	-0.169	-0.695*
		p						0.489	**0.001**
	CEA-R	r						1	0.459†
		p							**0.048**
Diparetic	CCD-R	r	0.847*	0.701†	0.005	0.565	-0.366	-0.738†	0.253
		p	**0.002**	**0.024**	0.989	0.113	0.299	**0.015**	0.480
	CCD-L	r	1	0.258	0.072	0.160	-0.670†	-0.314	0.002
		p		0.471	0.844	0.680	**0.034**	0.376	0.995
	MI-R	r		1	-0.033	0.828*	0.171	-0.953*	0.449
		p			0.928	**0.006**	0.638	**<0.001**	0.193
	MI-L	r			1	-0.051	0.049	0.212	-0.841*
		p				0.896	0.893	0.556	**0.002**
	AI-R	r				1	0.487	-0.782†	0.335
		p					0.184	**0.013**	0.378
	AI-L	r					1	-0.100	-0.025
		p						0.783	0.945
	CEA-R	r						1	-0.536
		p							0.110
Control	CCD-R	r	0.677*	0.115	0.125	-0.232	-0.094	-0.205	-0.110
		p	**<0.001**	0.576	0.542	0.254	0.648	0.316	0.592
	CCD-L	r	1	0.319	0.126	-0.194	-0.095	-0.176	-0.030
		p		0.112	0.540	0.343	0.645	0.389	0.885
	MI-R	r		1	0.762*	0.427†	0.374	-0.667*	-0.504*
		p			**<0.001**	0.029	0.060	**<0.001**	**0.009**
	MI-L	r			1	0.432†	0.598*	-0.551*	-0.729*
		p				**0.028**	**0.001**	**0.004**	**<0.001**
	AI-R	r				1	0.761*	-0.620*	-0.678*
		p					**<0.001**	**0.001**	**<0.001**
	AI-L	r					1	-0.551*	-0.742*
		p						**0.004**	**<0.001**
	CEA-R	r						1	0.729*
		p							**<0.001**

AI: acetabular index; **CCD:** caput-collum-diaphyseal angle; **CEA:** center edge angle; **MI:** migration index; **L:** left side; **R:** right side.*p<0.001, †p<0.05.

The mean AI angle between 5 to 11 years of age was reported to be 12.9±4.5° and the upper limit for normal coverage was accepted as 22°.[20] The AI angle was reported to be similar in spastic patients with stable and normal hips.[26] In our study, the mean AI angle was found to be within the normal limits in all groups. However, the AI parameter of hemiparetics was found to be higher in both hips than in the control group. Also, it was found to be higher in right of the diparetics than the control group (**Table 2**). Although normal limits were found in the study, the greater values in CP patients reflect the higher acetabular inclination and the predisposition to hip displacement.

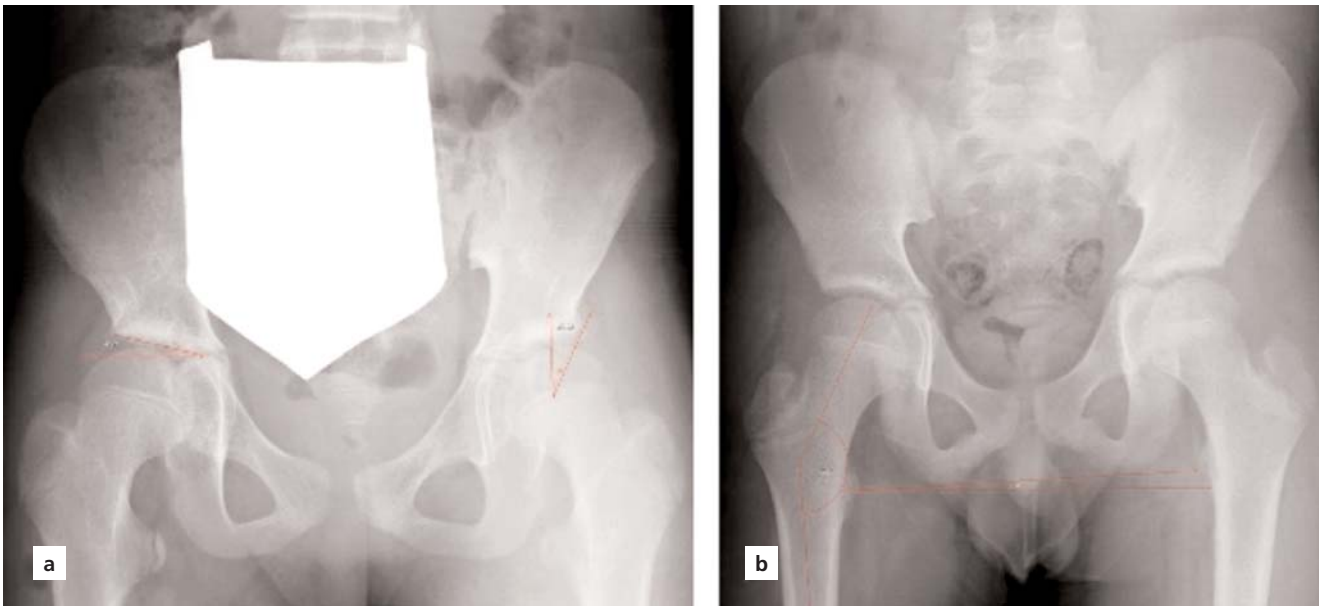

Figure 2. (**a**) AI on the right side and the CEA on the left side in diparetic CP girl aged 9 years. (**b**) The CCD on the right side and the PO on right side in hemiparetic CP boy aged 9 years. **CCD:** caput-collum-diaphyseal angle; **CEA:** center edge angle; **CP:** cerebral palsy; **PO:** pelvic obliquity.

The normal value of CEA for 5 to 10 years of age is reported to be 25.2±5.2° and 30±5.6° between 11 to 15 years of age. The lower limit of this angle between 5 to 10 years is 15° and 19° for 11 to 15 years of age. The CEA is reported to be a little bit higher in left hips, which is explained by the weight bearing differences between two sides.[20] In this study, interestingly the CEA was found to be lower in both hips of hemiparetic CP children and in the right hips of diparetic CP children than in the control group. Terjesen indicated the relationship of MI with CEA and AI in CP patients.[12] In the present study, negative correlation between CEA and MI parameters was observed in both hips of hemiparetics and diparetics. Similarly, negative correlation was found in both hips of hemiparetics and in the right hip of diparetics between CEA and AI (**Table 3**).

In comparing treatment outcomes, choosing children at the same age and the same GMFCS level is suggested in spite of normal children as control group.[7] In this study, comparison of hemiparetic and diparetic CP children in terms of hip sides revealed no statistically significant difference, except for the CCD angle in left hips. Therefore, we thought that the hip parameters of CP children at the same age and the GMFCS level I revealed similar morphology independent of the CP subtype.

Evaluation of the hemiparetic CP patients according to GMFM-66 revealed lack of ability in stability, kneeling and standing up activities. However, these cases were reported to have better activity scores when compared to other types of CP. According to gross motor development, diparetic CP patients were observed to have less ability, especially in standing and walking activities. In the same study, scores of GMFM-66 dimensions C, D and E for hemiparetic CP patients were 97±4.17%, 84.78±10.72% and 84.61±8.32%, and for diparetic CP patients 44.88±33.31%, 28.95± 27.97% and 30.45± 33.11%, respectively.[27] Significant differences between the scores were reported in that study as topographic distribution and GMFCS levels were ignored in evaluation phase. The results of this study demonstrated higher scores of GMFM-66 in the GMFCS level I hemiparetic and diparetic CP children (**Table 4**), in accordance with the previous report of Oeffinger et al.[3]

The "C" dimension of GMFM-66 includes more upper extremity involving activities than "D" and "E". Although, hemiparetic CP patients are known to have better abilities in walking and lower extremity functions, they are reported to have lower capacity in upper extremity functions than diparetic CP patients.[2] In this study, scores of hemiparetics in dimension "E" were found to be significantly higher than diparetics and scores of diparetics in dimension "D" were found to be significantly higher than hemiparetics. However, no significant difference was observed between the two groups by means of "C" scores (crawling and kneeling activities). According to GMFM-66, hemiparetic children in GMFCS level I were found to have higher ability in

Table 4
Comparison of GMFM-66 dimensions C-D-E between the hemiparetic and diparetic CP groups.

	Right hemiparetic (n=24)		Diparetic (n=10)		
	Min-max	Median (25–75%)	Min-max	Median (25–75%)	p-value
C- crawling and kneeling	81–100	100 (95–100)	76–100	98 (81–100)	0.163
D-standing	64–100	70 (68–74)	54–100	87 (69–95)	**0.010**
E-walking	74–100	92 (82–96)	46–100	75 (52–97)	**0.029**

walking, running and jumping activities than diparetics. Our study results were compatible with this report (**Table 4**).

The present study has an important limitation regarding less number of study populations. Due to having q mild form of CP with GMFCS level I children need less interventional treatments. Nevertheless, finding CP children without botulinum toxin injections, cognitive impairments and hypotonic/dyskinetic types is difficult. In further studies, a larger study population or community based registry will be evaluated within all GMFCS levels.

Conclusion

Threshold values of certain radiographic parameters of the CP children with GMFCS level I were determined in the present study. The radiographic findings revealed that development of the femoral head and acetabulum was not different from the control group and the literature data. Accordingly, these results support the effect of walking on hip development and the protective role in hip biomechanics according to GMFM-66 scores.

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Conflict of interest statement: No conflicts declared.

Original Article

www.anatomy.org.tr
Received: February 24, 2019; Accepted: March 12, 2019
doi:10.2399/ana.19.019

anatomy
An International Journal of Experimental and Clinical Anatomy

Radiographic evaluation of bone maturations in children and adolescents living in Erzurum using Greulich-Pyle method

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Abstract

Objectives: Bone age (BA) is used in age determination for a number of medico-legal reasons. One of the most commonly used radiographic methods in BA assessment is to compare hand-wrist radiographs with a standard reference. In this study, Greulich-Pyle (GP) method was used to determine the bone maturation values of a population of children and adolescents living in the city of Erzurum, Turkey.

Methods: Hand and wrist radiographs of 507 individuals (243 boys and 264 girls) aged between 7–19 years were evaluated and the BA was estimated by a radiologist using the GP method. Difference between estimated BA and chronological age (CA) was analyzed.

Results: In boys, except for the ages of 12,13 and 17 years, BA was significantly lower than the chronological age. In girls, BA was significantly lower than CA at 7, 8, 18 and 19 years of age, but not statistically different in the other ages.

Conclusion: According to the GP atlas, it is suggested that many factors including high altitude, cold climate, environment, nutrition, genetics, ethnics and socio-economic diversities might cause differences between BA and CA. Our results obtained in this study suggest that this method can guide the determination of bone age in children living in Erzurum, Turkey. However, in certain age groups for both genders, the GP method significantly underestimated skeletal age.

Keywords: adolescent; bone age; bone maturation; child; Erzurum

Anatomy 2019;13(1):21–26

Introduction

Determination of the degree of skeletal maturation is generally defined as skeletal age or bone age (Bone Age = BA) which is different from chronological age (CA) calculated by the date of birth of a healthy individual.[1] In medicine, BA determination is used to provide the closest estimate of CA of a person.[2,3] However, the processes of bone development might be adversely affected with various diseases, in addition to factors including race, genetic inclination, ethnicity, socio-economic status, geographical factors (altitude, climate) and gender.[4–12]

Although different methods are used in the determination of BA, the most commonly used ones are the Greulich-Pyle (GP), Tanner-Whitehouse (TW) 2 and TW3 methods. Greulich and Pyle published the "Radiographic atlas of skeletal development of the hand and the wrist" in 1959 and the GP method was developed based on the bone maturation values of children and adolescents with high socio-economic status residing in the United States. The BA is determined by a comparison between the left hand-wrist radiograph of the subject to the nearest matching reference radiograph. The GP method is a relatively practical and frequently used method.[1] On the other hand, TW2 method was developed in British children, in 1950s, based on European standards and descendants of Europeans. TW3 is a quantitative alternative of this method based on the skeletal maturity scores for each ossification center in the bones of hand and wrist.[13,14]

The GP atlas is applicable to most of the populations; however, some studies questioned the accuracy of the GP method, particularly in less developed countries including Turkey. Erzurum is a province located in the Eastern Anatolia with a cold climate and high altitude (approximately 2000 m). It is a region where the production and consumption of animal foods is common and socio-economical level is moderate. This study was carried out to obtain information about the bone maturation in this region by referring to the GP atlas in children and adolescents.

Materials and Methods

This study approved by the review board of Ethics Committee. A total of 507 individuals, 243 males and 264 females aged between 7–19 years, born and raised in Erzurum region were included in the study. Individuals with a disease history that could adversely affect the bone development were excluded from the study population. The age of individuals was identified as day-month-year. The day values were then converted to the month value by ± 15 days. A minimum of 15 girls and 15 boys were included in each age group. Evaluation of bone age by hand and wrist radiographs over 2 years of age is a well-accepted method of evaluation.[4]

In this study, left hand and wrist postero-anterior radiographs of the children between the ages of 7–19 were used for investigation. Radiographs were obtained by focusing on the metacarpal per tube-film distance of 60 cm.[1] The bone age was determined by the same radiologist by comparing the closest standard bone age in the same gender group in the GP atlas. The subjects were divided into two main groups as boys and girls. Each group was divided into chronological age (CA) groups. BA averages and standard deviation (SD) values of each group were determined. The difference between CA and BA was calculated for each case. The arithmetic mean and standard deviation of this difference were determined for each age group. The collected data were subjected to statistical evaluation. The values of BA in each group and SD and CA were determined by using paired Student's t-test. The confidence interval for the BA and CA averages of the groups was determined with a level of 95%. CA and BA correlations and significance were found in all age groups and genders. A statistical regression method was used for each of the female and male gender groups showing the least squares mean and linear correlation coefficients between BA and CA.

Results

This study was performed on a total of 507 individuals, consisting of 243 males and 264 females. The mean chronological ages (CA) of all groups in the 7–19 age range for males are shown as months in **Table 1**. In this table, average values of BA as well as the minimum and maximum values of BA are indicated in all age groups. The mean CA values for girls in each age group and mean, minimum and maximum BA values were calculated as months and shown in **Table 2**. CA values, mean BA values, difference of BA and C values and paired samples (p) values of each age group in males are shown in **Table 3**.

Table 1
Mean values of chronologic (CA) and bone ages (BA) in males.

	BA (months)		
CA (months)	Min.	Max.	Mean
92.15	60	96	79.38
98.86	72	108	88.00
115.16	96	138	109.55
125.52	108	132	122.28
134.90	108	138	128.10
151.65	132	162	151.20
162.52	132	192	160.73
173.90	162	186	172.00
187.21	168	192	183.47
195.35	180	204	192.00
209.50	204	228	211.20
218.33	204	216	214.66
230.89	192	228	221.00

Table 2
Mean values of chronologic (CA) and bone ages (BA) in females.

	BA (months)		
CA (months)	Min.	Max.	Mean
91.93	69	94	86.43
101.60	82	106	97.60
114.80	106	132	117.12
125.00	106	144	131.10
137.15	132	156	141.47
150.95	132	156	150.54
170.24	156	198	172.80
171.68	156	180	171.27
184.85	162	216	188.42
196.61	180	204	198.28
208.09	168	216	204.57
219.66	180	216	208.00
230.00	216	228	221.14

Table 3
The differences between BA and CA in males.

		Chronologic age (CA)		Bone age (BA)		BA-CA differences		%95 confidence interval			
Age (year)	n	Mean	SD	Mean	SD	Mean	SD	Min.	Max.	t	p
7	13	92.15	3.51	79.38	11.52	-12.76	11.8	19.91	5.62	3.895	0.002
8	15	98.86	2.89	88.00	12.55	-10.86	10.8	16.85	4.87	3.891	0.002
9	18	115.16	3.22	109.56	9.85	-5.61	9.7	10.46	0.75	2.440	0.026
10	21	125.52	3.32	122.29	8.99	-3.23	8.1	6.96	0.48	1.814	0.085
11	20	134.90	2.86	134.90	2.86	0.00	8.5	10.78	2.81	3.569	0.002
12	20	151.65	2.32	151.20	9.04	-0.45	7.9	4.16	3.28	0.252	0.804
13	19	162.52	3.33	160.74	11.23	-1.78	10.1	6.67	3.09	0.770	0.452
14	21	173.90	3.52	172.00	6.92	-1.90	7.4	5.27	1.46	1.179	0.252
15	19	187.21	2.82	183.47	7.56	-3.73	6.9	7.07	0.39	2.349	0.030
16	20	195.35	3.81	192.00	7.53	-3.35	6.4	6.34	0.35	2.337	0.031
17	20	209.50	4.11	211.20	8.16	+1.70	5.7	0.99	4.39	1.322	0.202
18	18	218.33	2.86	214.67	3.88	-3.66	4.7	6.01	1.31	3.290	0.004
19	19	230.89	2.88	221.06	10.05	-9.84	9.1	14.26	5.41	4.674	0.001

The difference between BA and CA values for males were significant at the ages of 7 years ($p<0.01$), 8 years ($p<0.01$), 9 years ($p <0.05$), 11 years ($p<0.01$), 15 years ($p<0.05$), 16 years ($p<0.05$), 18 years ($p<0.01$) and 19 years ($p<0.001$). On the other hand, no significant difference was observed at the 10, 12, 13, 14 and 17 age groups. These data indicated that the mean BA of males between 7–19 years was 4.27 months lower than the chronological age in Erzurum. The standard deviation values of the difference of BA and CA were between 4.7 and 11.8 for males, whereas they were between 5.42 and 13.28 in females, indicating some individual variations in the values.

Following a similar approach, mean CA, BA, difference between BA and CA values and p-value for females are shown in **Table 4**. The difference between BA and

Table 4
The differences between BA and CA in females.

		Chronologic age (CA)		Bone age (BA)		BA-CA differences		%95 confidence interval			
Age (year)	n	Mean	SD	Mean	SD	Mean	SD	Min.	Max.	t	p
7	16	91.93	2.90	86.43	7.58	-5.50	6.01	8.70	2.29	3.660	0.002
8	15	101.60	3.10	97.60	9.47	-4.00	7.16	7.96	3.41	2.163	0.048
9	16	114.81	2.80	117.12	9.74	+2.31	9.00	2.48	7.11	1.027	0.321
10	20	125.00	3.19	131.10	11.77	+6.10	9.90	1.46	10.73	2.753	0.013
11	19	137.15	3.45	141.47	7.56	+4.31	8.26	0.33	8.30	2.276	0.035
12	22	150.95	3.48	150.54	6.90	-0.41	5.68	2.93	2.11	0.337	0.739
13	25	170.24	9.18	172.80	10.39	+2.56	9.84	1.50	6.62	1.301	0.206
14	22	171.68	3.28	171.27	8.22	-0.40	7.76	3.85	3.03	0.247	0.807
15	28	184.85	3.43	188.42	13.35	+3.57	13.28	1.57	8.72	1.423	0.166
16	21	196.61	4.16	198.28	7.21	+1.66	7.24	1.62	4.96	1.055	0.304
17	21	208.09	3.74	204.57	11.04	-3.52	10.47	8.29	1.24	1.541	0.139
18	18	219.66	3.89	208.00	9.20	-11.66	9.10	16.19	7.13	5.435	0.001
19	21	230.00	2.60	221.14	6.08	-8.85	5.42	11.32	6.38	7.482	0.001

CA values for females were significant at the ages of 7 ($p<0.01$), 8 ($p<0.05$), 10 ($p<0.05$), 11 ($p<0.05$), 18 ($p<0.001$), and 19 ($p<0.001$). However, in age groups of 9, 12, 13, 14, 15, 16 and 17 years, differences were not significantly different. In this table, mean BA was 1.06 months underestimated than the average CA in the categories of 7–19 years of age.

A series of statistical procedures and regression analyses were performed for a global relationship between BA and CA. For males, the test statistics were calculated by using the formula of $y=a+bx$ in which x = CA, y = BA, and $t=38.586$, $p<0.001$, $y=-9.924+1.035x$. For females, $b> 0$, t: 29.158, $p<0.001$ were formulated as $y = 6.062 + 0.956x$. The linear correlation coefficient r was 0.996 for males and 0.994 for females, displaying a strong correlation in both genders.

Discussion

In this study, we aimed to investigate the accuracy and reliability of the GP method in a local population of children and adolescents living in the city of Erzurum, Turkey. The difference of the mean BA and CA was generally negative in all age groups. Especially at 7, 8 and 19 year age groups, differences were more significant. Among the investigated age groups, the difference between the average BA and CA values were positive only in the 17-year-olds. However, this difference was not statistically significant. In this study, mean bone ages of 7–19 year-old males estimated by the GP-method were 4.27 months behind the CA values.

The differences between BA-CA values in males were negative between the ages of 7–11. This negativity was more prominent between ages 18–19, but less between ages 11–17. Although, the mean differences of BA and CA values in females between the ages 9–16 showed relatively positive values in general, it was negative in the age groups of 7–8 and 18–19. Despite the fact that the age of puberty in Turkey is generally accepted as in the range of 12–20 years in males and 10–18 in females, bone maturation develops earlier with the values of 11–17 years in males and 9–16 years in females, reflecting the effect of pubertal period[15] In studies conducted in other countries, BA in males and females usually showed negative values in the preadolescent period.[8,16–18] However, in adolescents, BA was found to be equal to CA in males and females, or even higher than those of CA values.[8,18–21]

Previous studies suggested that bone maturation is enhanced in both females and males in specific age groups. Especially during the puberty, the effects of sex hormones accelerate bone maturation in females and males, although female sex hormones might be more effective than the male hormones.[22]

Our results also showed a regression in BA during the pre-adolescent period in both males and females. There was a significant negativity in boys at the age of 7 and 8. Similarly, a significant negativity was also observed in girls at 7 and 8 years old. In Erzurum, the mean BA of 7 to 19 year-old females was 1.06 months behind the CA. This indicates that BA value is close to CA value in girls. On the other hand, the highest standard deviation of the BA was roughly one year in both males and females. While documenting the age identity of individuals, declaration of persons was taken into consideration, but minor error margins should be taken into consideration, due to possible delays in the official recordings of date of birth certificates. In spite of the absence of reliable information, this study suggests that GP method provides valuable data in predicting the CA values and gives a basic information.

