Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta Morphologica et Anthropologica, 31 (1-2)
Sofia ● 2024

Morphological Study of the Tongue during Acupuncture

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The aim was to investigate the morphological changes occurring in the tissues of the rat tongue during acupuncture. Acupuncture needles on the rat's tongue were placed at an acupuncture point corresponding to the human acupuncture point Ex-HN-10 (Juquan). We used a methodology developed by us. In the needle tract formed after acupuncture, we observed a violation of the integrity of the epithelium, the lamina propria, the deep muscles and nerve fibers of the tongue. We scrutinized nerve fibers in the vicinity of the needle tract, some of them were unaffected by the needle and others were destroyed. We detected mast cells (MCs) in the tissues near the blood vessels and nerve fibers as well as in the nerve fibers themselves. Some of the MCs in the vicinity of the needle showed signs of degranulation with released granules found a considerable distance from the cell.

Key words: acupuncture, acupoint, tongue, needle, mast cells

Introduction

Acupuncture is one of the main areas of Traditional Chinese Medicine (TCM). This is a method for beneficial effects on health, in which acupuncture needles are placed in acupuncture points on the body. Tongue acupuncture is part of (TCM) and is becoming increasingly popular as a method of treatment. The doctor who is scientifically researching this area in the present time is Prof. Sun, practicing mainly in Hong Kong. Autism is one of the diseases in which the effectiveness of tongue acupuncture is studied [19]. Depression is another disease affected by acupuncture on the tongue. Most studies have shown a positive effect of these procedures, with difficulties in the methodology and selection of homogeneous groups [7]. The clinical effect and application of electroacupuncture on the tongue in the treatment of patients with lingual hemangiomas have been studied, and the results of this method of treatment have been

reported [9]. According to some authors, due to the close relation of the tongue to the brainstem and cerebellum, its stimulation can increase the associated nerve pathways to the motor/somatosensory cortex, leading to an improvement in motor function, even with a short course of acupuncture treatment on the tongue [15].

The human tongue assists in a variety of functions; the anterior tongue plays a prominent role in speaking and mastication, while the posterior tongue performs a role in upper airway maintenance [20]. The tongue is innervated by hypoglossal nerve (CN XII), trigeminal (CN V3) and glossopharyngeal (CNIX) nerves; autonomic nerve fibers which stem from the intermedio-facial nerve (CN VII), the glossopharyngeal nerve, and the vagus nerve (CN X); and sensory nerve fibers. Characteristic features of the tongue in rodents are the elongated body of the tongue and the presence of a prominence known as the "intermolar" prominence. The dorsal surface of the mucosa of the tongue is covered by lingual papillae. The tongue papillae with its taste buds in humans and mammals are the receptors for the sensation of taste [18].

The dorsal surface of the tongue in rats is covered by partially keratinized stratified squamous epithelium. The lamina propria is located under the epithelial layer and is represented by the loose connective tissue that includes the blood vessels and nerves. The lamina propria is fused with the underlying connective tissue of the tongue muscles without a sharp boundary. The large number of papillae, which are different in size, shape, and localization on the dorsal surface of the tongue. The connective tissue of the tongue includes a large number of vessels and nerves [2].

Mast cells (MCs) are found in surfaces in contact with the external environment, in all tissues of the body, but are widespread near blood vessels and nerves. Their function is related to immunity, protection against parasitic infections, immunomodulation, tissue repair and angiogenesis [11]. Specific connective tissue cells called mast cells, which are missing in the brain, are found in the skin and mucous membranes [14]. In mammals, they are associated mainly with blood vessels and nerve fibers, and with the boundaries between the organism and the external environment. Mast cells (MCs) are also found in the tongue of rats. The authors describe in rat tongue a number of MCs scattered in the submucosal area adjacent to nerve bundles, blood vessels, and skeletal muscle of the tongue. MCs are found in bundles containing both myelinated and unmyelinated nerves in the tongue of rats [1]. Studies of the distribution of MCs in healthy rats were performed. MCs were found in rat tongue. MCs have also been observed in other internal organs in rats, with increased numbers of MCs in inflammatory lesions. The role of MCs and the changes that occur in them as a result of acupuncture at certain points on the body have been studied by many authors [3, 4, 5]. The results described in the literature by some authors in combining classical acupuncture with electroacupuncture suggest that peripheral nerves play an important role in the convergence and degranulation of MCs [8]. There is still insufficient research on the effects of acupuncture on the tissues, MCs and nerves of the tongue. The aim of our study was to investigate the morphological changes occurring in the tissues of the tongue during acupuncture.

Materials and Methods

Wistar rats were used for experimental animals in the present study. The tongues of a total of 25 Wistar rats of both sexes weighing between 220-350g and 3 months of age

were studied. The rats were provided by project 13/2017 at the Medical Faculty of the Trakia University. All studies were conducted in accordance with the regulations for work with experimental animals in the Republic of Bulgaria and are in accordance with the ethical standards specified in the rules of the Department of Anatomy at the Medical Faculty of the Trakia University and comply with the European Animal Welfare Directive, with the Commission on Ethical Treatment of Humans and Animals.