In another study conducted in Turkish children, SD values were found over a year.[23] In general, BA is used for age determination in forensic medicine. Especially for medico-legal and forensic interests, it is important to draw erroneous conclusions of adulthood based on the finding of full skeletal maturity by radiographic methods. It is stated that the age of consent in law starts with the substitution of 18 years of age.[2] Therefore, errors in the estimation of age could result in children being considered by the legal system as adults and *vice versa*. This study displayed several instances of children under 18 years of age having attained full skeletal maturity, as well as individuals 18 years and older with immature skeletons.

Although, our study population is different from the reference population originally studied by Greulich and Pyle in several ways, our findings suggest that reliance on the GP method as evidence for age determination in defendants of uncertain age lacks a basis in the scientific literature.

In boys, the difference between BA and CA at the onset of age of consent (18 years) and at the age of 19 years were -3.66±4.7 and -9.84±9.1 months, respectively. However, in girls, there was a very significant difference between BA and CA in both at the age of consent (-11.66±9.10 months) and also at the age of 19 years (-8.85±5.42 months). Individual differences are found in these values presented in **Tables 3** and **4**, due to factors affecting bone maturation in determining age determination, in our study population in Erzurum, the bone maturation level of boys at the age of 18 displayed a delay of approximately 8 months in comparison to girls. However, this difference between

males and females decreased at other ages. In the literature, differences in bone maturation between boys and girls were also reported similar to our study.[6,7]

In this study, males showed a delayed maturation of 3.21 months compared to females with an average of 7–19 ages. In Ireland, bone maturation age of males was reported as 2.3 years behind of females; but this difference was lower in countries such as Denmark, Greenland and Australia.[3,6,7,24] In a longitudinal study, the genetic slope was lower at the first 3 months of life, associated with the breast-feeding period. The growth rate was depressed in countries with low-middle socio-economic backgrounds, in comparison to North American and European standards.[5] This study indicates that socio-economic factors are more effective at the 7–19 years of age.

The effects of living in high altitude also has a special importance on child growth and development. In general, people born and raised at a high altitude tend to have lower birth weight, slower growth rate, longer period of growth, poorly defined adolescent growth spurt and delay in psychomotor development, compared to children living at the sea level.[11] In Erzurum, the high altitude up to 2000 meters might cause a delay in bone maturation by affecting the genetic slope. Bone maturation was 4.27 months behind the CA of males and 1.06 months behind for females in this study. Even more negativity was detected in the sampled population, as related to the socio-economic level. On the other hand, consumption of adequate dairy products might have a positive effect on bone mineralization and might have decreased the extent of delay in bone maturation.[25] As a matter of fact, in a number of studies, it has been emphasized that nutrition and socio-economic factors affect bone maturation significantly.[5,9,16,26–28] In another study conducted in Turkey around Sivas province, which also has a high altitude and cold climate, partial retardation was detected in comparison to Malatya which has a lower altitude.[29] Therefore, climate, nutrition and socio-economic factors are thought to play an important role on the growth rate of children. It is also pointed out that warm climate and hot environment cause premature maturation.[12] In China, Harbin, BA values of urban children were found ahead of the CA and ahead of the United Kingdom's standard.[8] Also, in Sweden with a cold climate, the BA was higher and further developed than those of in the United Kingdom.[30] In spite of the cold climate, genetic influences and socio-economic factors might exert stronger effect on the growth rates rather than the effects of climate and nutrition. The individuals living in Erzurum have a genetic inclination for about 1000 years of people living in the geography of this region and also a genetic affinity of the middle Asia inhabited by long years.

BA under the age of 3-year displays more genetic tendency; however, BA of the individuals are affected more from the influences of the socio-economical factors after the age of 3. Since this study covers the children and adolescents at the 7–19 age group, the effects of genetic tendency was less influential than the socio-economic factors.

Conclusion

In this study, the mean BA of children between the ages 7–19 in Erzurum was calculated using the GP method and found approximately 4.27 and 1.06 months behind the mean CA of males and females, respectively. Due to the fixed ethnic and socio-economic groups of children selected for the generation of GP atlas, its applicability varies in different parts of the world. The differences between BA and CA are thought to be caused by many factors such as high altitude, cold climate, environment, nutrition, genetic, ethnic and socio-economic factors. The findings of our study suggest that the GP method method is useful in determining the bone maturation of children and adolescents living in Erzurum, Turkey. However, in certain age groups for both genders, the GP method might significantly underestimate the skeletal age.

Acknowledgement

This manuscript was prepared from the PhD thesis of Cengiz Öztürk.

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Conflict of interest statement: No conflicts declared.

 Please cite this article as: Öztürk C, Özdikici M, Diyarbakır S. Radiographic evaluation of bone maturations in children and adolescents living in Erzurum using Greulich-Pyle method. Anatomy 2019;13(1):21–26.

Original Article

www.anatomy.org.tr
Received: February 16, 2019; Accepted: March 9, 2019
doi:10.2399/ana.19.020

anatomy
An International Journal of Experimental and Clinical Anatomy

Effects of chronic unpredictable stress on intestinal morphology in Wistar rats

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Abstract

Objectives: Stressful events might cause immune dysfunction and trigger various disorders. Adverse effects of acute or chronic stress exposure on the gastrointestinal system have been shown previously in several studies. In this experimental study, we used chronic unpredictable stress (CUS) paradigm to better mimic effects of the intermittent exposure to daily life stress and investigated the morphometric alterations occurring in the small intestines of rats.

Methods: Male Wistar rats were randomly divided into stress and control groups (n=8, each). While stress group was subjected to chronic unpredictable stress protocol for 21 days, control group remained undisturbed. Intestinal tissue samples were obtained from two different regions; one was 3-6 cm away from the pylorus and the other one 3-6 cm prior to the ileocaecal valve. Tissue sections were obtained from paraffin blocks at the thickness of 3 micrometers and stained with hematoxylin-eosin (HE) or periodic acid-Shiff (PAS). The lengths of villi were measured from the basal membrane to the top of the villus. The ratio of degranulating and non-degranulating mast cells per unit area were estimated by point counting method.

Results: The mean villi length in the stress group were significantly higher ($p<0.01$) than those of the control group. Degranulation to non-degranulation ratio of the mast cells were 40% and 54% in the control and stress groups, respectively.

Conclusion: Animals exposed to chronic unpredictable stress protocol displayed a significant elongation in the villi of small intestines and an increase in the number of degranulating mast cells in the intestinal mucosa. Since activation of mast cells causes releasing of various chemical mediators and growth factors, it is plausible that stressed animals developed an adaptation mechanism to enhance the capacity for absorption and digestion per unit length of the guts.

Keywords: chronic unpredictable stress; gastrointestinal system; mast cell; villus length

Anatomy 2019;13(1):27–32

Introduction

Stress is a usual part of daily living; however, if its level exceeds the adaptive capacity of an individual, it might predispose to illnesses in multiple systems including the gastrointestinal organs.[1,2] It has been shown that diverse stressors have a major influence on the gastrointestinal secretion, motility, epithelial permeability, and inflammation.[3] Also, in different animal models, stress exposure leads to intestinal pathology such as increased ion secretion, macromolecular permeability, microscopic inflammation, visceral hypersensitivity, dysmotility, and even bacterial penetration.[4]

The chronic unpredictable stress (CUS) model is originally used to study mechanisms underlying the stress response.[5] One of the main advantages of this protocol is better imitating the intermittent exposure to daily life stress. It also has a greater face validity than the other animal models of stress exposure.[6] Multiple experimental observations have suggested that endogenous gastrointestinal secretions and structural changes are important mechanisms for adaptation to stress exposure. Among them, mast cells have a special importance since they are associated with diverse modulatory effects in innate and adaptive immunity.[7] The mast cells of rodents and humans are numerous, and if grouped together they would make an organ equal to the size of the spleen.[8] Upon activation, mast cells can release a variety of chemical mediators stored in their secretory granules into the

extracellular environment within minutes, a process known as degranulation.[9] Therefore, in this study, we used CUS model to investigate the morphological changes in the small intestines of rats and specifically aimed to analyze alterations in the mast cells located in the guts.

Materials and Methods

Healthy male Wistar rats were obtained from the breeding colony at the Eskişehir Osmangazi University Animal Care Facility and maintained under constant temperature (21°C) and light (12:12 h light/dark cycle) conditions. Experimental procedures were performed in accordance with protocols approved by the Institutional Animal Usage Committee (Protocol number: 2016/526). Chronic unpredictable stress (CUS) model was applied to the stress group (n=8). During 21 days, rats were randomly exposed to stressors such as change of dark / light cycle, 45 degree tilted cage, wet bedding, crowded conditions, cold environment, predator odor, isolation in steel cage, exposure to bright light, water and food deprivation; two times a day (**Table 1**). Any intervention was not applied to the animals in the control group (n=8) except weekly body weighting. At the end of 21th day, rats were perfused intracardiacally with neutral phosphate buffer saline (pH=7.4) and then with 4% paraformaldehyde solution.

Intestinal tissue samples were obtained from two different regions; 3-6 cm away from pylorus, 3-6 cm prior to the ileocaecal valve. They were dehydrated, cleared, and then embedded in paraffin; 3 µm thick sections were obtained from each sample. The sections were mounted on slides, dried, and subsequently deparaffinized in three changes of xylol and rehydrated in three changes of 95% ethyl alcohol and distilled water. Histomorphological assessment was carried out by staining the sections with hematoxylin-eosin (HE), and periodic acid-Shiff (PAS) staining was used for mast cell counting. The sections were then rinsed and blotted carefully, dehydrated through 95% ethanol, absolute alcohol and xylene. For each animal, 10 sections in which the villi can be identified clearly were selected for the morphometric analyses. Measurements were done in at least 10 different areas from each section. Villi lengths were measured from the basal membrane to the top of the villus in a straight line by the StereoInvestigator software (Version 11; Microbrightfield Inc., Williston, VT, USA). A total of 1600 villi lengths were measured from stress and control groups. The number of non-degranulating and degranulating mast cells were estimated with point counting method using a grid. Systematic random sampling method was applied in the selection of areas under the high power objectives and both degranulating and non-degranulating mast cells per unit area were counted on 10 different sections obtained from each rat (**Figure 1**).

Table 1
The list and the application time of each stressor. Two of the stressors were applied randomly per day to each rat.

Stressor type	Application duration
Change of dark / light cycle	12 /12 h
45 degree tilted cage	8 h
Wet bedding	8 h
Crowded conditions	8 h
Cold environment	15 min
Predator odor	15 min
Isolation in steel cage	8 h
Exposure to bright light	12 h
Water deprivation	4 h
Food deprivation	8 h

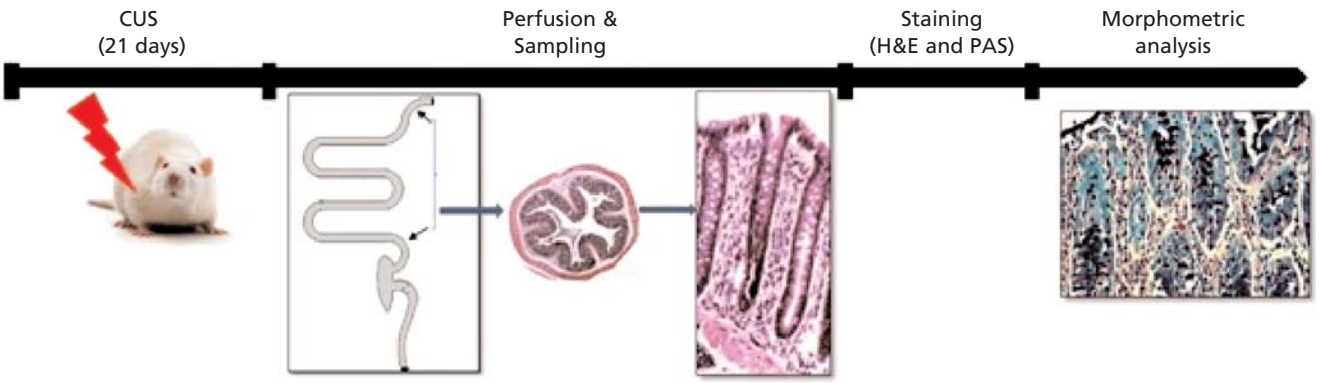

Figure 1. Experimental design. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

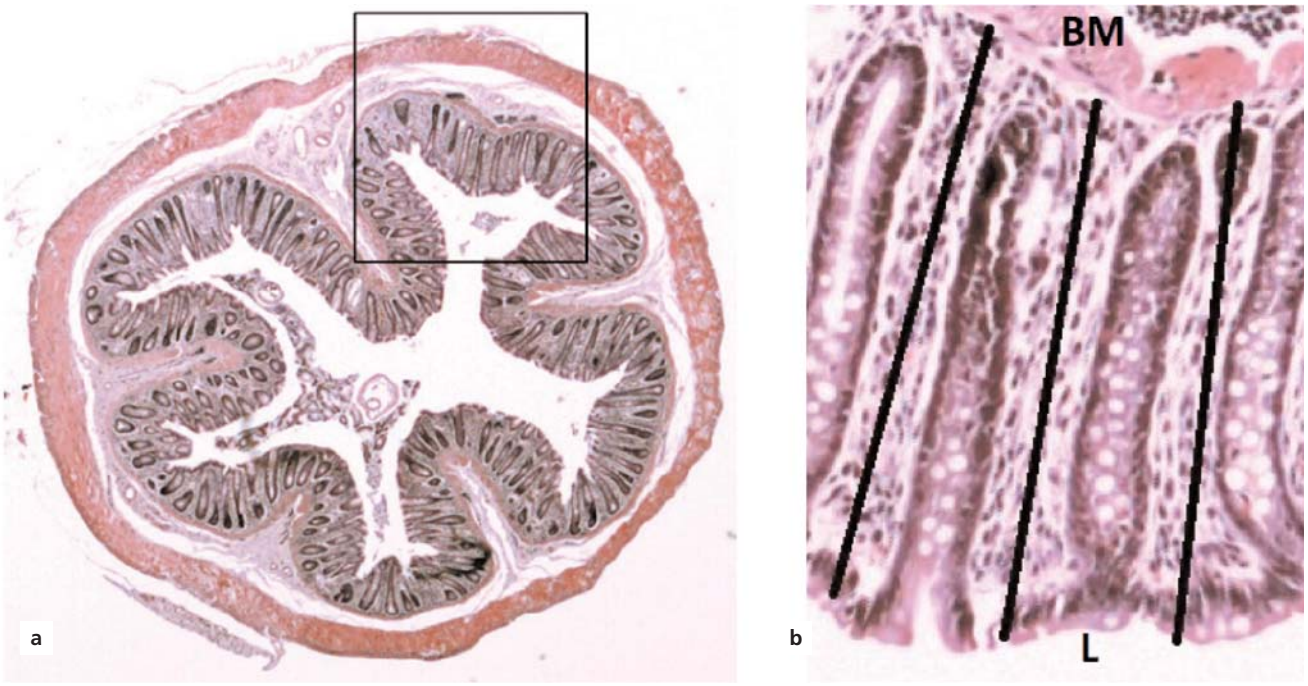

Figure 2. Photomicrographs obtained from the small intestinal tissue. (**a**) Cross sectional view of small intestine (10×) is shown; (**b**) High power view of outlined area (**black box**). Black straight lines in B are used for the villus length measurements. **BM:** basal membrane; **L:** lumen. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

Results

The cross sections obtained from the intestinal tissue of animals were stained and examined under the light microscopy. In both control and stress groups, the integrity of the intestinal mucosa was maintained and there were no irregularities in the glandular structures (**Figure 2a**). No inflammatory infiltration or ulceration was seen in the intestinal wall. Analysis at higher magnifications allowed the measurement of the villus length, from the basal membrane to the tip of the villus (**Figure 2b**). The mean length of the villi was 292.4 µm and 229.1 µm in the stress and control groups, respectively; significantly higher ($p<0.01$) in the stress group compared to those of controls (**Figure 3**). At the same time, a significant increase in the density of mast cells was detected in the stressed group. Degranulating mast cells were detectable in the histological sections stained by PAS method with numerous extracellular meta-chromatic granules. On the other hand, non-degranulating mast cells were characterized by rich intracytoplasmic granule contents. Average density of degranulating and non-degranulating mast cells were estimated by using a counting grid. In the high power view of degranulating mast cells, pink-purple color granules were easily discernable (**Figure 4**). When degranulation to non-degranulation ratio of mast cells were calculated by using the percentages of cells per unit area, the control group had lower (40%) degranulating mast cells than the stress group (54%) in the intestinal mucosa (**Figure 5**).

Discussion

In recent years, increasing number of publications has indicated that stress plays a major role in the gastrointestinal pathophysiology.[10] Stress-related functional

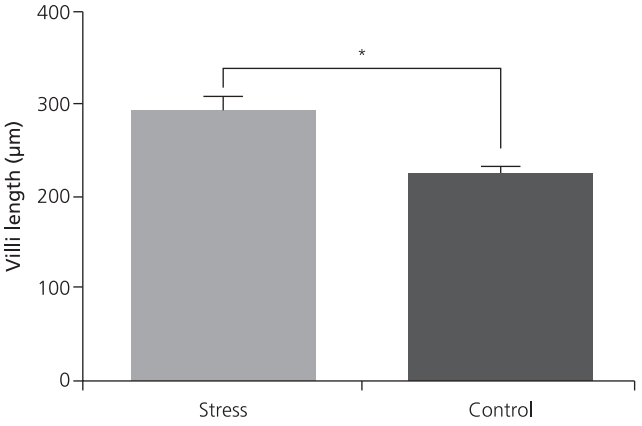

Figure 3. Comparison of villi lengths. The mean lengths of villi are significantly higher in the stressed animals than those of the control (*$p<0.01$).

intestinal diseases are becoming more common in modern human life, almost like the flue infections. Both of these are the most frequently seen causes of the labor loss due to the illness.[11] Experimental studies showed that after repetitive stress exposure, gastrointestinal inflammation is activated.[12] The influence of stress on the clinical course of some intestinal diseases, e.g. irritable bowel syndrome (IBS) a highly prevalent disorder in developed countries, is increasingly being recognized, but the underlying mechanisms are largely unknown.[12–15] Evidence from several studies indicate that mucosal mast cells play an important role in the stress related intestinal diseases, possibly by activating neurons that release corticotropin-releasing hormone and/or acetylcholine.[15] The main function of the intestinal mucosa is to exchange the nutrients with waste products between intestinal lumen and blood.[11] Epithelial cells act as a physical and functional barrier that limits the uptake of luminal antigens and pathogens.[12] Mast cells play an important role in the regulation of epithelial transport in both human and rodent intestine and there is clear evidence that nerve and mast cell interactions are responsible in intestinal epithelial dysfunction.[12]

Mast cells are effector cells of the immune system, found principally in all organs and vascularized tissues which are in contact with the outer environment[16] and regulate adaptive and innate immunity.[16,17] Mast cells could be seen in the skin, the gastrointestinal and the respiratory tracts, and also found in the peritoneum and synovium.[16] They are highly active cells of hypersensitivity reactions and allergic disorders, as seen in the allergic or parasitic inflammations.[13,18] Mast cells produce various inflammatory and immunoregulatory molecules called cytokines and chemokines.[18] While the first identified signaling mediator molecule of mast cell is heparin, more than 200 mediators are produced by mast cells all around the body.[17] In addition, mast cells produce biogenic amines (histamine, serotonin), interleukin (IL-1 to IL-6), leukemia inhibitory factor, tumor necrosis factor, interferon, transforming growth factor, granulocyte-microphage colony-stimulating factor, enzymes (acid hydrolyzes, chymase, phospholipases, rat mast-cell protease I and II, trypase), lipid metabolites (prostaglandins, leukotrienes, platelet-activating factor), ATP, neuropeptides (vasoactive intestinal peptide), growth factors (nerve growth factor), nitric oxide, and heparin.[16] These multifunctional biochemical messengers were collected in the cytoplasmatic granules of mast cells and released via a very rapid process called as degranulation.[10,16] Mast cells are present in all tissue layers of the gastrointestinal tract.[13,19] Human and rodent mast cells derive from

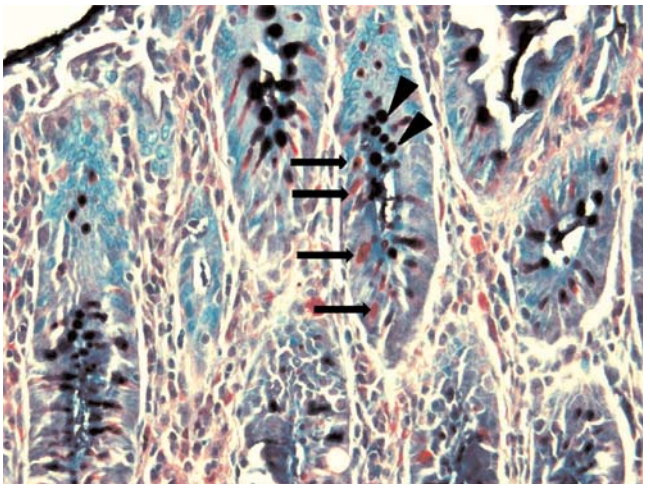

Figure 4. PAS stained histological sections showing degranulated and non-degranulated mast cells in small intestinal villi. The black arrows point the granulated and the black arrowheads indicate the non-degranulated mast cells. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

pluripotent hematopoietic progenitors in the bone marrow and reach to the gastrointestinal system *via* the blood stream during the 16th-22nd week of fetal life.[13,16] After completing their maturation, they are mostly located in the lamina propria of the mucosal layer and in the submucosal layer.[19] It is reported that the intestinal mucosa of irritable bowel syndrome patients contains an increased number of mast cells, also most of these patient have food allergies or adverse reactions to food.[10,18]

The pathophysiological mechanisms of chronic stress exposure on the intestinal tissue remains uncovered.[2] Traumatic experiences during childhood have been shown

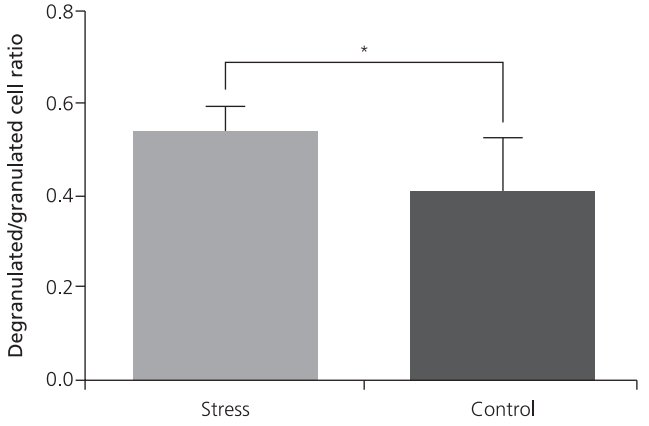

Figure 5. The ratio of degranulated/non-degranulated mast cells in stress and control groups. The stressed animals have significantly more degranulating mast cells than those of control animals (*p<0.01).

to increase the risk of IBS development.[14] Mast cells have been shown to play an important role in the responses of intestinal tissue to acute stressors in humans and rats.[2] During the last decade, studies focused on mast cells and various groups have highlighted the importance of the mast cells in stress-related changes in the intestinal tract.[11,14] The pathological changes caused by mast cell degranulation, goblet cell secretion and endothelial cell membrane alterations in the intestinal tissue are similar to changes produced by oxidative stress.[11] Mast cell activation results in ion transport changes in human and rat intestinal system.[2] Researchers report that all these stress-induced abnormalities seem to rely on biological mediators released by activated mast cells.[4,10] Thus, in adult rats, repeated stress has increased the amount of colonic mucosal mast cells, as demonstrated by using mast cell deficient rats or mast cell stabilizers.[14] Under light microscopy, mast cells were observed in the colon of these rats, but repetitive stress exposure significantly increased the number of mast cells in the mucosa of the colon.[10,12]

Various stress models were used to evaluate the effects of stress on the gastrointestinal tract and gastrointestinal disorders. Crowding, neonatal maternal deprivation, water avoidance stress, immobilization stress, cold pain stress, acquired intestinal infection or intestinal irritation, mild environmental stress such as change of rooms are the most commonly used stress models. Researchers have demonstrated that a mild environmental stress affects the intestinal mucosa by increasing the degranulation of mucosal mast cells, the activation of goblet cells and altering the capillary endothelial ultrastructure.[11,13] It is reported that crowding is reproducing naturalistic psychosocial stress. All these single acute or repetitive homotypic stress models affect the intestinal pathobiology such as increased ion secretion and permeability, inflammation, visceral hypersensitivity and bacterial penetration.[4] Stress has been shown to reactivate colitis in animal models. There are evidences that severe physical stress can cause gastrointestinal dysfunction and pathology. The central nervous system has the ability to modulate intestinal mast cell activity and that mast cells play a role in stress-related gut mucosal dysfunction.[15] The proximity of degranulated mast cells to enteric glia has suggested that stress activates the enteric nervous system and attracting and activating mast cells. In stressed rats gastrointestinal mucosal mast cells were observed as hyperplastic. Stress causes epithelial barrier defects and mucosal mast cell activation in rats.[17]

Conclusion

In this study, animals exposed to chronic unpredictable stress protocol for 21 days displayed a significant increase in the number of degranulating mast cells in the intestinal mucosa. In addition, villus length of stressed animals was significantly higher than the controls. It is known that degranulation of mast cells causes releasing of various chemical mediators, neutral proteases, a group of growth factors and vasoactive intestinal polypeptide. Therefore, structural changes detected in the villi might be associated with these mediators released by activated mast cells. It is also plausible that animals exposed to stress develop an adaptation mechanism characterized by elongation of villus and deepening of crypts, which increases the capacity for absorption and digestion per unit length.

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Conflict of interest statement: No conflicts declared.

Original Article

www.anatomy.org.tr
Received: February 21, 2019; Accepted: March 26, 2019
doi:10.2399/ana.19.022

anatomy
An International Journal of Experimental and Clinical Anatomy

Clinical evaluation of the temporomandibular joint anatomy using hologram models: a retrospective study

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Abstract

Objectives: Temporomandibular joint (TMJ) is the only synovial joint in head region and is exposed to very strong pressure, especially during chewing. Disorders of this joint are quite common and occur with severe pain. Recent studies focused on the ideal surgical procedures to manage disorders and pathologies of TMJ. The number of studies on prosthesis implantations for the condylar process is also increasing; therefore, the three-dimensional anatomical organization of the TMJ becomes important.

Methods: Computed tomography images of 160 healthy individuals (82 women, 78 men) submitted to the Radiology Department of Balıkesir University Hospital from 2016 and to the first three months of 2018 were evaluated retrospectively to describe the detailed three-dimensional anatomical organization of this joint. The anterior, posterior and superior articular spaces between the condylar process and the temporal bone were measured. Anteroposterior condyle diameter and condyle height were also evaluated. Data were compared for age and gender.

Results: The mean value of superior articular distance was measured as 2.39 mm, anterior articular distance 1.83 mm, posterior articular distance 1.99 mm and diameter of the condylar process 10.38 mm. Statistical results indicated that there were gender differences among the parameters.

Conclusion: The results of the present study point out to the importance of the gross anatomy of the TMJ and revealed the differences between genders and individuals. These data may guide surgeons for planning the ideal surgical protocols during managing of joint disorders.

Keywords: computed tomography; mandibular surgery; radiologic anatomy; temporomandibular joint

Anatomy 2019;13(1):33–39

Introduction

The temporomandibular joint (TMJ) is a condylar-type synovial joint in the head region between the mandibular fossa, the articular tubercle of the temporal bone, and the head of the mandible. Since the TMJ has complex anatomical properties and is exposed to severe workload in daily life, it is very sensitive to pathological changes.[1]

TMJ disorders, which are seen very frequently, can cause serious morphological changes, are seen more in women than in men.[2,3] They occur with clinical signs such as limited jaw movement, pathological sounds coming from the joint, or the locking of the jaw as a result of degeneration in the trabecular bone tissue and the erosion of the joint disc. Although etiology has been implicated in many factors, the cause of TMJ disorders has not yet been fully elucidated.[4]

Studies that include patients with clinical symptoms in the TMJ region with the aim of explaining the biomechanical mechanisms of the etiologies of TMJ disorders have reached no definitive conclusions regarding the eti-

ology. Another study that compared the condyle morphology of healthy individuals and patients suffering from TMJ disorders revealed morphological differences between genders, and age groups, and stated that three-dimensional imaging methods give more accurate results in the diagnosis compared to two-dimensional imaging.[5,6] Furthermore, it has been reported that using advanced imaging techniques during diagnosis can be beneficial and will form a more reliable differential diagnosis list.[1,7]

Surgical studies revealed changes in TMJ morphology following mandibular osteotomy operations. These studies also defined risk factors to prevent progressive condyle resorption, which was particularly likely in the post-operative period. Furthermore, their results indicated that morphological changes in the TMJ that were formed after surgery caused condyle resorption.[8,9]

A study that compared the morphometric properties of the TMJ using X-ray images reported differences among populations.[10] TMJ was studied in a Turkish population to investigate the effects of TMJ disorders on the joint morphometry;[11] however, morphometric properties of the TMJ in healthy individuals in the Turkish population were not well-described. Therefore, the main aim of the present study was to evaluate the anatomical organization of the TMJ using computed tomography (CT) images and hologramic images of healthy individuals in the Turkish population.

Materials and Methods

The ethical board of Balıkesir University approved this study (Decree Number: 171; Date: 12/27/2017). The study started upon gaining ethical approval and finished in July 2018. The study was performed according to the principles of the Helsinki Declaration (2008).

The CT image series of the temporal bones of 160 healthy individuals (82 women and 78 men) submitted to Radiology Department of Balıkesir University Hospital between 2016 and the first three months of 2018 were retrospectively evaluated. The mean age of the participants was 40.79 years (range: 12–75). The patients included in this study had no history of surgery or trauma against the head or mandible. Patients that had any bone deformation, TMJ disorders, surgery, or trauma history were excluded from the study.

A 64-slice CT scanner (Aquillon 64, Toshiba, Otawara, Japan) was used for image acquisition. Images were obtained in the axial plane from the frontal sinuses to the nasal floor. Continuous non-overlapping sections of temporal bone CT scan were obtained with acquisition parameters of 1 mm-slice thickness, 120 kV, and 200 mAs. The pixel spacing was 0.3×0.3 mm. Images were sent to the workstation (Aquarius Intuition edition version 4.4.6, TeraRecon, Foster City, CA, USA) for assessment. Reformatted images in the sagittal and coronal planes were constituted in addition to the axial plane with the same resolution characteristics. Images were evaluated with both bone and soft-tissue algorithms.

All CT images were obtained from the Picture Archiving and Communication System (PACS) at the University Hospital. Measurements were completed by radiology professor with a twenty-year experience, an anatomy professor with twenty six-year and experience and an anatomy specialist with six-year experience using Osirix-Lite version 9 (Pixmeo, SARL, Switzerland). Furthermore, ready-to-print three-dimensional images were obtained from the CT image series using Blender and Meshmixer software (Autodesk Inc, San Rafael, CA USA). These programs can be downloaded from the manufacturer's official websites for free. All parameters were measured from these ready-to-print three-dimensional images using free licensed Meshlab software that allows the spatial measurement of the distance between two selected points in three-dimensional images. There was no statistically significant difference between the measurements.

The Frankfurt horizontal plane, which lies from the inferior margin of the orbit to the superior margin of the external acoustic meatus, was determined. A parallel line to the Frankfurt horizontal plane from the most inferior point of the posterior border of the articular tubercle of the temporal bone to the anterior border of the condylar process of the mandible was drawn to measure the anterior articular space in sagittal sections. The posterior articular space was evaluated by drawing a parallel line to the Frankfurt horizontal plane from the most posterior point of the condylar process to the temporal bone in sagittal sections. The condylar process height was measured by drawing a parallel line to the posterior border of the condylar process from the tip of the condylar process to the deepest point of the mandibular notch. The diameter of the condylar process was evaluated by drawing a parallel line to the Frankfurt horizontal plane from the most anterior to the most posterior point of the head of the mandible in sagittal sections. The angle between the Frankfurt horizontal plane and the posterior border of the condylar process was also examined in sagittal sections (**Figure 1**). Finally, the superior articular space was measured by drawing a vertical line between the tip of the condylar process and the mandibular fossa in axial sections (**Figure 2**). All parameters were measured in three-dimensional images using Meshlab software (Autodesk Inc, San Rafael, CA USA) (**Figures 3** and **4**).

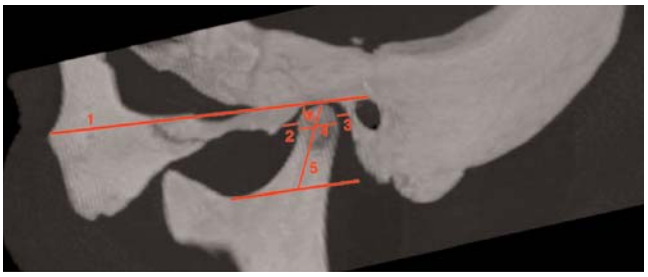

Figure 1. Radiological measurements in sagittal images. **Line 1:** Frankfurt horizontal plane; **Line 2:** anterior articular space; **Line 3:** posterior articular space; **Line 4:** condylar diameter; **Line 5:** condylar height; **Red star:** condylar angle. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

Statistical analyses were performed using IBM SPSS Statistics for Windows (Version 21, Armonk, NY, USA). Determining the normal or non-normal distribution of all measurements was evaluated using the Kolmogorov–Simirnov and Shapiro-Wilk tests. Descriptive analyses were performed to demonstrate all variables' mean values and standard deviations. Comparisons between genders and measurements were performed using Student's t test and the Mann-Whitney U test for normal and non-normal distributed measurements, respectively. The paired Student's t test was utilized to compare measurements between the right and left sides. While investigating the associations between age and the measured data, Spearman's rho test was used to calculate the correlation coefficients and their significance at the 5% Type-I error level. Measurements with p-value <0.05 were considered statistically significant.

Results

The detailed information of the morphometric measurements, including their mean values, standard deviations and minimum and maximum values are summarized in **Table 1**.

The distance between the condylar process and the mandibular fossa (the superior articular space) was as short as 0.41 mm in women and 0.4 mm in men. The superior articular space was longer in men than women on both sides (p<0.001). The lowest condyle height was 11.9 mm in women and 11.1 mm in men. There were statistically significant differences in the right (p=0.02) and left (p=0.01) condyle height between genders in favor of women.

The diameter of the condylar process was only statistically different on the right side and men had a wider condylar process (p=0.01). The condylar angles were wider in men on the right (p=0.02) and left (p<0.001) sides.

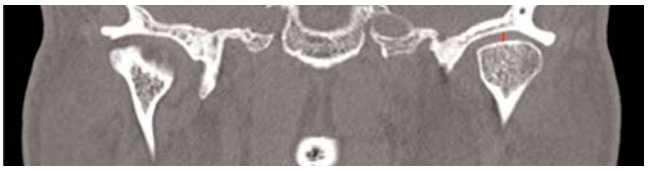

Figure 2. Measurement of superior articular space in axial images.

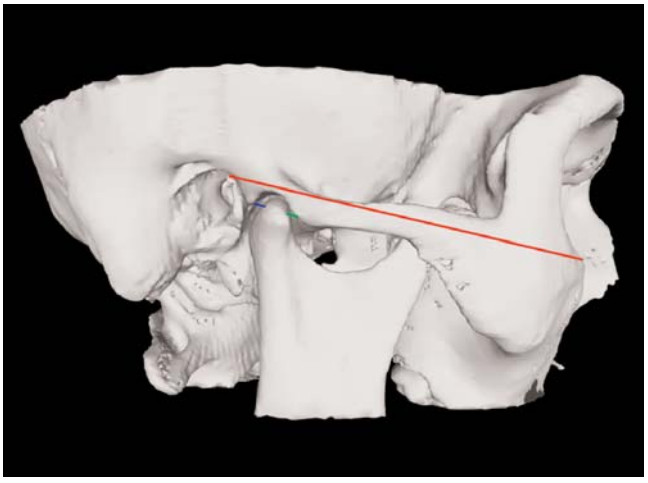

Figure 3. Lateral view from the right side of the virtual reality image that used for spatial measurements. **Red line:** Frankfurt horizontal line; **Blue line:** posterior articular space; **Green line:** anterior articular space.

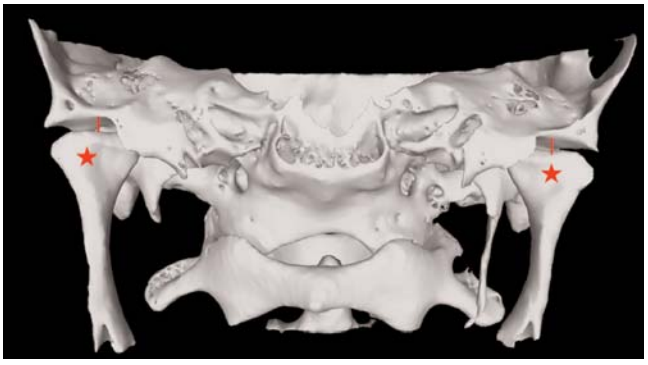

Figure 4. Anterior view of the coronal section of the virtual reality image. **Red lines:** superior articular space; **Red stars:** mandibular condyle.

The closest anterior articular space was 0.57 mm in women and 0.7 mm in men. The anterior articular space (p=0.002) and condylar height (p=0.001) were longer on the right side. However, the anterior articular space was not statistically different between genders for both sides (p=0.16 on the right and p=0.32 on the left side). In contrast, the diameter of the condylar process was larger on

Table 1
Results of morphological measurements. Mean values with standard deviations, minimum and maximum values (mm).

	Women		Men	
Parameter	**Right**	**Left**	**Right**	**Left**
Anterior articular space	1.83±0.73 (0.57–4.24)	1.71±0.8 (0.72–4.54)	2.03±0.86 (0.7–4.9)	1.74±0.67 (0.7–3.73)
Posterior articular space	1.98±1 (0.65–6.32)	1.9±1.08 (0.58–6.4)	2.07±0.9 (0.75–6.73)	2.05±0.87 (0.71–6.02)
Superior articular space	2±0.8 (0.6–4.14)	2.09±1.01 (0.41–6.66)	2.74±0.84 (0.4–5.03)	2.7±0.87 (0.65–5.78)
Condyle height	20.15±3.75 (13–29.8)	19.74±3.83 (11.9–28.5)	18.82±3.67 (11.5–28.7)	18.33±3.4 (11.1–25.7)
Anteroposterior condyle diameter	10±1.18 (6.8–12.4)	10.37±1.47 (6.36–13.9)	10.5±1.37 (7.1–15.1)	10.68±1.67 (7.2–15.6)
Angle of the condyle	70.04±5.4 (57–84.32)	69.32±6.29 (52.08–88.54)	72.18±6.16 (54.04–85.8)	72.16±6.13 (55.38–87.5)

the left side (p=0.01). There were no statistical differences between the right and left sides for other measurements.

The closest posterior articular space was measured as 0.58 mm in women and 0.71 in men. However, there was no statistically significant difference between genders for both sides (p>0.05).

Correlation analyses were also performed to evaluate the relationships between the variables. According to the correlation analyses results, the posterior articular spaces on either side narrowed with age (right: r=-0.28, p<0.001; left: r=-0.28, p<0.001). The superior articular space had a negative weak correlation with age only on the left side (r=-0.16, p=0.04). The diameter of the left condylar process increased with age (r=0.22, p=0.004). Statistically significant correlations between the other measurements were shown in **Table 2**.

Discussion

Several reported studies have evaluated gender differences in the TMJ; women have a greater tendency to suffer from TMJ disorders.[12,13] Morphometric properties of the TMJ also show differences among various populations.

Table 2
Correlation summary for the parameters.

Parameter	Correlation coefficient (r)	Significance (p)
Right superior and posterior articular spaces	0.32	p<0.001
Right and left anterior articular space	0.467	p<0.001
Right superior articular space and condyle height	-0.164	p=0.03
Right superior articular space and condyle angle	0.193	p=0.01
Right and left posterior articular spaces	0.569	p<0.001
Right and left superior articular spaces	0.711	p<0.001
Right and left condyle angle	0.68	p<0.001
Right and left condyle heights	0.9	p<0.001
Left superior articular space and condyle height	-0.189	p=0.01
Left condyle height and condyle angle	-0.237	p<0.001

The effects of radiologic tools for an accurate diagnose were reported.[14] Appropriate surgical techniques are of crucial importance for both patients' post-operative period and their life quality through the rest of their lives. Therefore, choosing the best surgical protocol is greatly important besides gaining knowledge of the gross anatomy of this joint. Furthermore, investigating the effects of the surgical methods on the TMJ after surgery may increase patients' life quality. Various published surgical reports have demonstrated the effects of surgery on the joint.[5,8,15] Iguchi et al.[9] reported gross changes in the TMJ after a ramus osteotomy of 39 patients using CT and magnetic resonance imaging (MRI). They compared the joint anatomy between the pre- and post-operative periods. According to their results, all articular spaces were narrower after surgery. Likewise, the heights of the condyle and ramus of the mandible also decreased after surgery in all patients. Nevertheless, the morphometric values were not statistically different between genders in either the pre-operative or post-operative periods. In contrast to mandibular measurements, the lengths of the joint elements on the temporal bone such as the articular fossa and articular height increased two years after orthodontic surgery, while the anteroposterior width decreased.[16]

The elements composing the TMJ have been assumed to play a significant role in joint functions. In contrast, a micro-tomography study using 16 fresh cadaver mandibles pointed out that the cartilage had no effect on either the joint's morphological functions or the trabecular microstructure of the mandible's condyle.[2] Furthermore, Ma et al.[17] reported that the articular disc was not affected by activator therapy.

This study's main purpose was to evaluate the bony structures of the TMJ. However, the TMJ has a complex

structure that consists of bone and soft tissue. Studies have reported the soft tissue properties of the TMJ using MRI;[18,19] these studies support our results to understand the anatomy of the TMJ in detail. Also, Safi et al.[20] retrospectively evaluated the bilateral condylar volume of 350 patients using cone-beam CT. According to their results, women had a smaller mandibular condyle than men, and age had no effect on the condylar volume. In contrast, our results demonstrate that aging correlates with posterior and superior articular spaces and condyle diameter.

Another morphological study of the TMJ that included CT images of 60 patients reported that the anterior articular distance was not statistically different between the right and left sides, while our results demonstrated significant differences for the anterior articular space.[21] The selection criteria were not well explained in that study, so their results may not be recognized as the morphometric values of the TMJ, because several studies have reported differences between healthy and pathological joints.

Detailed anatomical knowledge of the TMJ including bone and soft tissues has crucial significance to the development of new surgical techniques for the treatment of joint disorders. Recent studies using flap implantation to mandibular reconstruction had successful results.[22–24] Furthermore, facial asymmetry had a direct effect on TMJ morphometry.[25] Therefore, it is very important for neurosurgeons to have knowledge about the normal morphometry of TMJ.

Technology provides great opportunities in medical science. Researchers can complete pre-operative simulations using virtual-reality options. Detailed remodeling of the TMJ has also been studied using three-dimensional reconstructed radiological images.[26]

Morphometric studies focused on the comparing the pathological conditions with healthy joints for detecting morphometric changes.[27,28] Our study had a larger patient cohort than previous studies, as we included healthy people to evaluate the anatomical properties of the TMJ, while other studies compared pre- and post-operative changes in patients suffering from specific pathologies. Bony structures can be easily detected and reconstructed three-dimensionally on CT images, so our study provided detailed bony measurements of the TMJs. Furthermore, technology improvements have greatly contributed to medical education.[29] Since this is an incontrovertible contribution of technology, we used virtual reality images that were directly obtained from real patients' CT images. We completed our study using such images with a 1:1 ratio with real patients.

Patients do not always remain at the same angle during the CT scans. Therefore, the orientation function of radiological imaging software may not provide the same positions among all patients, and morphometric measurements may not be possible according to standard points. However, the Frankfurt horizontal plane is drawn according to bony landmarks, the inferior border of the orbit, and the superior border of the external acoustic meatus, which can be easily identified on the CT images. Hence, this plane helps researchers complete their morphometric studies without considering the patients' resting angles during radiologic scans. We performed our study using the Frankfurt horizontal plane, thus avoided subjectivity and made objective measurements using three-dimensional images. The angle between the Frankfurt horizontal plane and the posterior border of the mandibular condyle was evaluated for the first time in the present study. We suggest that the results of this study can make an important clinical contribution to the management of TMJ disorders.

Recent studies have mostly focused on monitoring the effects of surgeries on the TMJ. These studies compared the morphometric properties and alterations to this joint before and after surgeries on patients suffering from TMJ disorders. However, the present study was completed on patients who did not suffer from any TMJ disorder. Since we evaluated healthy joints, our results may be beneficial to create a more accurate differential diagnosis list.

The exact etiology of TMJ disorders remains unclear. Our results have shown that women had smaller articular spaces than men; this result may be an important reason for the high prevalence of TMJ disorders in women. Evaluating morphometric properties of TMJ of healthy individuals has some advantages. Knowing the morphometric properties of a healthy TMJ could be beneficial during diagnosis and for selecting an appropriate surgical method for treatment of TMJ disorders. Our results also provide the anatomical properties of the TMJ which will be useful for designing the appropriate prosthesis for condylectomy patients. Thus, life quality of these patients can be increased in the post-surgical period.

Conclusion

Knowing the range of the anatomical values that belong to the TMJ is crucial for patients' post-surgery life quality. Although the morphometry of the TMJ was widely studied, the present study was the first report that evaluated the morphometry of the healthy TMJ using three-dimensional holographic images obtained from CT

images of real patients. Our results will be beneficial for diagnosis and comparison between different races and contribute the literature for TMJ morphometry. Development of the technology provides us to investigate the morphometric values in more detail; thus, we completed our morphometric evaluations on three-dimensional models and between precise selected points. The main limitation of the present study was the lack of cadaveric measurements due to the insufficient number of specimens in our department. The other limitation was the lack of participant's information such as height, body weight, and body mass index, because the study was retrospective.

Acknowledgement

The authors acknowledge the support of Balıkesir University Scientific Research Foundation (Project No: 2018/047). There is no conflict of interest.

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Conflict of interest statement: No conflicts declared.

anatomy
www.anatomy.org.tr
An International Journal of Experimental and Clinical Anatomy

Original Article

www.anatomy.org.tr
Received: March 9, 2019; Accepted: March 28, 2019
doi:10.2399/ana.19.030

Variation of the brachial plexus roots in the interscalene groove: relevance in interscalene blocks

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Abstract

Objectives: The interscalene block is utilized for regional anesthesia of the upper limb, targeting the roots and trunks of the brachial plexus in the interscalene groove. The prevalence of variation, which may affect the success of this block, has not been documented in detail with respect to side and sex, nor has a classification system been proposed.

Methods: Seventy-nine embalmed bodies were dissected bilaterally. The position of the roots and the subclavian artery relative to the anterior scalene muscle was documented and variations were classified according to prevalence. Differences in the prevalence of variation between left and right sides and between males and females were investigated.

Results: The standard position of the nerves and subclavian artery in the interscalene groove (Type 1) was present in 31.6%. Variant positions included the following passing through the belly of the anterior scalene: C5 and C6 roots (Type 2) (46.8%), C5 root (Type 3) (15.2%), C5 and C6 roots as well as the subclavian artery (Type 4) (3.8%), and lastly, C5, C6 and C7 roots (Type 5) (2.5%). Variant anatomy was statistically more prevalent in females on the right side only.

Conclusion: Variant locations of the roots and subclavian artery external to the interscalene groove were common, suggesting that ultrasound should be used to visualize variations prior to performing interscalene blocks. A classification type of variant positions has been developed for standardization.

Keywords: anatomical variation; anterior scalene muscle; brachial plexus; brachial plexus roots; interscalene block

Anatomy 2019;13(1):40–48

Introduction

The roots and trunks of the brachial plexus can be anesthetized by the interscalene block, the most commonly used brachial plexus block for procedures on the shoulder and arm.[1,2] Interscalene groove, the space between the anterior and middle scalene muscles, is the landmark for this block. The roots and trunks of the brachial plexus along with the subclavian artery are located in the interscalene groove between these two muscle.[1]

Complications of the interscalene block include temporary blockade of the recurrent laryngeal nerve, stellate ganglion and phrenic nerve, occurring from the spread of the injected anesthetic, resulting in hoarseness, Horner's syndrome and hemidiaphragmatic paresis, respectively.[3,4]

Although the roots and trunks of the brachial plexus are described as being located in the interscalene groove in anatomical textbooks,[1] several authors have reported variant positions of these nerves in relation to the scalene muscles.[5–11] In a dissection study, Harry et al.[7] observed the standard position of the roots in the interscalene groove in only 35% of individuals, of which most were bilateral, suggesting that variation is common. Variant patterns may consist of the C5 and C6 roots passing anterior to the scalene muscle or even through the muscle belly, either individually or coursing together.[6–10] The subclavian artery has also been observed passing anterior to the muscle[12] or through the fibers of the belly,[7] instead of being deep to it.

As the interscalene block is performed in the interscalene groove, the effectiveness of this block may be dimin-

ished in individuals with some or all of the roots or trunks of the brachial plexus located outside this groove.[13] Variations in the position of the roots, trunks and subclavian vessels in relation to the scalene muscles have also been implicated as a cause of thoracic outlet syndrome (TOS).[10,14]

There are few published studies reporting the prevalence of variation in the position of the roots relative to the scalene muscles,[6,7,9,10] and there is little information available about whether differences exist between left and right sides, or between males and females. To the best of our knowledge, there are no studies reported from Africa, nor has a classification system been proposed for the variant positions. The prevalence of variations, as well as the positions most likely to be encountered, are relevant to emergency physicians and anesthetists performing interscalene blocks, as the lack of knowledge may explain a reduced rate of success of the block in some patients.

The aim of the study was to document the prevalence of variation in the position of the roots of the brachial plexus and the subclavian artery relative to the scalene muscles, classify these variations as types, and to determine whether there were any statistically significant differences in prevalence between left and right sides, as well as between males and females.

Materials and Methods

A cross-sectional cadaveric study was undertaken during the years 2011 and 2012 in The University of Cape Town, Cape Town, South Africa. The posterior triangle of the neck was dissected bilaterally in 80 embalmed bodies, of whom 36 were female and 44 were male. The bodies were dissected by second- and third-year medical students, with the principal investigator completing exposure of the relevant structures. The neck was dissected to expose the posterior triangle. A midline skin incision was made, followed by lateral reflection of the skin, superficial fascia and platysma muscle, exposing the sternocleidomastoid muscle. The sternocleidomastoid and omohyoid muscles were reflected and the clavicle was disarticulated at the sternoclavicular joint, exposing the scalene muscles, clavicular part of the brachial plexus, and the subclavian vessels. Connective tissue was cleared from the surfaces of the anterior and middle scalene muscles as well as from the roots, trunks and divisions of the brachial plexus, and the subclavian artery and vein. The position of the roots and subclavian artery relative to the anterior scalene muscle was documented, and any variations in position were noted and photographed. The prevalence of variation was determined and expressed as percentages. The number of sides displaying variation was compared by means of a Fisher's exact test using the Statistical Package for Social Sciences (SPSS for Windows, version 25.0, IBM Corporation, Armonk, NY, USA) to determine whether there were any statistically significant differences between left and right sides, as well as between males and females. Statistical significance level was accepted as $p<0.05$.

A classification system was developed by allocating Type 1 as the standard position. Variant positions were assigned as subsequent types according to their prevalence.

Individuals displaying pathology or signs of surgical intervention in the posterior triangle of the neck were excluded from the analysis. The position of the phrenic nerve relative to the anterior scalene muscle and the roots of the brachial plexus could not be determined in this sample due to previous dissection by medical students.

Consent was obtained from the body donors prior to donation for teaching and research purposes; therefore, it was not necessary to seek ethical approval from the Human Research Ethics Committee of our institution. Permission was obtained from the Department of Health, Western Cape for the indigent individuals. The study was conducted in accordance with the Declaration of Helsinki (1964).

Results

Two right sides from male cadavers were excluded, resulting in a final sample of 158 sides, comprised of 44 left sides and 42 right sides from male cadavers, and 36 left sides and 36 right sides from female cadavers.

The standard location of the roots and trunks of the brachial plexus in the interscalene groove between the anterior and middle scalene muscles was observed in 50 out of the 158 sides (31.6%) (**Figure 1**, **Table 1**). Variant positions were observed in 108 sides (68.4%), in order of prevalence as the following structures passing through the belly of the anterior scalene muscle: the C5 and C6 roots in 74 sides (46.8%) (**Figure 2**), the C5 root in 24 sides (15.2%) (**Figure 3**), the C5 and C6 roots and the subclavian artery in six sides (3.8%) (**Figure 4**), and finally, the C5, C6 and C7 roots in four sides (2.5%) (**Figure 5**).

Variant positions were statistically more prevalent on the right sides of females ($p=0.005$), while there were no differences between sexes on the left side ($p=0.328$) (**Table 2**).

Variations were observed bilaterally (on both sides of the same individual) in 45 individuals and unilaterally (on one side only of the same individual) in nine individuals

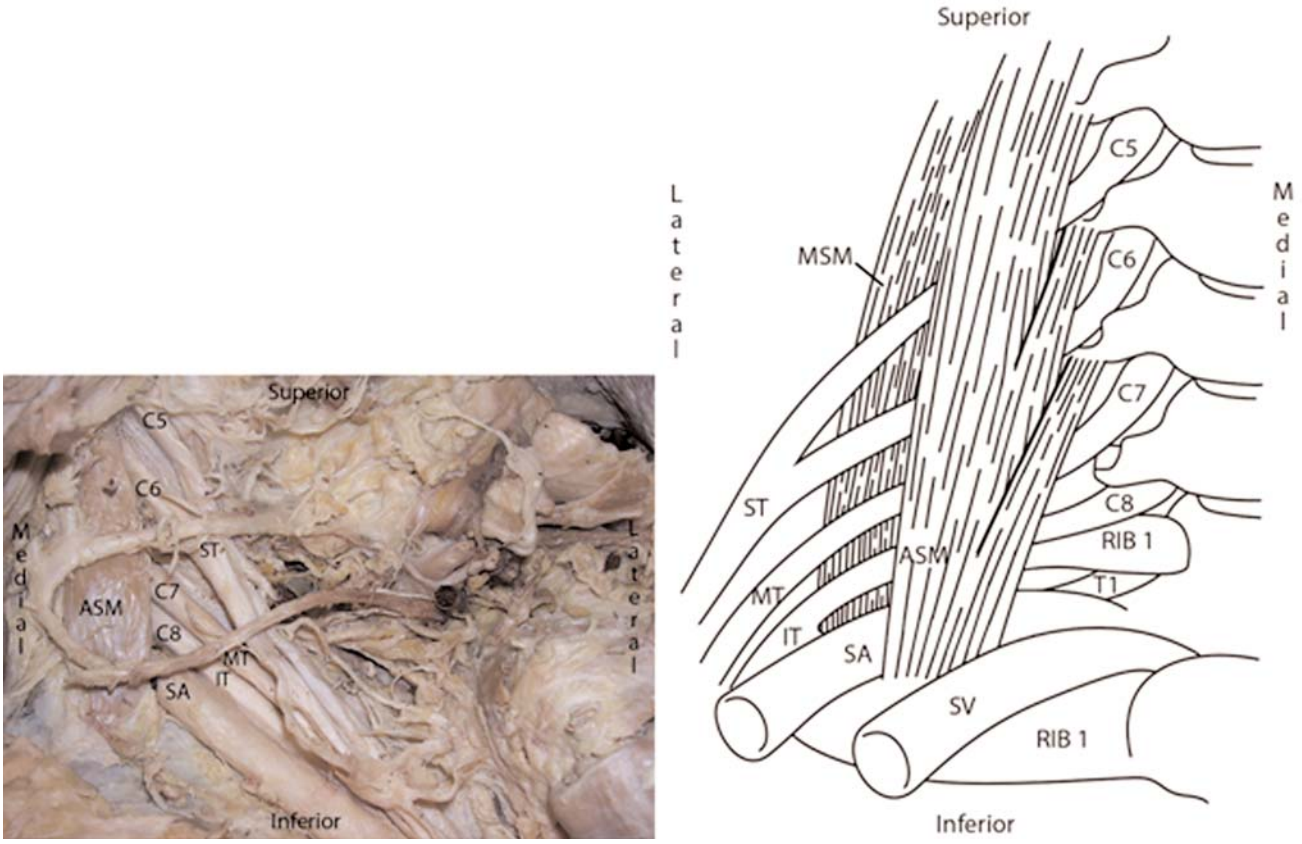


Figure 1. Location of the roots and subclavian artery (**SA**) in the interscalene groove (Type 1), deep to the anterior scalene muscle (**ASM**) in a photograph (**a**) of the left side and in a schematic illustration (**b**) of the right side. **IT:** inferior trunk; **MSM:** middle scalene muscle, **MT:** middle trunk; **ST:** superior trunk; **SV:** subclavian vein. Schematic illustration adapted from Harry et al.[7] [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

(**Table 3**). The standard location of the roots in the interscalene groove was observed bilaterally in 21 individuals, and unilaterally in four.

In individuals who displayed unilateral variations, the standard pattern was significantly more prevalent on the left side (p=0). With respect to structures piercing the anterior scalene the following were observed: C5 and C6 was more prevalent on the right side; C5 was roughly equal in prevalence for both sides; C5, C6 and the subclavian artery were more common on the right side; C5, C6 and C7 were more prevalent on the left side.

According to the prevalence of variant positions observed, the following classification system was developed: Type 1: standard position of all roots and the sub-

Table 1

Prevalence of the variant positions of the roots relative to the anterior scalene muscle as observed with respect to side and sex (n=158).

Position of the roots relative to the anterior scalene muscle	Total n (%)	Number of left sides (%)	Number of right sides (%)	Number of male sides (%)	Number of female sides (%)
Roots located in the interscalene groove (usual position)	50 (31.6)	29 (18.4)	21 (13.3)	37 (23.4)	13 (8.2)
C5 and C5 roots piercing the anterior scalene	74 (46.8)	34 (21.5)	40 (25.3)	32 (20.3)	42 (26.6)
C5 root piercing the anterior scalene	24 (15.2)	12 (7.6)	12 (7.6)	12 (7.6)	12 (7.6)
C5 and C6 roots and subclavian artery piercing the anterior scalene	6 (3.8)	2 (1.3)	4 (2.5)	2 (1.3)	4 (2.5)
C5, C6 and C7 roots piercing the anterior scalene	4 (2.5)	3 (1.9)	1 (0.6)	3 (1.9)	1 (0.6)

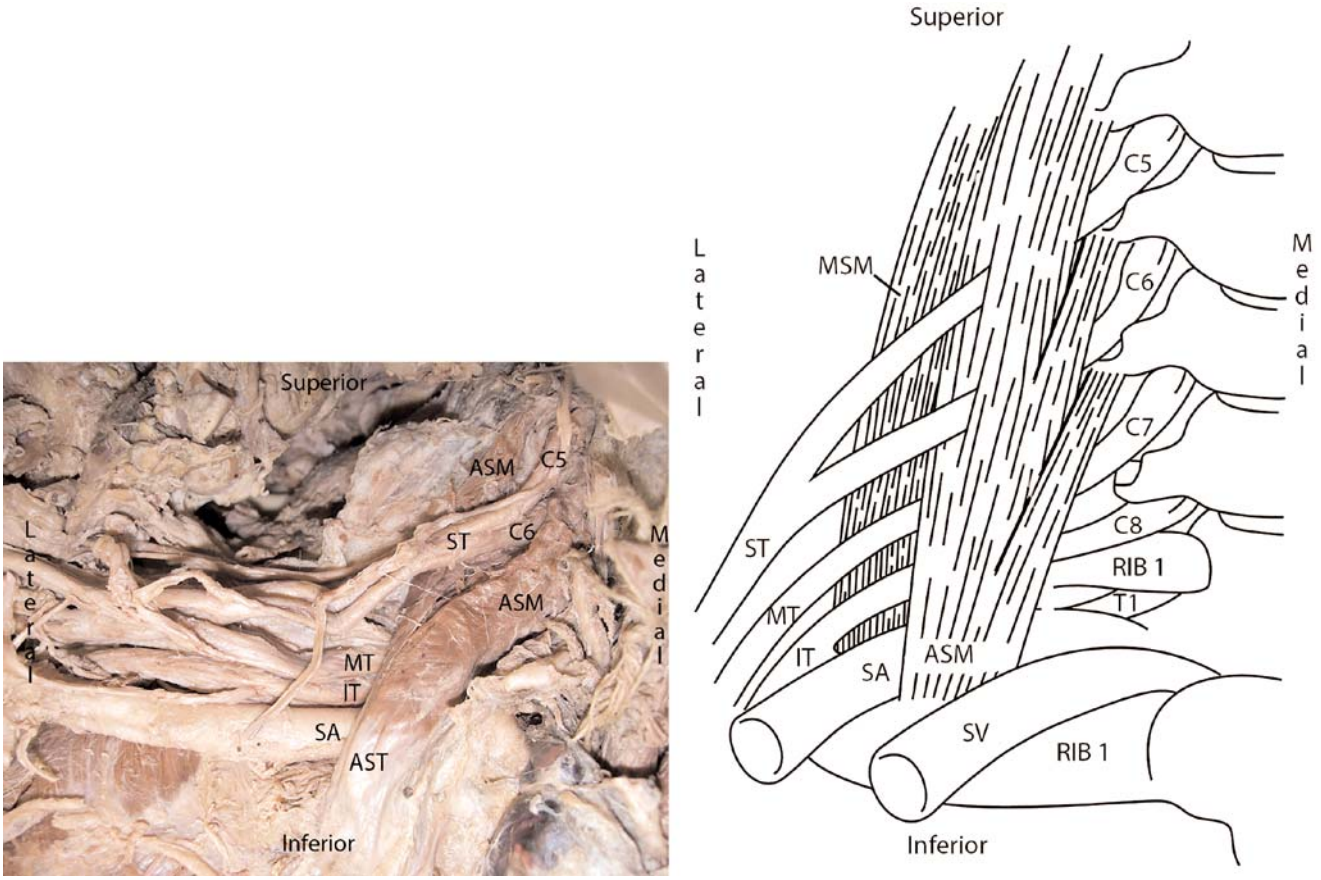


Figure 2. C5 and C6 roots passing through the belly of the anterior scalene muscle (**ASM**) (Type 2) as observed on the right side during dissection (**a**) and represented in a schematic illustration (**b**). **AST:** anterior scalene tendon; **MSM:** middle scalene muscle. **MT:** middle trunk; **IT:** inferior trunk; **SA:** subclavian artery; **ST:** superior trunk; **SV:** subclavian vein. Schematic illustration adapted from Harry et al.[7] [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

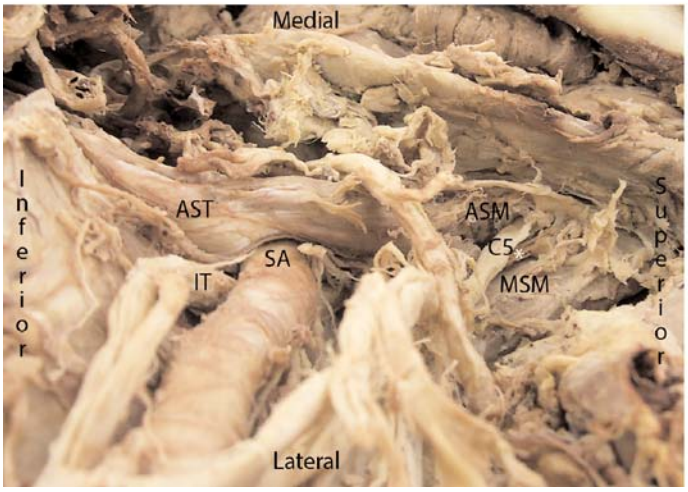
clavian artery located posterior to the anterior scalene muscle; Type 2: C5 and C6 roots passing through the anterior scalene muscle; Type 3: C5 root passing through the anterior scalene muscle; Type 4: C5 and C6 roots and the subclavian artery passing through the anterior scalene muscle; Type 5: C5, C6 and C7 roots passing through the anterior scalene muscle.

Discussion

Variant positions of the roots of the brachial plexus and the subclavian artery relative to the anterior scalene muscle were present in 68.4%, which was more common than the standard position of these structures in the interscalene groove observed in 31.6%. There are no other African studies with which to compare these results; however, this prevalence is similar to that reported by Harry et al.[7] who observed the standard position in 35% of sides in a study done in the United States. Gutton et al.[6] observed variation in 49% in an ultrasound study that was based in France and Italy. Five different position types were observed in this study.

Statistically significant differences in the prevalence of variation between males and females were found on the right side only. The standard position of the roots in the interscalene groove was more common in males, while C5 and C6 roots piercing the anterior scalene was more prevalent in females. The other variant patterns were present with similar frequencies between males and females. To the best of our knowledge, there are no other studies that compare the prevalence of variation in the position of the roots and the subclavian artery in relation to the anterior scalene muscle between sides and sexes.

Most of the variations from the standard pattern were observed bilaterally (83%), with nine individuals (17%) displaying variation on one side only. This is similar to findings of Harry et al.[7] reporting that 32% of individuals displayed bilateral variation, and only 3% displayed unilateral variations.



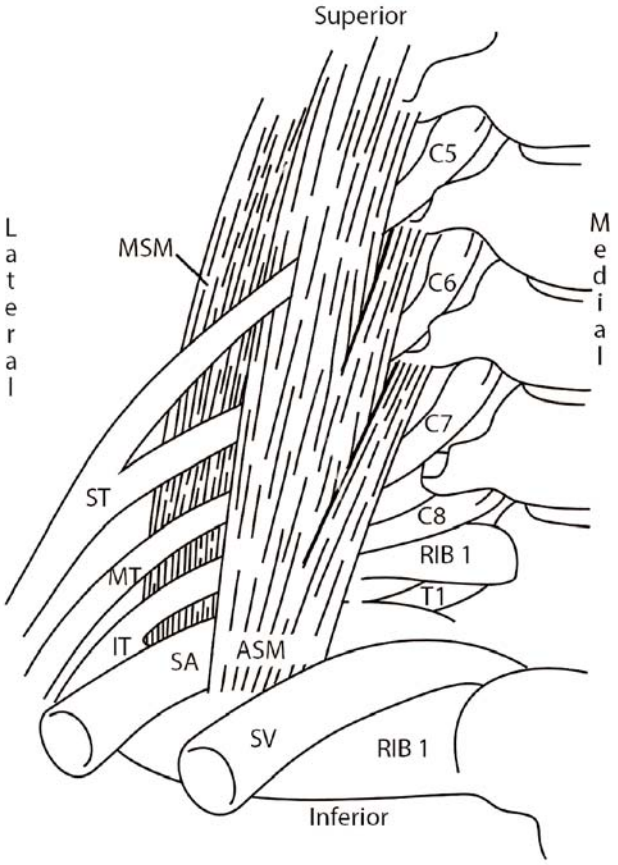


Figure 3. C5 root passing through the belly of the anterior scalene muscle (**ASM**) (Type 3). *Indicates the slip of muscle deep to C5 in a photograph of the dissection (**a**) on the left side; (**b**) is a schematic illustration of the variation on the right side. **AST:** anterior scalene tendon; **IT:** inferior trunk; **MSM:** middle scalene muscle; **MT:** middle trunk; **SA:** subclavian artery; **ST:** superior trunk; **SV:** subclavian vein. Schematic illustration adapted from Harry et al.[7] [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

The C5 and C6 roots piercing the anterior scalene was the most commonly observed position in this study (46.8%), with a higher prevalence than that reported in the literature. Harry et al.[7] observed this position in 15%, Matejcik[9] in 1% of a Slovakian sample. Nair and Sahoo[15] and Chin et al.[5] both described case reports of this position in two patients from India and Canada, respectively. The anterior scalene muscle inserting on either side of the subclavian artery, in combination with the C5 and C6 roots piercing this muscle was observed in 3.8% in this study, higher than the 1% observed by Harry et al.[7] Inuzuka,[12] in a case report, described the anterior scalene muscle situated posterior to the subclavian artery unilaterally in one Japanese individual , while in 1928, Adachi[16] reported the incidence of this variant position as 0.6% in Japanese and 1.2% in European individuals.

The C5 root has been described in the literature as piercing the anterior scalene, or more rarely, passing anterior to this muscle.[6,7,9,10,17] In this study, the C5 root was observed as passing through the muscle in 15.2%, similar to the 13% reported by Harry et al.,[7] but higher than the 3.3% recorded by Kessler and Gray.[17] The C5 root passing anterior to the anterior scalene was not observed in the current study; however, Gutton et al.[6] observed this position in 8%, Kessler and Gray[17] in 6.5%, Harry et al.[7] and Natsis et al.[10] in 3%, Matejcik[9] in 1%. Loukas et al.[8] described this position in a case report of a Caucasian cadaver. Natsis et al.,[10] who studied 93 cadavers in Greece, observed the C5 root passing anterior to the anterior scalene and the C6 root piercing the muscle in one individual. This variation was not observed in the present study.

The C5, C6 and C7 roots pierced the anterior scalene muscle in 2.5% in the present study. To the best of our knowledge, there is no other study that reports this variation. Other positions, such as all of the roots passing anterior to the scalene muscles, have also been reported in case reports.[11] Natsis et al.[10] observed the incidence of the

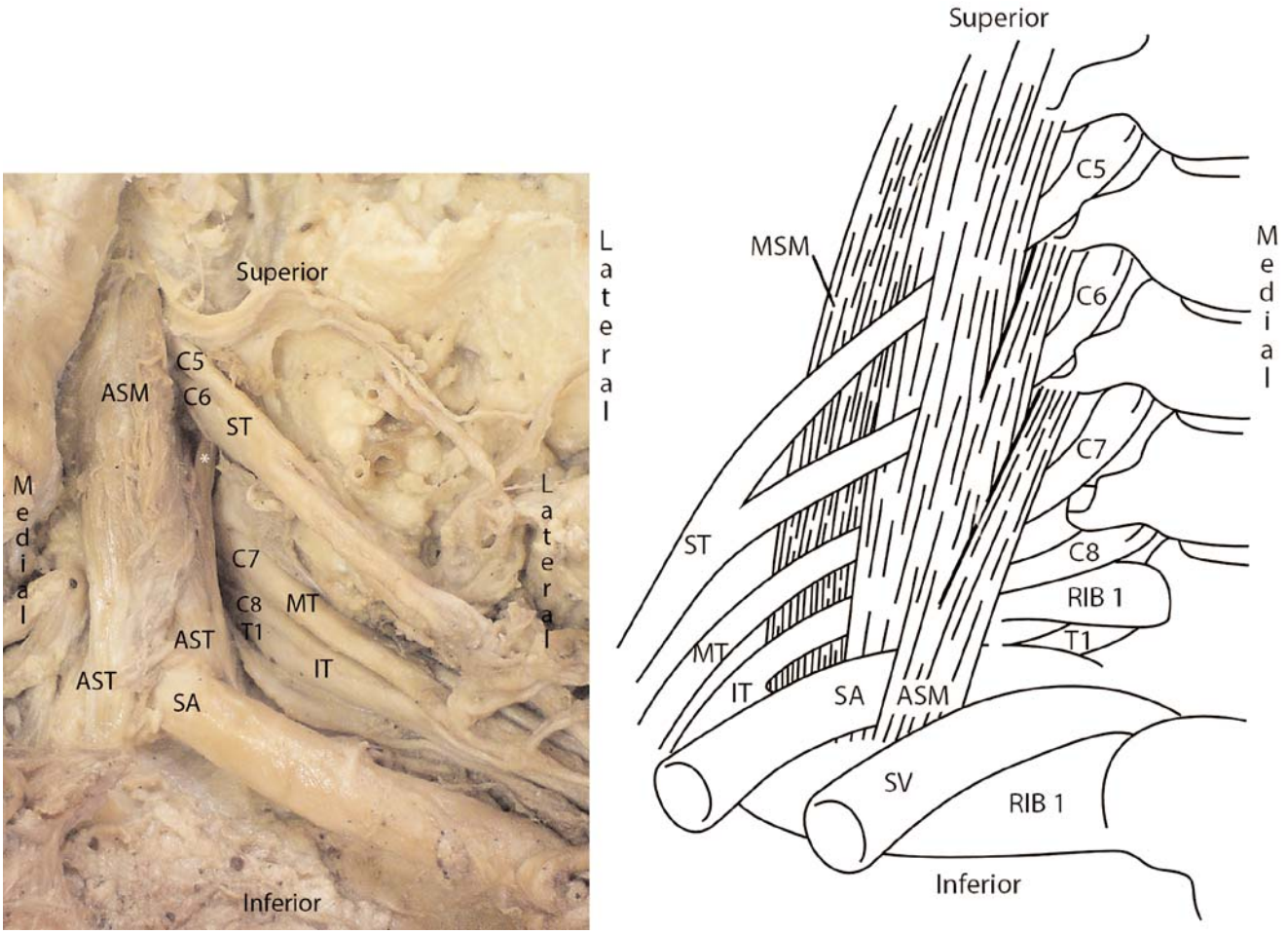


Figure 4. C5 and C6 roots piercing the anterior scalene muscle (**ASM**), while the anterior scalene tendon (**AST**) inserted on either side of the subclavian artery (**SA**) (Type 4). ***Indicates the slip of muscle deep to C5 and C6 on the left side (**a**); (**b**) is a schematic illustration of this variation on the right side. **IT:** inferior trunk; **MSM:** middle scalene muscle; **MT:** middle trunk; **ST:** superior trunk; **SV:** subclavian vein. Schematic illustration adapted from Harry et al.[7] [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

superior trunk passing anterior to, or through the anterior scalene as 2.2% and 6.5%, respectively. In a study from the United States, Leonhard et al.[18] reported that the superior trunk pierced the muscle in 33.3% of individuals, while the middle trunk showed this position in only one side.

Variation in the position of nerves relative to muscles may occur as a result of changes in cell signaling in mesodermal tissue during embryological development. Paraaxial mesoderm differentiates into muscle tissue during the fifth week of development, while growth cones of axons are guided along their path of development by chemicals known as chemo-attractants and chemo-repulsants. Signaling between the growth cones and mesoderm determines the anatomical relationship that will form between the developing nerve and muscle tissue. Changes in the standard chemical signaling may result in variation in the position of nerves relative to adjacent muscles.[19]

The subclavian artery is the continuation of the seventh intersegmental artery of the dorsal aorta, which becomes the dominant artery entering the upper limb bud.[20] Occasionally, other intersegmental arteries may become dominant, such as the sixth, eighth or ninth, resulting in a variant position of the subclavian and axillary artery in relation to the nerves of the brachial plexus.[20] Inuzuka[12] described the subclavian artery lying anterior to the anterior scalene muscle; the artery in this case originated from the eighth intersegmental artery.

There are implications of variations in the position of the roots of the brachial plexus relative to the anterior scalene muscle when performing an interscalene brachial plexus block. In cases where the roots are located outside

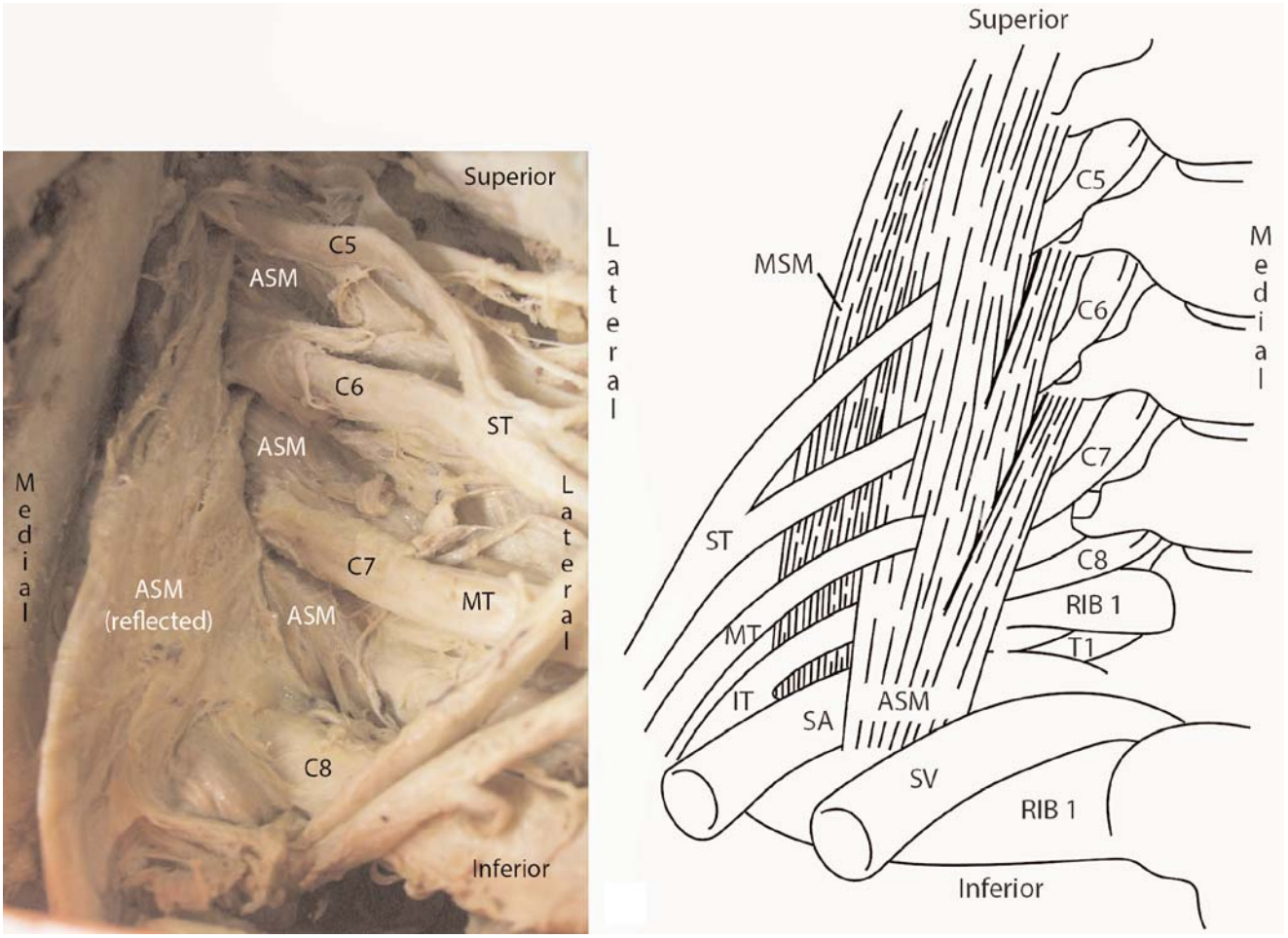


Figure 5. C5, C6 and C7 roots piercing the anterior scalene muscle (**ASM**) (Type 5) as observed on the left side during dissection (**a**); (**b**) is a schematic illustration of this variation on the right side. **IT:** inferior trunk; **MSM:** middle scalene muscle; **MT:** middle trunk; **SA:** subclavian artery; **ST:** superior trunk; **SV:** subclavian vein. Schematic illustration adapted from Harry et al.[7] [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

of the interscalene groove, which is the injection site for this block, the block may not adequately anesthetize the upper limb.[13] These cases include the variant positions observed in this study where the C5, C6 or C7 roots pierced the anterior scalene, instead of passing posterior to the muscle in the interscalene groove. As the standard position of all the roots in the interscalene groove was observed in approximately one-third of this sample,

Table 2

Prevalence of the variant positions of the roots relative to the anterior scalene muscle as observed between the left and right sides of both sexes (n=158).

Position of the roots relative to the anterior scalene muscle	Left sides of males (n=44) (%)	Right sides of males (n=42) (%)	Left sides of females (n = 36) (%)	Right sides of females (n = 36) (%)
Roots located in the interscalene groove (usual position)	20 (45.5)	17 (40.5)	9 (25.0)	4 (11.1)
C5 and C5 roots piercing the anterior scalene	16 (36.4)	16 (38.1)	18 (50.0)	24 (66.7)
C5 root piercing the anterior scalene	5 (11.4)	7 (16.7)	7 (19.4)	5 (13.9)
C5 and C6 roots and subclavian artery piercing the anterior scalene	1 (2.3)	1 (2.4)	1 (2.8)	3 (8.3)
C5, C6 and C7 roots piercing the anterior scalene	2 (4.5)	1 (2.4)	1 (2.8)	0 (8.0)

Table 3
Prevalence of the variant positions of the roots relative to the anterior scalene muscle with regards to bilateral or unilateral presentation in individuals (n=79).

Position of the roots relative to the anterior scalene muscle	Total	Bilateral (no. of individuals)	Unilateral (no. of upper limbs)
Roots located in the interscalene groove (usual position)	50	21	8
C5 and C5 roots piercing the anterior scalene	74	34	6
C5 root piercing the anterior scalene	24	8	8
C5 and C6 roots and subclavian artery piercing the anterior scalene	6	2	2
C5, C6 and C7 roots piercing the anterior scalene	4	1	2

emergency physicians and anesthetists should be aware of the possibility that the interscalene block may not be effective in a considerable percentage of individuals presenting for regional anesthesia of the upper limb. Depending on the area of the upper limb requiring anesthesia, an alternative block, such as the supraclavicular approach may be preferred. However, variations in the position of the roots and trunks in relation to the scalene muscles can be easily identified on ultrasound imaging.[13,21] Ultrasound guided visualization of the roots can be employed, and if variations are identified, the interscalene approach can be utilized with anesthetic injected into adjacent areas in addition to the interscalene groove, ensuring that these variant nerves are blocked and adequate anesthesia is obtained.[2,21] Kessler and Gray,[17] upon detecting variant passage of C5 either anterior to or through the anterior scalene, perform the interscalene block 1 to 2 cm caudal to the cricoid cartilage to ensure adequate blockade.

The passage of the roots of the brachial plexus and the subclavian artery through the anterior scalene muscle may lead to the compression of these structures, resulting in thoracic outlet syndrome (TOS).[10,14,22] Neurogenic TOS is characterized by numbness and tingling of the upper limb, while vascular causes of TOS include symptoms of pallor, coolness, fatigue, muscle cramps and swelling.[22] Muscle weakness may be present in the deltoid, biceps brachii, triceps brachii and rotator cuff muscles as well as the extensors of the forearm.[10] Pain is often described in the neck; however, it may radiate to the rhomboid, suprascapular, trapezius, deltoid and lateral arm areas.[10] In addition, pain in the pectoral region may be experienced as "pseudoangina". Thus, it is important for clinicians to distinguish between TOS and angina.

All but one of the 219 patients with TOS examined by Redman and Robbs[14] had some form of brachial plexus anomaly or variation. Although the majority of these cadavers were postfixed with T2 contributing to the inferior trunk and subsequently prone to being stretched over rib one, some patients had variant positions of the roots in relation to the scalene muscles.[14] Compression of the nerve roots by the anterior scalene may disrupt blood flow, resulting in ischemia of the nerves.[10] Patients may experience symptomatic relief when these muscles are relaxed.[22] Abnormal fibromuscular bands attaching onto the first rib have also been implicated in neurogenic TOS.[10,14,22] Conduction speed tests of the median nerve may assist in diagnosing neurogenic TOS.[14] The passage of the subclavian artery through the anterior scalene may play a role in TOS, although further studies are required.

Although the sample size was small, this study found variant positions to be more prevalent in females on the right side, which may increase the risk of above-mentioned symptoms and complications in this subset. Emergency physicians should be aware of the possibility of asymmetrical variations in patients.

A classification system has been proposed according to the prevalence of variant positions observed in this study. This system may assist anatomists and clinicians with describing variant positions observed during dissections, surgery or medical imaging. As further variant patterns are revealed in future studies, the classification may be updated accordingly.

The origin and position of the phrenic nerve were not able to be determined in this sample. We are currently documenting the phrenic nerve in detail in a separate study, to determine whether variations in its origin and /or course may increase the risk of iatrogenic blockade during interscalene blocks. There was no medical history available for the cadavers, and it was not known whether any of the individuals suffered from TOS during their lifetimes. The study was retrospective and studies assessing interscalene anatomy in patients for whom

interscalene blocks were unsuccessful may reveal more information about which variant positions result in a reduced efficiency of peripheral blocks.

Conclusion

Variation in the position of the roots of the brachial plexus relative to the anterior scalene muscle occurred with a higher prevalence than the standard position in the interscalene groove. Variant patterns included the C5, C6 and or C7 roots piercing the anterior scalene muscle, which have implications for a successful interscalene brachial plexus block. The subclavian artery was also observed passing through the anterior scalene muscle. Patients should be assessed for variant anatomy with ultrasound in cases where interscalene blocks are not effective. Variant positions of the roots and subclavian artery in relation to the scalene muscles may be a contributing factor in neurogenic TOS. A classification of variant positions has been developed to assist anatomists and surgeons with the description of variations.

Acknowledgments

This study was supported by The National Research Foundation (NRF) of South Africa. We would also like to acknowledge here the body donors and their families.

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Conflict of interest statement: No conflicts declared.

 Please cite this article as: Keet K, Louw G. Variation of the brachial plexus roots in the interscalene groove: relevance in interscalene blocks. Anatomy 2019;13(1):40–48.

Teaching Anatomy

www.anatomy.org.tr
Received: July 4, 2018; Accepted: September 8, 2018
doi:10.2399/ana.18.054

anatomy
An International Journal of Experimental and Clinical Anatomy

Cadavers in anatomy classes: opinions of the students of Bursa Uludağ University School of Medicine

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Abstract

Objectives: Informing the medical students about the ethical aspects related to human cadavers has been an important issue that should be taken into consideration by anatomists. The purpose of the study was to evaluate the opinions of the students of the School of Medicine of Bursa Uludağ University, Turkey on the cadavers, prior to the anatomy education, and to determine the changes in attitudes towards the dead human body after basic and compulsory anatomy courses.

Methods: The first-year class students of the School of Medicine of Bursa Uludağ University were included in the study. The questionnaire was applied before and after the education on cadavers in Topographic Anatomy Committee. The on-line survey form was created and the link address was shared with e-mail and social media. Statistical analyses of the obtained data were performed using the SPSS 22.0 program.

Results: The first questionnaire was answered by 297 students and the second questionnaire by 212 students. In the survey applied before the anatomy education in Topographic Anatomy Committee, the percentage of positive opinions (agree and strongly agree) on "Human is a valuable asset. For this reason, the human body must be valued and respected during life and after death" was found to be 83.8% and this percentage increased to 95.3% in the later survey.

Conclusion: Anatomy education can not be effective without cadavers. We believe that the current study will be useful for educators in order to contribute to the medical students' awareness of humanity and privacy.

Keywords: cadaver; ethics; medical students; survey

Anatomy 2019;13(1):49–55

Introduction

The thought of life after death and immortality led to the idea of preserving the human body after death in some societies. In addition, it has been possible to see that many scientists in history have been working on human or animal bodies with the aim of recognizing the human body.[1–3] Andreas Vesalius, one of the important names of anatomy, laid the foundations of medical education today with his cadaver dissections accompanied by his students in public theaters in Padua. The lesson on cadaver with an instructor has been a kind of continuation of modern anatomy education in our era.[4]

Because anatomy is a common language in medicine and clinical practices, anatomy education has been important for all health professionals, especially radiologists and surgeons.[5] It has also been a science based on visually that sometimes requires spatial imagination. Because of its threee-dimensional structure, working with cadavers, especially with the presence of atlas and models, has increased the efficiency of practical courses of anatomy.[6] In this way, it has been aimed to reinforce the theoretical education that has been learned with these training materials.[7,8] The close sense of reality has made the cadaver a unique educational material for medical students. Despite its importance, it is difficult to supply cadavers for educational purposes.[3]

The aim of anatomy education with cadavers has not been only to get to know the human body, but also to show the students that human body is a respected entity

within the frame of ethics.[9] For these reasons, informing the students about ethical behaviors towards the cadaver has been a matter to be taken into consideration before the anatomy classes. Also, the initial contact with cadaver in the anatomy classes of the students can be important in the name of continuation of the medical education.[10]

Therefore, the aim of this study was to evaluate the students' opinions towards cadavers, used as educational material in the courses, prior to the anatomy education and to determine the changes in their attitudes after anatomy classes.

This study will enable educators to have ideas about increasing the effectiveness of anatomy classes with cadavers by taking student opinions prior to and after anatomy education.

Materials and Methods

The study was approved by the Uludağ University Ethics Committee (approval number 2017-13/93). Term I students who just started to Bursa Uludağ University School of Medicine were included in the study. The questionnaire was applied before (September 9th, 2017) and after (February 5th, 2017) the education on cadavers in the Topographic Anatomy Committee starting on January 2nd 2018. At the beginning of the Topographic Anatomy Committee, a panel presentation was made with the students on February 5th by anatomy and deontology professors. This panel was about the human cadavers, donations and the medical ethics. The on-line survey form was created and the link address "https://goo.gl/forms/8ySM8f9fQgDAeNng2" was shared with e-mails and social media. The first questionnaire was answered by 297 students and the second one by 212. Questionnaire consisted of 14 questions with 2 demographic information in Section A, four questions about "Cadaver and Ethics Education" in Section B, and "Opinions about Cadaver" in C Section using a 5-point Likert-type scale. In terms of the transparency of the answers to the questions, no data regarding the personal information of the volunteer was requested. The frequency distributions were analyzed using the Statistical Package for Social Sciences (SPSS for Windows, version 22.0, Chicago, IL, USA). and the answers given to the questionnaires' mean values were compared. In addition, Kruskal-Wallis and Mann Whitney-U analyses were used whether there were any differences before and after education and $p<0.05$ was considered significant in statistical analysis.

Results

Of the 297 students (before the anatomy lessons), female participants' distribution was 49.50% and the male participants' distribution was 50.50%. Of the 212 students (after the anatomy lessons) it was 54.70% and 45.30%, respectively (**Figures 1** and **2**). All students who answered the questionnaire were aged 18–24.

In response to questions about Cadaver Ethics in Section B, for the item "Have you ever lost a relative before" question, the rate of the answers as "Yes" was 69.40% in the first survey and 72.60% for the second survey. The answers as "No" responds were 30.60% and 27.40%, respectively (**Figures 3** and **4**).

For the item "Do you know the difference between dead body and cadaver?" the rate of the answers as "Yes" was 66.30% and "No" was 33.70% in the first survey. In

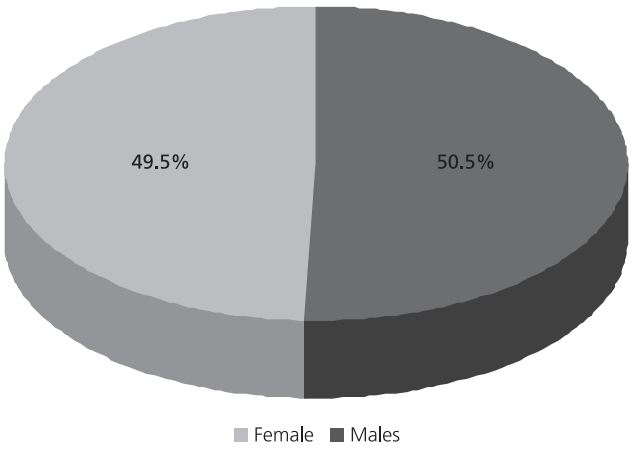

Figure 1. Age distribution of the participants of before the anatomy lessons.

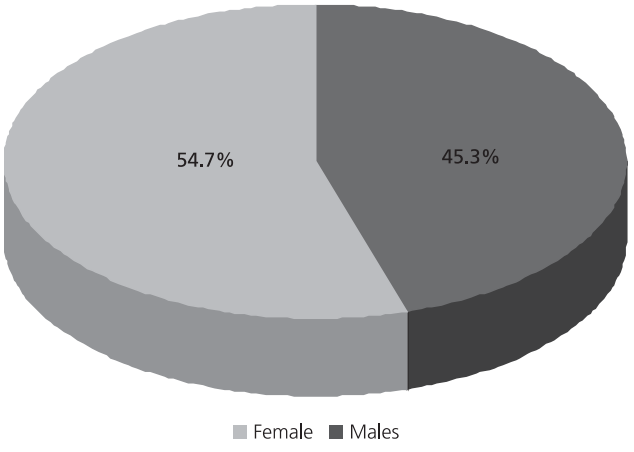

Figure 2. Age distribution of the participants of after the anatomy lessons.

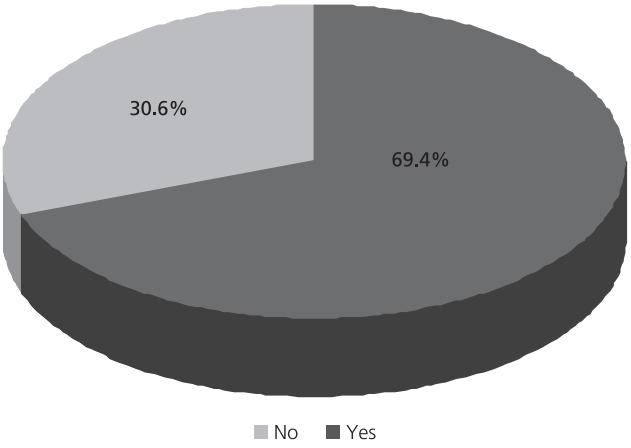

Figure 3. "Have you lost a close relative before?" answers for the first survey.

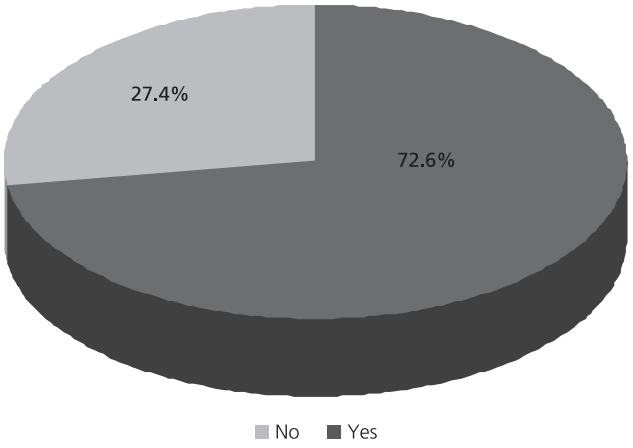

Figure 4. "Have you lost a close relative before?" answers for the second survey.

the second one, the frequency of the answers as "Yes" was 98.10%, "No" answers were 1.90%. (**Figures 5** and **6**).

Of respondents 82.50% answered as "Yes" and 17.50% answered as "No" to the item "Have you seen the cadaver before?" in the first survey. In the second survey, answers as "Yes" were 41% and "No" were 59% (**Figures 7** and **8**).

In response to the item "Is there any effect of having cadaver and cadaver training in Uludağ University School of Medicine in your choice of university?", the rate of the answer of "Yes" was 32.30% and "No" was 67.70% in the first, In the second survey, the rate of the "Yes" answers were 39.60% and the "No" answers were 60.40% (**Figures 9** and **10**).

The mean and standard deviation values, before and after the Committee of the responses obtained using the Likert type scale regarding the opinions about the cadaver in the C section were given in **Table 1** and the frequency distribution percentage values are given in **Table 2**.

Discussion

Despite the developing technology, cadaver has an importance as an indispensable material for anatomy classes for medical students and educators.[11] Particularly, the attitude of the students towards the concept of death and the moment of meeting a dead body are of the factors that can influence their education period.[10,12] It has been a necessity to inform the students about humanity and medical

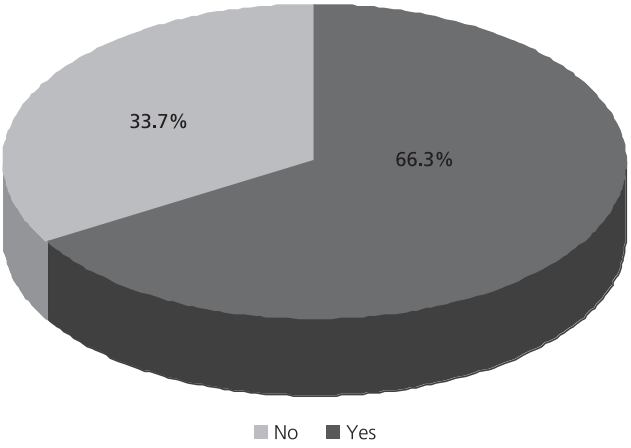

Figure 5. . "Do you know the difference between dead body and cadaver?" answers for the first survey.

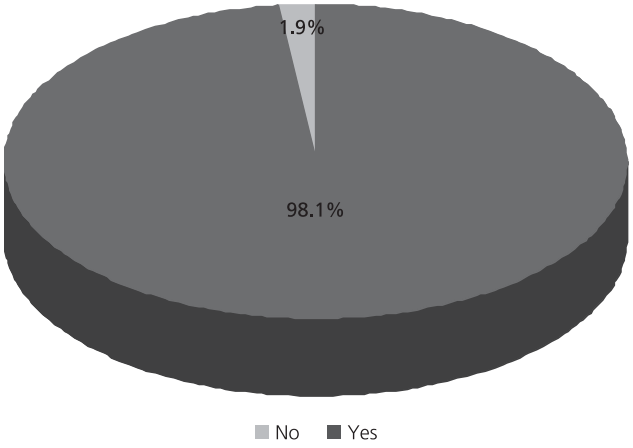

Figure 6. "Do you know the difference between dead body and cadaver?" answers for the second survey.

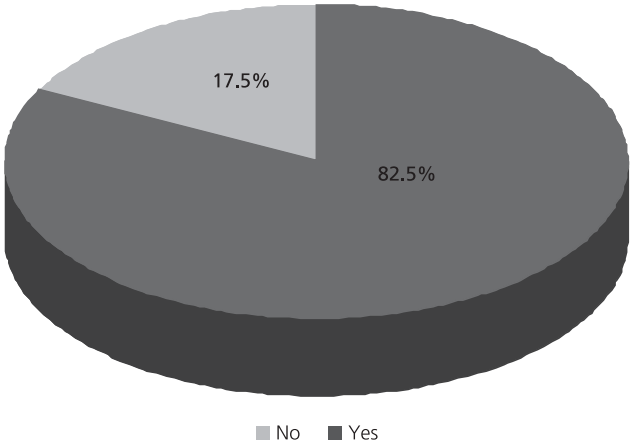

Figure 7. "Have you ever seen a cadaver before ?" answers for the first survey.

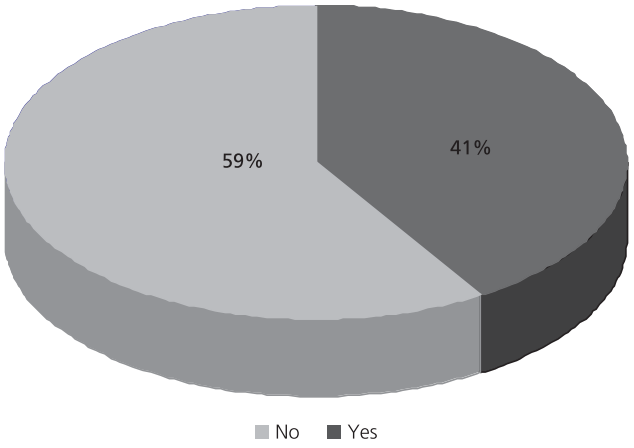

Figure 8. "Have you ever seen a cadaver before?" answers for the second survey.

ethics and to make them feel that they should show ethical behavior in accordance with their awareness during the medical education.[13] With this survey, we have tried to determine the attitudes of the students towards human cadavers and ethical awareness and to examine whether the pre-judgments have changed throughout their education.

For the item that "Human is a valuable asset. For this reason, the human body must be valued and respected during life and after death" while the number of students who answered "Strongly I agree" is 52.2% in the first survey, this percentage reached to 76.6% after the Committee. In the study conducted by Babacan et al. with the students of Uludağ University, the number of respondents who answered positively to the similar question is 88.4%.[14] The rate of positive response is 83.8% in the first survey and 95.2% in the second survey which we have done only with term I students. In another study by Babacan et al. they directed the same question to academicians who are working in different universities in Turkey, they have reported that they received 97.6% positive responses.[15] We believe that ethical education would be more effective in increasing the value of cadavers accompanied by anatomists who accept human dignity in a positive way. It is interesting that the students who attend the classes regularly answered the same question, mean value is 3.85±0.91 and the students who do not attend regularly at the average values is 4.22±0.78

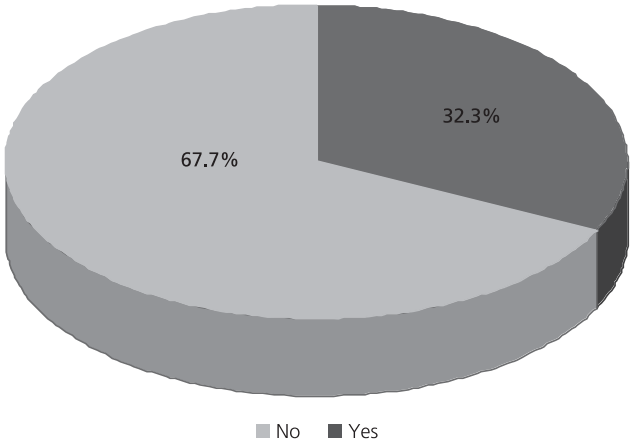

Figure 9. "Is there any effect of having cadaver and cadaver training in Uludağ University School of Medicine in your choice of university?" answers for the first survey.

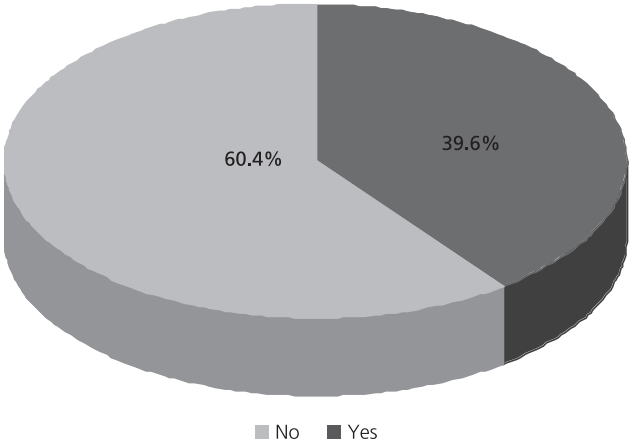

Figure 10. "Is there any effect of having cadaver and cadaver training in Uludağ University School of Medicine in your choice of university?" answers for the second survey.

Table 1
Mean and standard deviation values before and after Committee.

Questions	Before	After	p-value
1. Human is a valuable asset. For this reason, the human body must be valued and respected during life and after death.	4.27±0.95	4.66±0.62	**0.001**
2. The cadaver should be in a more respected position because it contributes to anatomy education.	4.44±0.71	4.63±0.55	**0.001**
3. The point of view towards the cadaver is as sensitive and important as approaching the patient.	4.01±0.95	4.05±0.92	0.650
4. On the right of privacy, covering the face of the cadaver during the dissection is a human delicacy that should not be neglected.	3.84±1.03	4.15±0.88	**0.001**
5. Memories taken from cadavers can be shared in social media	1.68±0.95	1.42±0.77	**0.001**
6. Cadaver dissections can be performed outside of anatomy rooms or surgical units in hospitals (congress center, hotel, etc.).	2.02±0.99	1.89±0.87	0.120
7. It makes me uncomfortable to see the photo or video of my acquaintance, who has donated his body, in social media.	4.35±0.89	4.58±0.73	**0.002**
8. I warn the people who share cadaver images in social media.	3.87±0.93	3.91±0.87	0.595
9. Cadaver dissections should not be performed except in the anatomy laboratories or surgical sciences in hospitals.	3.96±0.98	4.06±0.92	0.266
10. I would prefer to dissect the donated body	3.95±0.85	3.90±0.87	0.515
11. I would prefer to dissect the body belonging unclaimed	2.69±0.90	2.42±0.85	**0.001**
12. Education, studies and researches related to cadaver should be done only in anatomy laboratories.	3.73±0.88	3.62±0.98	0.187
13. The acquisition of the sense of ethics and privacy related to the cadaver is important in terms of medical ethics and patient privacy.	4.37±0.69	4.44±0.65	0.241
14. Lessons made with a cadaver whose integrity is impaired are affecting my education negatively.	3.43±1.06	3.81±1.11	**0.001**

after the anatomy lesson in a different study.[5] Erbay et al. also asked the same question and they reported the average value of the students who responded to the same item is 4.27±1.04.[10] In our study we found the same values but after education this value has increased to 4.66± 0.62 and this result shows us the importance of Committee.

Parallel to the first item; it was anticipated that an increase in the number of positive respondents to "The cadaver should be in a more respected position because it contributes to anatomy education." is an expected result. "The point of view toward the cadaver is as sensitive and attractive as approaching the patient" item's results show that there is no statistically significant difference between before and after Committee. This results bring up to mind that students still do not have a patient's sensitivity to cadaver even they have respect.

"On the right of privacy, covering the face of the cadaver during the dissection is a human delicacy that should not be neglected" it is still a controversial subject and is sometimes criticized by students during the courses. For this item, Erbay et al. 2015 reported that mean value is 3.27±1.19 over 5, Ogenler et al. 2014 reported data as 6.01±3.30 over 10.[9,10] In our study, the values we have determined were 3.84±1.03 before Committee and 4.15±0.88 after education. This results have showed us the importance of the Committee once again.

The sharing of photographs taken with cadaver in social media has been one of the issues that has been still being discussed ethically however seen that such violations continue. The rate of the answers given negatively to the item "Memories taken from cadavers can be shared in social media" before education was 7.1% and after course this value decreased to 4.8%. This results showed that some of the students' ideas have changed about cadaver. It was seen that this value was 1.2% in the study conducted with the academicians.[15] In parallel with this item, it was expected to see the same result as "It makes me uncomfortable to see the photo or video of my acquaintance, who has donated his body, in social media". It was encouraging that the attitudes of the students in warning the photographers are also positive in the pre-training period.

When the answers to the question asked about "Where the cadaveric dissections should be done" were examined, it was seen that the idea of "Dissections could be done not only in the anatomy rooms but also in the surgical units, but that no dissection should be done except these units" were positively at two surveys (77.1% and 79.2%, respectively). Regarding the situation on the cadavers they work on; it was seen that working with orphaned cadavers disturbed the students after committee while cadaver donation did not affect the students in the before Committee surveys. But the number of students who were undecided about this issue was not negligible. The rate of students who thought about the orphaned cadaver was negatively 47.7% and the number of undecided students was 45.7%.

Table 2
Frequency distribution before and after Committee percentage values.

Questions		Strongly Disagree	Disagree	Undecided	Agree	Strongly Agree
1. Human is a valuable asset. For this reason, the human body must be valued and respected during life and after death.	Before	2.1%	4.7%	9.4%	31.6%	52.2%
	After	0.5%	0.5%	3.8%	22.6%	72.6%
2. The cadaver should be in a more respected position because it contributes to anatomy education.	Before	1.2%	1.2%	3.2%	41.2%	53.2%
	After	0.1%	0.9%	0.9%	31.6%	66.5%
3. The point of view towards the cadaver is as sensitive and important as approaching the patient.	Before	1.4%	6.7%	16.2%	40.7%	35.0%
	After	0.5%	7.1%	16.0%	39.6%	36.8%
4. On the right of privacy, covering the face of thecadaver during the dissection is a human delicacy that should not be neglected.	Before	2.7%	8.1%	22.2%	36.0%	31.0%
	After	1.5%	4.2%	11.3%	43.4%	39.6%
5. Memories taken from cadavers can be shared in social media.	Before	55.2%	30.0%	7.7%	5.1%	2.0%
	After	68.4%	25.9%	1.9%	2.4%	1.4%
6. Cadaver dissections can be performed outside of anatomy rooms or surgical units in hospitals (congress center, hotel, etc.).	Before	34.3%	39.4%	18.9%	4.4%	3.0%
	After	37.3%	41.5%	17.4%	2.4%	1.4%
7. It makes me uncomfortable to see the photo or video of my acquaintance, who has donated his body, in social media.	Before	1.7%	4.0%	6.1%	33.3%	54.9%
	After	0.9%	1.9%	3.4%	25.9%	67.9%
8. I warn the people who share cadaver images in social media.	Before	1.7%	6.4%	21.9%	43.1%	26.9%
	After	0.9%	5.3%	21.2%	46.7%	25.9%
9. Cadaver dissections should not be performed except in the anatomy laboratories or surgical sciences in hospitals.	Before	3.7%	4.4%	14.8%	45.5%	31.6%
	After	1.9%	4.7%	14.2%	43.4%	35.8%
10. I would prefer to dissect the donated body	Before	0.0%	5.1%	23.6%	42.1%	29.2%
	After	0.5%	5.2%	25.0%	42.0%	27.3%
11. I would prefer to dissect the body belonging unclaimed	Before	11.8%	23.2%	50.8%	11.8%	2.4%
	After	17.0%	30.7%	45.7%	6.1%	0.5%
12. Education, studies and researches related to cadaver should be done only in anatomy laboratories.	Before	1.0%	8.8%	23.2%	49.5%	17.5%
	After	1.9%	12.3%	25.4%	42.0%	18.4%
13. The acquisition of the sense of ethics and privacy related to the cadaver is important in terms of medical ethics and patient privacy.	Before	0.7%	1.7%	3.4%	47.7%	46.5%
	After	0.9%	0.5%	1.9%	46.2%	50.5%
14. Lessons made with a cadaver whose integrity is impaired are affecting my education negatively.	Before	5.4%	11.8%	32.7%	34.3%	15.8%
	After	4.2%	9.0%	20.7%	34.0%	32.1%

In a study conducted with two groups of the second term students of the School of medicine of Ege University. One group was given an education on cadaver, donation, life and death prior to the Anatomy class and another group wasn't given that education. When the situation students were examined at the end of the year, it was seen that the students who took the course were more successful.[16]

In the study based on the opinions of School of Medicine of Bursa Uludağ University students' on cadaver in 2003, 92.2% of the students reported that they could not be educated well on anatomy without cadaver.[17] In the study conducted with the students of the School of Medicine, Akdeniz University, 84.3% of the first term students indicated that cadaver had an important place in anatomy education, also 96.8% of the students of the second term responded positively on the same idea.[18] These results show that the awareness of the students has been increasing in the education process.

Conclusion

Anatomy education not only teaches the morphological aspect of the human body but also contributes to the conception of patient confidentiality and medical ethics through attitude towards cadavers. Ethical education courses can be given to the students to ensure a respectable attitude against the cadaver. Remembering that every person is a respectable being is a rule that should not be forgotten for all of us. With this survey study, we intended to reveal the effects of the concept of ethics on medical students. Furthermore, this study also investigates the demographic information, especially the gender factor, which has an effect on attitudes and behaviors using the statistical analyzes.

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Conflict of interest statement: No conflicts declared.

 Please cite this article as: Tuncel Çini N, Babacan S, Kafa İM, Erer Kafa S. Cadavers in anatomy classes: opinions of the students of Bursa Uludağ University School of Medicine. Anatomy 2019;13(1):49–55.

anatomy
www.anatomy.org.tr
An International Journal of Experimental and Clinical Anatomy

Teaching Anatomy

www.anatomy.org.tr
Received: January 8, 2019; Accepted: February 15, 2019
doi:10.2399/ana.19.016

Impact of a gynecologic oncology cadaveric dissection course for surgical training

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Abstract

Objectives: The aim of this study was to measure the educational efficacy of a gynecologic oncology cadaveric dissection course on fellows and specialists.

Methods: After the radical and reconstructive vulvar and abdominal gynecologic cancer surgery cadaveric course, a post-course survey was applied to evaluate the improvement in topographic surgical anatomy and the effect on broadening the surgical experience.

Results: Totally 10 and 16 participants attended to the vulvar cancer surgery cadaveric dissection course and abdominal gynecologic cancer surgery cadaveric dissection course, respectively. All participants stated that they had an improvement in topographic surgical anatomy and they found the cadaveric workshop beneficial to broaden the surgical experience. All participants suggested this kind of courses to learn the proper techniques of a surgical procedure in a comfortable and non-stressful setting and improve the surgical skills for rare and complicated surgeries.

Conclusion: Cadaveric dissection courses in the field of gynecologic oncology tailor the surgical anatomy education and improve the training.

Keywords: anatomy; cadaveric dissection; education; gynecologic oncology; surgery

Anatomy 2019;13(1):56–60

Introduction

There is a trend shifting towards problem-based learning in surgical anatomy education. Facing with difficult cases increased the need for a detailed knowledge of anatomy, and many residents and junior post-graduate surgeons know little about the relevant anatomical planes of the procedure they perform.[1] Many post-graduate surgical anatomy courses which are organized nowadays brought the solution to this problem.

Cadaveric dissection constitutes the cornerstone of anatomy education since centuries; however, integration of 3D printed models and computer-based virtual and augmented reality programs improved the general teaching and learning utilities which lead to a more structured design of education.[2,3] Despite the improvements in software programs and models, cadaveric dissection provides a different point of view especially for surgical education and training.[4]

Researchers in the field of anatomy investigated the role of many teaching tools like software, advanced imaging or multimedia-based programs, 3D printed material; however, it is widely accepted that there is no one particular way of teaching anatomy.[5] Combination of traditional and novel innovative methods provides an excellent way of teaching and learning anatomy; nonetheless, the group you intend to teach is the major determinant for the way you need to select during anatomy education.[6] In that point, the educational activities differ due to the target group. Surgeons need to learn the 3D oriented anatomy and close anatomical landmarks of the field they operate, and management of potential complications, additionally they need to practice to gain skills and confidence. On the basis of these needs, the cadaveric dissection studies give them

many vulnerable options. This study evaluated the educational results of a 'Radical and reconstructive vulvar and abdominal surgery cadaveric course' on learning anatomy and gaining surgical experience.

Materials and Methods

Second annual cadaveric course on radical surgical procedures in gynecologic oncology was held in September 2018 at Department of Anatomy, Faculty of Medicine in Bahçeşehir University. The venue was in Istanbul, an ancient city with lots of historical and modern sightseeing places.

The course was separated into two workshops; Radical and Reconstructive Vulvar Cancer Surgery Workshop on August 31, 2018 and Radical and Reconstructive Abdominal Gynecologic Cancer Surgery Workshop on September 1-2, 2018. Both were European Society of Gynecological Oncology (ESGO) endorsed meetings. Professor Ate Van der Zee, Professor Christina Fotopoulou (ESGO Council Member) and Dr. Kamil Zalewski (President of European Network of Young Gynae-Oncologists) were the international faculty members, with collaboration of 20 national (Turkish) faculty members. Turkish Society of Obstetrics and Gynecology supervised the courses. Department of Anatomy in Bahçeşehir University supported the workshop and a clinical anatomist - Professor Çağatay Barut - was the faculty member both for theoretical lessons and cadaveric dissection. Ten participants attended to the vulvar cancer workshop in which three totally fresh-frozen cadavers were used; 16 participants attended to the abdominal surgery workshop in which four totally fresh-frozen cadavers were used. A heterogeneous group of gynecologic oncology fellows and specialists from Turkey, Azerbaijan and Europe attended the course.

The vulvar cancer workshop was a one-day program. Theoretical lessons finished in early afternoon and three hours were spent in the anatomy laboratory for cadaveric dissection. Radical vulvectomy and inguinofemoral lymph node dissection with alternative flap reconstructions were performed at the master table; so, all the participants watched the operation live for this rare vulvar cancer surgery by a video recording system. Afterwards, participants performed the dissection and surgical procedures under the supervision of seniors (**Figure 1**).

Radical abdominal surgery workshop consisted of theoretical lessons for the first day and cadaveric dissection for the whole second day. Theoretical lessons covered all the advanced procedures for pelvis and upper abdomen with special sessions targeting ovarian cancer surgery, management of complications and reconstruction techniques. At the end, all the faculty discussed 'what is not written in books, tips and tricks of surgical principles' interactively. Coffee breaks were beneficial for networking and a specially organized dinner was held after the end of first and second days which presented the beauty and history of Bosphorus in a lovely climate.

Second day focused on practical sessions with four fresh frozen cadavers covering all the open surgical procedures that will be applied in open abdomen. Additionally, participants had enough time to make dissections and perform the surgical procedures for tailoring the surgical practice. Anatomy laboratory was well designed to maintain a clean working environment. **Table 1** shows the subjects of cadaveric dissections.

After the end of the course, a questionnaire was replied by all of the participants to measure the efficacy of the course, with the answers 'Yes' or 'No'. The post-

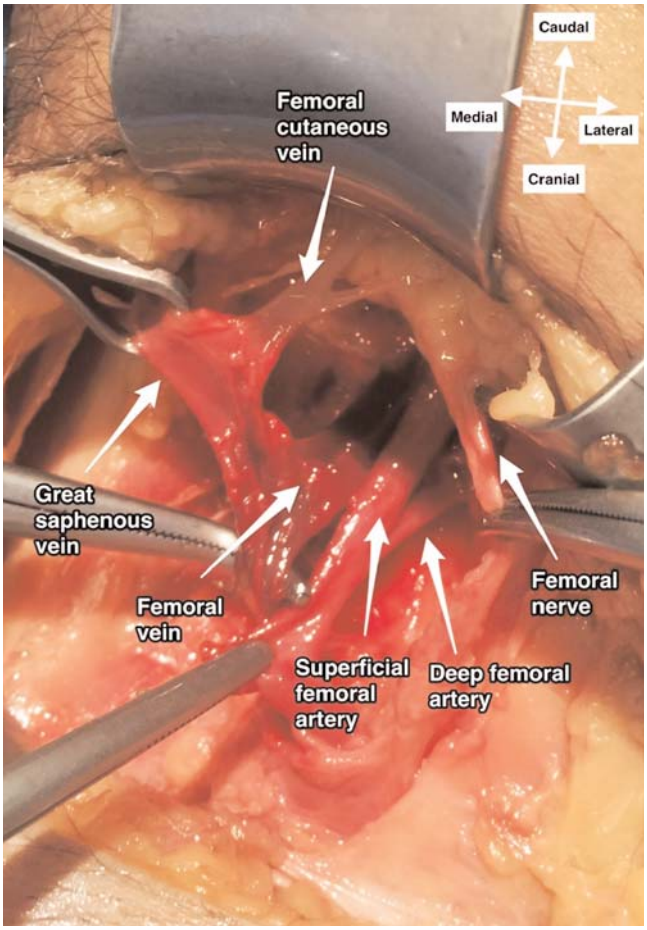

Figure 1. Right inguinofemoral lymph node dissection and femoral vessels on a fresh-frozen cadaver. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

Table 1
Subjects of procedures performed during cadaveric dissections.

Vulvar cancer surgery cadaveric workshop
• Vulvar dissection
• Radical vulvectomy and wide local excision
• Skinning vulvectomy
• Dissection of uretra and anus, excision and repair
• Inguino-femoral dissection, femoral vessels
• Vaginal surgery
• Vulvar/vaginal reconstruction
• Vulvar flaps and step by step flap techniques
Abdominal gynecologic cancer surgery cadaveric workshop (open surgical procedures)
• Anatomy of abdominal wall, different incision types
• Mobilizations of right and left hepatic lobe
• Identification and dissection of hepatoduodenal ligament and portal triad
• Pringle maneuver
• Step by step segmental hepatectomy and pont hepatique
• Cholecystectomy
• Diaphragm stripping and resection
• Repair of diaphragm and chest tube insertion
• Cardiophrenic lymph node excision
• Mobilization of stomach, dissection of subpyloric area and partial gastric resection
• Bursa omentalis, lesser sac and celiac trunk dissection
• Splenectomy and pancreas tail excision
• Omentectomy
• Retroperitoneal right side medial visceral rotation; Kocher and Cattel-Braasch maneuver
• Retroperitoneal left side medial visceral rotation, Mattox maneuver
• Resection/anastomosis of small and large bowel, rectum
• Techniques in opening stoma
• Mobilization of kidney, nephrectomy and adrenal gland resection
• Pelvic lymphadenectomy
• Para-aortic lymphadenectomy
• Radical hysterectomy and nerve sparing radical hysterectomy
• Total mesometrial resection
• Laterally extended endopelvic resection
• Trachelectomy
• Radical oophorectomy en-bloc rectosigmoid resection and pelvic peritonectomy
• Total pelvic exenteration (isolated anterior/posterior)
• Mobilization and injury repair of vena cava, aorta and common iliac vessels
• Partial resection and repair of ureter and bladder
• Radical cystectomy
• Continent urinary pouch and neo-bladder

course survey aimed to measure the improvement in topographic surgical anatomy and surgical experience. Results were analyzed with a computer-based calculation system.

Results

All of the participants, 10 for the Radical and Reconstructive Vulvar Cancer Surgery Workshop and 16 for the Radical and Reconstructive Abdominal Gynecologic Cancer Surgery Workshop, stated that they had an improvement in topographic surgical anatomy (100%) and found the cadaveric workshop beneficial to broaden the surgical experience (100%) (**Table 2**).

Additionally, all of the participants suggested this kind of courses to learn the proper techniques of a surgical procedure in a comfortable and non-stressful setting (100%) and improve the surgical skills for rare and complicated surgeries (100%) (**Table 2**).

Discussion

Irrespective of the medical specialty, anatomy education is the supplementary part of clinical practice. Despite many ways of teaching anatomy, simulation based hands-on models are becoming an increasing demand among the surgical residents and post-graduate surgeons. There are many deficiencies that surgical residents come across during the residency or post-residency. Most importantly, feeling fully prepared to perform a surgical procedure or manage the surgery independently is lacking among the junior surgeons. This deficiency arises from the gap in anatomy education and projecting the anatomic knowledge into surgical practice.[7–9] This study focuses on the effect of gynecologic oncology cadaveric dissection course on surgical anatomy knowledge and practice.

The results of this study revealed that a single cadaveric course improves the topographic surgical anatomy knowledge and broaden the surgical experience. However,

Table 2
Post-course evaluation of educational efficacy.

	Vulvar cancer workshop (n, %)		Abdominal surgery workshop (n, %)	
	Yes	No	Yes	No
Improvement in topographic surgical anatomy	10 (100%)	None	16 (100%)	None
Beneficial to broaden the surgical experience	10 (100%)	None	16 (100%)	None
Suggest this course to learn proper techniques of surgical procedures in a comfortable and non-stressful setting	10 (100%)	None	16 (100%)	None
Suggest this course to improve surgical skills for rare and complicated surgeries	10 (100%)	None	16 (100%)	None

the limited number of the study population and measuring the effectivity subjectively are the major limitations of this study.

The practical way of surgical training is shaped on a learning method in which the skills are gathered from the senior surgeons during the operations performed on patients. The educational curriculum of surgical residency does not involve anatomy rotation and the ethical dilemma of performing a procedure for the first time on a patient reveals restrictions in surgical education. First of all, residents watch the surgical operations many times, afterwards they assist the first surgeon, lately they perform the procedure under mentorship and finally they perform the procedure independently. However, occasionally they do not find the opportunity to perform a surgical procedure independently. The study by Gultekin et al.[10] which analyzed the gynecologic oncology training systems in Europe showed that many of the countries did not have a dedicated gynecologic oncology training program (47%) and 41% of the fellows do not have an access to advanced laparoscopic surgical procedures. This brings the feeling of anxiety during the junior post-graduate period while operating. Moreover, many of the residents do not observe management of complications during the surgery, by the way they will be unexperienced to manage a difficult case or complication during the surgery.

Post-graduate courses are held with the aim of improving the surgical anatomy knowledge and surgical skills. Nevertheless, the most beneficial courses which contribute to the surgical education are hands-on practical activities. Cadaveric dissection courses are the practical way of understanding the anatomy and surgical planes that the participants watch the anatomical dissection of the mentor and they find the chance to self-practice and explore. On the other hand, the difficulties to maintain a cadaver, high-costs and limited number of participants per cadaver are the major drawbacks of cadaveric dissection courses.

Barton et al.[11] stated that there was a significant improvement in surgical anatomy knowledge of pelvis and upper abdomen among the gynecologic oncology fellows after the cadaveric workshop of surgical procedures in gynecologic oncology and all of the participants found that the course was valuable and recommendable. After the anatomy of complications workshop to improve the training of gynecologic oncology fellows, Hammond et al.[12] reported that cadaveric dissection courses provided practice of less common surgical procedures and assisted to learn how to manage complications. Additionally, Hammond et al.[13] performed a similar study to shape an educational program for obstetricians and gynecologists. They designed a course based on interactive surgical anatomy lectures, practical surgery videos, cadaveric dissection and specific learning tasks, and aimed to teach how to deal with unexpected injuries and complications via cadaveric dissection. This combination of observational and practical learning improved the knowledge, confidence, competence and performance. Heisler[14] emphasized the point of gross anatomy education at the laboratory which maintains discovering the anatomic landmarks and their relationships, developing tissue planes, good handling of instruments and self-dissection under mentorship. Despite the lacking objective criteria to evaluate the clinical application of gross anatomy education, there are many evidences that a structured anatomy education during junior residency or post-graduate period will improve the anatomical competence and surgical capability.[15,16]

There may be an option to mimic some surgical procedures where a fully-equipped material defining nearby anatomical landmarks is not needed, like performing a loop electrosurgical excision procedure.[17] On the other hand, gynecologic oncology practice is related with performing major surgical procedures on abdominal visceral organs and aorta or inferior vena cava or branches and tributaries of them. Based on this reality, simulating operations like retroperitoneal lymphadenectomy, splenectomy, bowel or liver mobilization, or resection after developing the accurate surgical planes is not easily applicable on models or will not be highly-effective, because these procedures need the three-dimensional view of relevant anatomic structures and dissection of surgical planes. Procedure-based learning provides many gains for the residents and fellows, therefore cadaveric dissections will yield an important area of self-practice for particular surgical procedures.[18] From a different point of view, simulation models or animal models will generate an area of practice for some abdominal procedures like ureteric or vascular repair or bowel anastomosis.[12] Thus, the target population, aim of the course and selected procedures are the key elements to determine the style of teaching. This study was based on a cadaveric model to teach the vulvar and abdominal surgical procedures after dissection of all relevant surgical spaces and planes.

Cadaveric dissection courses may also tailor the surgical practice due to the needs of participants and may focus on a specific issue by performing proper dissections. Cadaveric dissection improves the basic surgical skills and confidence; yet, transfer of skills from the laboratory to the operation room is questionable. It is not easy to evaluate the gains which were reflected to the operation room while performing a procedure independently. Considering

this, the degree and level of the skills and abilities transferred to the operation room are subjective and not totally measurable.[19,20] However, it is obvious that cadaveric dissection courses improve the topographic surgical anatomy knowledge and dissection techniques used in surgery to develop proper tissue planes, which is the main result of this study.

One important issue as the limitation of cadaveric courses is that, since a single cadaver is shared by three or four participants, generally none of the participants complete all the steps of a single surgical procedure individually. However, all of the participants observe the master table cadaveric dissection, perform dissections under mentorship and apply the procedures by sharing the steps.

Conclusion

Cadaveric dissections improve the anatomical knowledge and surgical experience. However, there is a need for further investigations to demonstrate the objective efficacy of these courses.

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Conflict of interest statement: No conflicts declared.

 Please cite this article as: Selçuk İ, Barut Ç, Çalışkan E. Impact of a gynecologic oncology cadaveric dissection course for surgical training. Anatomy 2019;13(1):56–60.

Review

www.anatomy.org.tr
Received: September 6, 2018; Accepted: January 28, 2019
doi:10.2399/ana.18.062

anatomy
An International Journal of Experimental and Clinical Anatomy

Sutural bones: a literature review

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Abstract

Using standard search engines PubMed, Scopus, Scielo, Cochrane, Science Direct and Medline, 433 articles were found to evaluate the information about sutural bones. Thirty-six articles that match our stated objectives about sutural bones were analyzed. Data were evaluated in the current medical literature for their historical aspects, anatomical classification, development, clinically significance and their presence in some bone dysplasias. There is still dispute on whether sutural bone development is influenced by genetic or external factors. Sutural bones are a known sporadic occurrence in the human cranium and do not predispose a person to any particular disease. Their presence, however, is commonly used as a useful marker of some underlying genetic disorders.

Keywords: cranium, human, interparietal bone, sutural bone, Wormian bones

Anatomy 2019;13(1):61–65

Introduction

The human cranium is the most studied and documented part of the vertebrate skeleton and also rather important and complex, possibly because of its relationship with the nervous system. It can be described, broadly, as a container for more or less rigid tissues, being the encephalon, organs of vision, smell and inner ear. It also serves to support the external organs of digestive apparatus and the respiratory apparatus and defines the direction of a human's movement.

The human cranium is composed of numerous bones that fuse together after birth in addition to the regular centers of ossification of the cranium. Sutural bones can be isolated or, occasionally, found in cranial sutures and fontanels. They are supernumerary, irregular, accessory and abnormal small bones interposed between cranial bones, most commonly located in the lambdoid suture (**Figure 1**). Sutural bones result from the formation of abnormal ossification centers in the cranium that develops in addition to those seen normally. They are poorly reported although they are quite common. Sutural bones have been documented on most mammals and in hominids, so they are not exclusive to the modern human cranium.

Sutural bones are studied and reported as ethnic variables, being of interest in matters of human anatomy, physical anthropology, forensic medicine and radiology.

Figure 1. Sutural fontanelle bones in lambdoid suture (With kind permission of the Museum Villa Julian Complutense University of Madrid, Spain). [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

The origin of sutural bones remains unclear, but mechanical, pathological and genetic factors have been proposed as the primary causes of their incidence. The main objec-

tive of this article was to review the information about sutural bones in current medical literature.

Materials and Methods

This study was undertaken because many aspects of sutural bones are not yet entirely clear. For the article, the authors proceeded as follows: The criterion for including articles was that they be freely available on the web, and on the other hand the exclusion criteria was that the data can obtained from paid subscription or related. The search engines PubMed, Scopus, Scielo, Cochrane, Science Direct and the Medline were consulted and 433 articles were reviewed thoroughly in the scientific literature in both English and Spanish. The following keywords were used: sutural bones, ossa Suturalia, Wormian bones, bone dysplasia and interparietal (Inca) bone. Subsequently, the authors decided to focus on 36 articles and a inferential statistical analysis was applied to data obtained for sutural bones, their historical aspects, anatomical classification, clinically significant development, and their presence in some bone dysplasias. The period of research articles was from 1965 to 2018.

Results

Sutural Bones: Historical Aspects

Sutural bones appear to be as old as man himself: they have even been observed in Australopithecine cranial fragments from Makapansgat.[1] They were named Wormian bones after the Danish anatomist, Olaus Wormius, a medical doctor at the University of Copenhagen.[2] In 1643, he provided a detailed description in a letter in Latin to his colleague Thomas Bartholin, then in Padua, Italy. After receiving the letter, Bartholin himself decided to call sutural bones "ossa Wormia" (Wormian bones). However, Wormian bones had been described even before Olaus Wormius' time. The first description is attributed to Hippocrates himself and the first diagrams of the cranial structure can be found in Avicenna's Canon.[3] Sutural bones were also mentioned by Paracelsus, who named a bone located in the posterior fontanel the "ossiculum antiepilepticum",[4] and D'Andemach Gonthier who described them in detail.[5,6] One of the first to associate sutural bones with cerebral disorders was Vesalius. In the international anatomical nomenclature, sutural bones are called ossa suturalia and are marked as A02.1.00.043.[7] However, these bones are still denominated "Wormian bones" in most research articles, e.g. in references of this review.

Anatomical Classification

Sutural bones are detached portions of the primary ossification centers of the adjacent membrane bones in the cranium. They articulate with the surrounding bones by sutures with indentations that are more complex on the outer surface of the human cranium than on the inner one.[8,9]

Sutural bones are found in both sexes, as well as on both sides of the cranium. They exhibit different irregular shapes: round, oval, oblong, triangular, quadrilateral and polygonal, and can vary in dimension from under 1 mm to 5×9 cm. The denominations used for sutural bones depend on the site where they actually appear: the names given are generally derivatives of the suture or sutures they are in contact with or the centre of ossification or fontanel where they originate. Although some locations of sutural bones are more common, these bones do not receive particular names in the international anatomical nomenclature, because they vary in number, size, shape and thickness from cranium to cranium. Notwithstanding the above, some authors name the preinterparietal bone or the Inca bone - a triangular sutural bone located at the previous site of the posterior fontanel. It is so called because of its incidence in Inca bones in Peruvian mummies.[10,11]

About half of the sutural bones are located in the lambdoid suture and fontanel (and the masto-occipital suture). The second most common site of occurrence (about 25%) is in the coronal suture.[12,13] The remaining occur in any remaining suture or fontanel.[14] Knowledge of this variation is very important for neurosurgeons, radiologists and anthropologists, among others.

Sutural bones are classified into true and false variants.[6,15] False sutural bones are ossification centers not welded to independent bones, such as occipital or temporal bones. True sutural bones are derived from one or many points of ossification. They consist of all supernumerary developed bony parts in the marginal part of the cranial bones.[15,16]

Depending on their location, sutural bones can be true sutural bones, fontanels or isolated ones.[17–19] True sutural bones may be sagittal bones (between the two parietal bones) or developing in the occipitoparietal sutures (**Figure 2**), fronto-parietal sutures, sphenoparietal sutures, petro-occipital sutures or inter-parietal sutural bones (**Figure 3**). Sutural fontanel bones can be found in almost all normal and abnormal craniums. They can be bregmatic, lambdoid, pteric or orbital.[6,19,20] The isolated sutural bones are those that develop at a distance from the sutures and fontanels. They frequently take up the entire thickness of the cranium, but can also be formed only at the expense of the outer table of the cranium (exocranial); more rarely, they are formed at the expense of the inner table of the cranium (intracranial).

The Development of Sutural Bones

Although the incidence of sutural bones is quiet common,[21] the observational data on them is poorly reported. The cranium of the human embryo begins developing between days 23 and 26 of gestation. It can be divided into the viscerocranium, which is derived from the first three branchial arches and forms the facial skeleton and the neurocranium; this surrounds the brain and develops from the surrounding mesenchyme.[13,22] The human cranial skeleton is composed of an assortment of neural crest- and mesoderm-derived cartilages and bones. The formation of the human cranium is a complex interaction between bony and meningeal elements. Any irregularity in these interactions may result in the incidence of sutural bones.[14] Since sutural bones belong to the neurocranium, they share its embryology. They appear as isolated ectopic islands of intramembraneous ossifications. In the fetus, the diploë is not formed yet, and thus Sutural bones are composed of a single layer of compact bone on the dural side.[12]

Sutural bones are thought to appear in connection with increased dural strain from mechanical force, as well as with increased sutural width. In some cases, sutural bones will appear at the same time as the normal ossification centers; in others, as with some ossification centers, they will appear as a necessity for them arises.[7] External factors, such as artificial cranial manipulations, are thought to play a significant role in the number of sutural bones present an individual cranium.These factors include mechanically induced stress owing to intentional deformation such as that practised in ancient cultures.[21,23] It is controversial whether the development of sutural bones is influenced by genetic or external factors; nevertheless their presence is commonly used as a useful marker of some underlying genetic disorders such as imperfect osteogenesis.[24] Researchers have shown that sutural bones located posteriorly have more of an external factor associated with them than anterior sutural bones.[25] There is no consensus in the literature regarding the degree to which the presence and frequency of sutural bones can be attributed to external or genetic influences.[25,26] Anthropologic research has shown that most sutural bones develop in anteroposterior deformed crania; non-deformed crania were shown to have lower incidence. Circumferentially deformed crania were found to have the lowest incidence of sutural bones.[27]

Clinical Significance

Sutural bones can be found in healthy individuals with an incidence of 8% to 15% of the population. In Bregman`s study, nearly 40% of craniums contain sutural bones in the vicinity of lambdoid sutures.[28] The incidence of sutural bones has also been studied and reported as an ethnic variable: there are differences between various ethnic groups. Chinese individuals exhibit the highest incidence - as high as 80%; the incidence in Indian individuals is 40% in Indian and 10% in Caucasian individuals.[14,22] Their incidence is useful for comparative studies as an anthropological marker or an indicator of population distance. Ethnic variation in sutural bones may suggest possible genetic influences, but environmental influences can

Figure 2. Occipitoparietal sutural bone (With kind permission of the Museum Villa Julian Complutense University of Madrid, Spain). [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

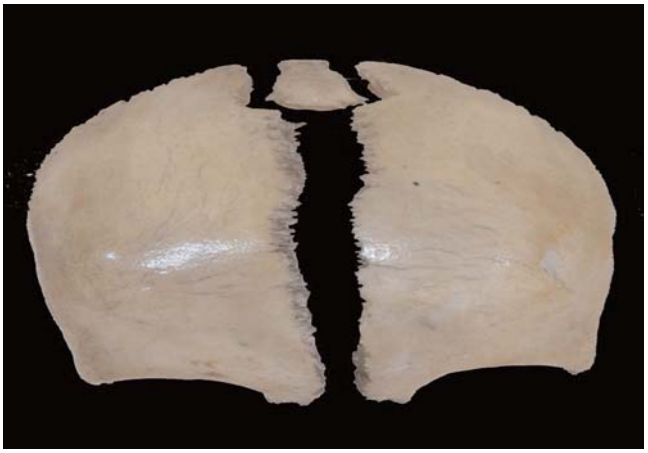

Figure 3. Interparietal sutural bone (With kind permission of the Museum Villa Julian Complutense University of Madrid, Spain). [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

also play a role. Some researchers argue that males are affected more than females, while others have concluded that there is no significant difference between genders.[7,12,24] The bones are generally found unilaterally and are more frequently located on the right side of the cranium.[12,24] The number of sutural bones is generally limited to two or three, but more than a hundred have been found in the cranium of an adult hydrocephalic skeleton.[19] In order to be regarded as pathologically significant, incidences of sutural bones must meet some definite criteria: they must be more than ten in number; the bones must be arranged in a general mosaic-type pattern; and their size must be larger than 6 mm by 4 mm.[29] The mere presence of sutural bones may be misleading: on X-ray images, they may be mistaken for fractures.

Sutural Bones in Some Bone Dysplasias

Sutural bones have been found in a diversity of congenital disorders like hypophosphatasia, imperfect osteogenesis, progeria, craniosynostosis, hypothyroidism (cretinism), cleidocranial dysostosis, rickets, pyknodysostosis (osteopetrosis acro-osteolytica), pachydermoperiostosis and others.[22,30] Among the more common congenital disorders with presence of sutural bones are imperfect osteogenesis and craniosynostosis, which are bone dysplasia. Imperfect osteogenesis commonly occurs owing to genetic mutations. The phenotypic presentation involves extremely breakable bones and deformations in the osseous structuring of the spine and cranium. In addition, imperfect osteogenesis can cause a slow deformation of the bones of the cranium, known as a basilar abnormality. It this condition, the soft bones of the cranium cannot support the normal weight of the cranium and brain.[31] One study has revealed that sutural bones were present in 89% of the patients with imperfect osteogenesis.[29] Although the incidence of sutural bones is not pathognomonic of imperfect osteogenesis, it may warrant further investigation of such disorders involving defective osteogenesis. Sutural bones are also present in cases of craniosynostosis, a bone dysplasia which is a cause of early closure of fontanels in infancy, because of the effects of early fusion of the sutures which, in turn, cause abnormal dural strain in the area of the opposing fontanel. This strain can give rise to the formation of bony islands in the membranous portion of the fontanel. A study of this aspect showed that sutural bones may occupy the anterior fontanel in at least 4% of the cases of isolated sagittal craniosynostosis: this can lead to the appearance of a closed fontanel.[32] It can occur as part of a syndrome or as an isolated defect. Craniosynostosis is called "simple" when only one suture is involved, and "compound" when two or more sutures are involved. If craniosynostosis occurs due to an intrinsic suture defect, it is named "primary craniosynostosis", while craniosynostosis resulting from another medical condition is name "secondary craniosynostosis".[13] Morphological knowledge of sutural bones can be important for the diagnosis of these disorders,[33,34] but sutural bones, in themselves, do not carry a negative prognosis,[35,36] and thus the prognosis will depend on the type and severity of the associated diseases.

Discussion

Sutural bones are formed due to additional ossification centers in or near sutures of the flat bones of the human cranium and are usually regarded as normal variants. They occur most frequently in the lambdoid and occipitomastoid sutures. They are found in both sexes, as well as in both sides of the human cranium. Sutural bones are classified by location in most cases, derivative from the suture next rather than shape, hence the name of this type of bone. When sutural bones occur as a normal variant, they tend to be smaller and less numerous than when they are associated with bone dysplasias.

Anatomical knowledge of sutural bones is clinically important, because their presence, and this refers mainly to bone dysplasia such as craniosynostosis and imperfect osteogenesis, is commonly used as a useful marker of some congenital disorders. Nevertheless, standing alone, their presence is not a clinically significant event for determination of any particular disease. Whether the development of sutural bones is influenced by genetic or external factors or both remains in dispute and it is still unclear why sutural bones are common in certain races.

Conclusion

Although sutural bones are poorly reported, a knowledge of them is of interest and useful to human anatomy, neurosurgery, physical anthropology, forensic medicine, craniofacial surgery, imaging medicine and legal medicine, medical research among others.

Acknowledgments

Authors thank to Professor Mérida Velasco, M.D., director of the Museum Villa Julian Complutense University of Madrid for allowed us to include in this article the images of the human cranium (**Figures 1–3**).

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Conflict of interest statement: No conflicts declared.

 Please cite this article as: Romero-Reverón R, Arráez-Aybar LA. Sutural bones: a literature review. Anatomy 2019;13(1):61–65.

anatomy
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An International Journal of Experimental and Clinical Anatomy

Review

www.anatomy.org.tr
Received: January 9, 2019; Accepted: March 3, 2019
doi:10.2399/ana.19.002

Morphological features of the ventral tegmental area: a brainstem structure related to attention deficit hyperactivity disorder

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Abstract

Attention deficit hyperactivity disorder (ADHD) is the most common behavioral disorder of the childhood and more interest is raised by clinical investigators nowadays. In spite of being the most studied neurobehavioral condition in child psychiatry, the pathophysiology of ADHD remains elusive. The ventral tegmental area (VTA) has been implicated in the etiology of ADHD. This part of the midbrain needs to be investigated further due to its complex cytoarchitecture and connectivity in order to gain insight into the neurobiology of ADHD. In this review, we will first briefly explain the history of the VTA researches and then summarize the anatomical features and connectivity of this region.

Keywords: attention deficit hyperactivity disorder; dopamine; mesocortical pathway; ventral tegmental area

Anatomy 2019;13(1):66–70

Introduction

Nowadays the ventral tegmental area (VTA) has become the focus of a major research area as being involved in the mechanism of attention deficit hyperactivity disorder (ADHD). On the basis of neurophysiological theories, the atypical development of the connectivity or activity among the midbrain, prefrontal cortex (PFC) and ventral striatum play a key role in the etiopathogenesis of ADHD.[1,2] Fronto-subcortical circuits in these structures are rich in catecholamines such as dopamine (DA) and noradrenaline (NA).[3,4] Therefore, DA and NA dysfunctions have long been implicated in the etiology of ADHD.[5,6] It is currently hypothesized that the symptoms of ADHD (inattention, hyperactivity and impulsivity) are due to the dysfunctions of the mesocortical dopaminergic pathway which plays a critical role in the various circuits of the PFC.[7]

VTA is a major structure of the mesocorticolimbic dopamine (DA) pathway that involves in executive function, attention and reward-related cognition.[8,9] In fact, dopaminergic neurons are aggregated in two neighboring midbrain regions; VTA and substantia nigra pars compacta (SNc).[10] However, the firing and projection patterns of neurons located in these two neighboring regions are different from each other.[11,12] These molecular, anatomic and electrophysiologic differences, are specifically important in understanding the intrinsic distinctness of the dopaminergic VTA neurons.[13,14]

Starting with the first study by Soemmerring[15] in 1792, research has been increasingly focused on cytoarchitectural features of the midbrain nuclei. Cytoarchitectural studies have provided valuable information for the functional demarcation of catecholaminergic neurons in the brainstem.

After demarcation of these nuclei, it has been shown that SN and VTA are associated with movement disorders and psychotic diseases, respectively.[16,17]

The immunohistochemical revelation of tyrosine hydroxylase (TH), the rate-limiting enzyme of DA synthesis, was a breakthrough in the identification of dopaminergic cells.[18] Immunocytochemical studies have revealed clusters of dopaminergic neurons and three main

nuclei in the midbrain have been identified: retrorubral field (A8), SN (A9), and VTA (A10). The A8 neurons are generally considered as the extension of the A9 cell group, since the rostral and ventral portion of the A8 cell group can not be clearly differentiated from the contiguous A9 neurons of the caudal and lateral SN. The A8 neurons are also continuous with the caudal and lateral portions of the A10 cell group.[19] The A9 neurons correspond to the nigral DA-cells, most of which are localized in the SNc, but also in the substantia nigra pars reticularis (SNr) and to a lesser extent in the substantia nigra pars lateralis (SNl). The A9 neurons project predominately to the dorsolateral striatum which corresponds to putamen and part of caudate nucleus in man and plays a crucial role in the control of movement and degenerates preferentially in Parkinson's disease.[20,21] A10 neurons are localized in VTA and give rise to mesocorticolimbic system. The mosocorticolimbic system innervates the frontal cortex, ventral striatum (nucleus accumbens), the bed nucleus of the stria terminalis and amygdala complex, and is also involved in motivation, reward and sustained attention processes.[19,22]

In terms of relative proportion of TH-immunopositive cells, the A8 cells account for about 5%, and the A9 and A10 cells account for about 95%, with a more or less equal distribution in rodents. It was reported that in the rat TH immunohistochemistry reveals 15,000-20,000 dopaminergic neurons on each side of the midbrain tegmentum, and about 9000 of these cells belong to the VTA.[19] Furthermore, subdivisions of VTA have been defined in some species, including human, rat, cat, and monkey.[23] The subgroups of VTA have been termed with different names for approximately 200 years. Tsai's descriptive anatomical study on the brain of the opossum is the keystone study about VTA structures. The ventromedial mesencephalic tegmentum was also first described and named as VTA by Tsai.[24] Phillipson has described five distinct nuclei: three of them are located in the midline or medial position (nucleus linearis rostralis, nucleus linearis caudalis, and nucleus interfascicularis) and two of them are described as lateral nuclei, nucleus paranigralis and nucleus parabrachialis pigmentosus.[23,25,26]

Cytoarchitectural Description of Components

Since the boundaries of VTA are not distinct, it is difficult to define its components. Therefore, it is necessary to use neuron-specific immunohistochemical staining for their identification. In Nissl stained sections, neurons of VTA were distinguishable from the red nucleus dorsally, and separated from the interpeduncular nucleus ventrally.[25,26] However, this adjacency of these structures shows differences in the sections of midbrain from caudal to rostral (**Figure 1**). In terms of ease of understanding, the local-

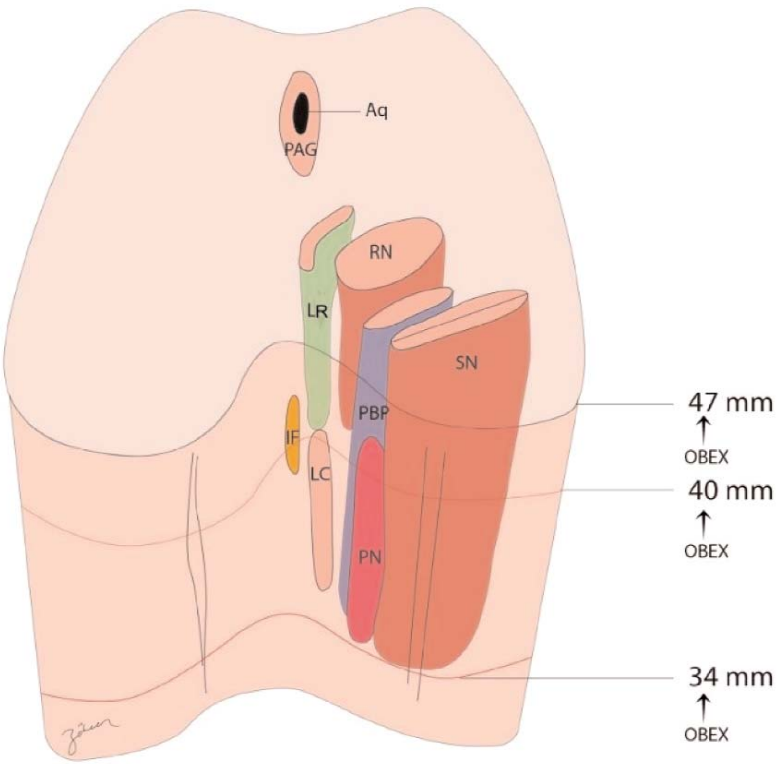

Figure 1. Schematic illustration of the human midbrain displaying VTA nuclei. The localizations of the nuclei are approximately displayed by using the Atlas of the Human Brainstem by Paxinos and Huang as reference. **Aq:** aquaduct; **IF:** nucleus interfascicularis; **LC:** nucleus linearis caudalis/centralis; **LR:** nucleus linearis rostralis; **PAG:** periaquductal gray; **PN:** nucleus paranigralis; **PBP:** nucleus parabrachialis pigmentosus; **RN:** red nucleus; **SN:** substantia nigra. Illustration design: Esat Adıgüzel; digitalization: C. Gökçen Köseli. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

ization of these structures were discussed by using the Atlas of the Human Brainstem by Paxinos and Huang that takes obex as a reference point.[27] The order of the structures is as follows; the most caudal VTA subdivision is the nucleus PN (paranigralis), seen 34 mm rostral from the obex. Nucleus PBP (parabrachialis pigmentosus), nucleus LC (linearis raphe caudalis/centralis), red nucleus (RN), IF (interfascicularis), and LR (linearis raphe rostralis) start appearing from obex at 35 mm, 35 mm, 38 mm, 40 mm and 40 mm, respectively; LC, IF and PN disappear at 40 mm 41 mm and 41 mm, respectively. LR and PBP continue from obex up to 47 mm rostral[27] (**Figure 1**). IF is located in the midline, LR and LC are in the paramedian zone, and others (PN, PBP) are located more laterally in humans. The LC curves lateral of PAG dorsally and extends to interpeduncular fossa ventrally. It neighbors the RN and PBP laterally.[19]

In the region of the human VTA, neuron sizes range from 10 to 53 μm.[19] PN neurons are small to medium in size, stellate, round, fusiform or spindle in shape, and lie ventrally in the VTA. The orientation of PN neurons in coronal sections is horizontal. In contrast, PBP neurons lies dorsally in the VTA. Some of these neurons are small, but most of them are medium-sized, and are more randomly arranged dorsal to the PN with no preferential orientation.[26] The IF neurons are usually very small particularly at the anterior pole, fusiform and oriented mediolaterally. LR and LC neurons are very variable based on their size, shape, and are often oriented ventrodorsally. They are clearly isolated from other neurons of the VTA because of the presence of neuromelanin pigment. The largest neurons of the five component nuclei are found in LR.[19,25]

Five component nuclei contain roughly 690,000 neurons, and approximately 80% of these neurons are dopaminergic in human.[10,19] However, these neurons are different from each other due to their properties. After Carlsson first developed the histofluorescence microscopic method that allowed sensitive visualization of a neurotransmitter in the neuron, the first detailed paper displaying the distribution of catecholamine (CA) containing neurons in the rat brain was published.[28–30] A new nomenclature for the monoamine-containing neuron groups was adopted based on Dahlström and Fuxe observations with CA histofluorescence. The CA class of monoamines was named "A" for the descriptive purposes. They identified twelve groups of catecholaminergic neurons (A1-A12) distributed from the medulla oblongata to the hypothalamus.[30]

The dopamine (DA) containing pathways of the midbrain were divided as A8, A9 and A10 neurons. A9 neurons are located in the SNc, whereas the A8 neurons are located dorsal and caudal to the SN. The A10 neurons are found in the VTA with some of them extend into the structures located at the midline.[31] The A10 neurons projecting from the VTA (five component nuclei) to the limbic and cortical areas along mesolimbic and mesocortical pathways.[32] Dopaminergic axon branches of the mesocortical pathways within the cortex reach more than one cortical areas.[33] Neocortex is extensively innervated by midbrain dopaminergic projections in humans. These projections to the PFC arise mostly from the VTA, mesocortical dopaminergic system, and play a critical role in executive functions, attention and motor components of the behavior.[34,35] Mesocortical dopaminergic system has received considerable attention due to its involvement in a range of psychological processes and neuropsychiatric diseases such as ADHD.[36,37]

Despite the presence of subdivisions within the VTA, their respective functions have not yet been clarified. This is more likely due to the difficulty in selectively manipulating these different groups of neurons, their relatively small size, proximity and mostly shared neurochemistry. However, behavioral evidence supports the presence of a major antero-posterior heterogeneity within the VTA.[38] In addition, this anteroposterior heterogeneity concerns the functionality as one of the most important symptoms of ADHD, the locomotor activity.[39] Accumulating evidence indicates that subdivisions of the VTA are also physiologically and characteristically heterogeneous. One group of neurons is centered in the medial VTA and projects to the medial PFC. The nucleus accumbens medial shell and core and basolateral amygdala are smaller and have fewer and shorter dendritic branches as compared to the lateral VTA neurons which are projecting to more lateral parts of the nucleus accumbens and ventrolateral caudat-putamen.[13,40–42] While the medial subdivisions of the VTA are fast-firing, display higher baseline activity profiles and have low DA transporter (DAT)/TH mRNA ratios, lateral VTA neurons have a slow firing pattern and express DAT more robustly.[13,41] Uniquely, VTA neurons that project to the medial PFC lack D2 receptor-mediated auto-inhibition and display lower levels of D2.[13] These neurons also have a very low expression of DAT when compared with mesolimbic projecting neurons.[43,44] Indeed, it has been shown that the medial PFC maintains higher concentrations of DA for longer amounts of time compared with the striatum.[45,46] Because of the decreased uptake for DA in VTA-medial PFC, DA neurons could have functional importance for the DA in working memory and executive functions in the cortex.

Conclusion and Perspectives

Psychostimulants affecting dopaminergic systems are the most frequently prescribed treatments for ADHD. The mechanism of these stimulants is to increase the DA level in the PFC by inhibiting the dopamine transporters.[36] Since VTA is the major dopamine source of the PFC, it has been that VTA plays an important role in ADHD. Nevertheless, the neuronal pathophysiology of ADHD is obscure, and further researches are needed.

We have reviewed the subdivisions of VTA neurons and highlighted the necessity for a better understanding of these subdivisions of VTA in terms of functionality. We would argue that the depth of our understanding of the functional role of the subdivisions of VTA will continue to increase, only if accompanied by continued elucidation of its neuroanatomical relationship. However, the subdivions of VTA sends functionally distinct DA projections to its targets. Recent advances in optogenetic techniques, projection- and cell-specific molecular profiling have opened up new avenues into addressing these issues. To better understand how these subdivisions of VTA convey it to their target sites, it is necessary to determine the organization of connectivity in the VTA and the functional nature of the synapses that are established by these neurons in upstream brain structures. The subdivions of VTA cell maps and identifying their connectivity pattern will be useful for future neurobiological studies on ADHD.

Acknowledgement

The authorsare thankful to Gökçen Köseli for digitalization of the drawing.

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Conflict of interest statement: No conflicts declared.

 Please cite this article as: Güngör Aydın A, Adıgüzel E. Morphological features of the ventral tegmental area: a brainstem structure related to attention deficit hyperactivity disorder. Anatomy 2019;13(1):66–70.

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