Animals were initially premedicated with ether for 5 minutes, followed by intraperitoneal injection of xylazine and ketamine (40-80 mg/kg, i.p). After maximal extension of the tongue the experimental animals were pierced with an acupuncture needle (acupuncture needles were inserted into the acupuncture point) 0.22×13-mm stainless steel needle, (Beijing Zohongyan Taihe Medical Instrument Co., Ltd., China) at point Ex-HN-10 (Juquan). The location is in the center of the tongue body and the needle was kept for 10 minutes. The depth differ form a few millimeters, up to pierce the entire tongue. Due to its elasticity, the tissues quickly regain their integrity after removing the acupuncture needle. The defect that occurs in the tissues is minimal in diameter and the needle tract is difficult to distinguish. In order to trace more precisely the depth of its penetration and to demonstrate the needle tract and the surrounding tissues, in a state as close as possible to the in vivo state, we altered the method by leaving the needle in the tissues during the processing of the taken material until the stage of "cutting". In this case, the observed needle tract shows the exact path of the needle and the deformation of the surrounding tissues at the time of insertion of the needle. The methodology developed by us allows visualization of the needle tract formed after acupuncture and the tissues located near the needle tract and the MCs contained in them [4, 5]. This technique was also used to study the effect of acupuncture in rat tongue. Together with the needle inserted at the test point (EX-HN-10), the rats were perfused through the ascending aorta with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.36). The tongue was cut near its root and a piece of tongue material together with the needle inserted into it was taken and placed for fixation in 10% formalin for 48 hours. The 10 × 10 × 10 mm tissue blocks were then placed under running water to wash away excess fixative, dehydrated through an ascending row of alcohols, and placed in Cedarwood oil until amber in color. In some of the experimental animals, material from point EX-HN-10 was taken without inserting a needle into the point in order to establish the normal morphological structure in it. The tissue blocks were embedded in paraffin and cut to a thickness of 7 μm. Classical histological staining such as Orcein, Van Gieson, Azan, Toluidine blue, Bismarck brown and immunohistochemical techniques to detect tryptase in mast cells have been applied.

The paraffin sections were washed in 0.1M PBS and placed in 1.2% hydrogen peroxide and methanol for 30 minutes. Antigen recovery was performed in buffer (pH 9.0) for 20 minutes. After washing with an EnVisionFlexWash Buffer, the sections were incubated in a humidified chamber overnight at 4°C with primary antibody: Monoclonal Mouse antihuman mast cell tryptase (Dako, Danmark) – ready to use. Then sections were washed with PBS and incubated with EnVision detection system (DAKO) for 24 hours at 4°C. The reaction was demonstrated with diaminobenzidine. The negative control was done by PBS instead of primary antibody. The samples were photographed with a research microscope (Leica DM1000) equipped with a digital camera (Leica DFC 290)

Results

Holding the needles in the tongue for 10 min in the anesthetized rats allows to track the changes occurring over time in the tissues and cells. This is especially important for the reaction of mast cells with their accumulation around the area of the needle tract and their degranulation. After removal of the acupuncture needle, rapid closure of the adjacent tissues in the vicinity of the needle tract is observed. The tissues quickly fill the defect caused by the acupuncture needle and the needle tract becomes difficult to detect after removing the needle without using the methodology developed by us. The long retention of the acupuncture needle in the tissues with our technique allowed for a good visualization of the needle tract during processing of the tongue samples. The stains we use visualize well the needle tract, MCs, epithelium, blood vessels, nerves and muscles of the tongue.

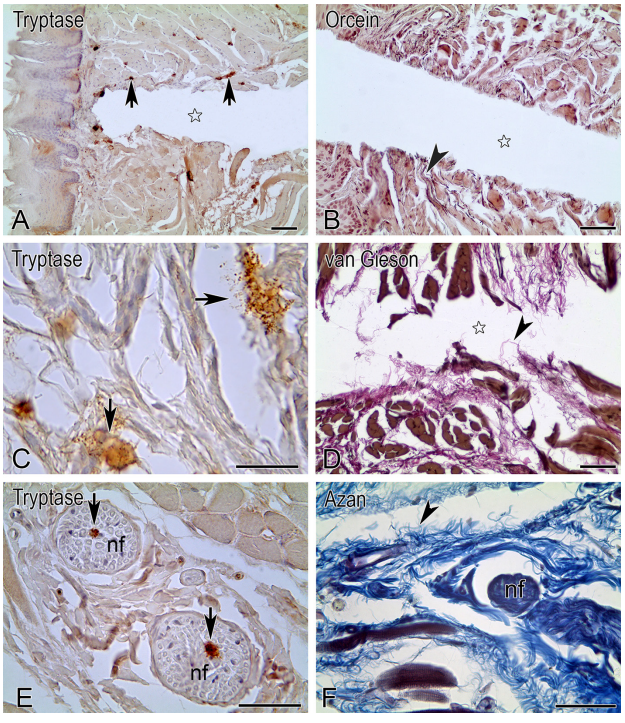

Fig. 1. (A, C, E) Triptase positive MCs in the vicinity of the needle tract. Photomicrographs stained with Orcein (B), van Gieson (D) and Azan (F) showing the tissue reaction in the vicinity of the needle tract formed after acupuncture. Needle tract (star), elastic fibers (arrowheads), collagen fibers (arrowheads), nerve fibers (nf). Scale bar = 50 μm.

After experimental acupuncture at point Ex-HN-10 in a rat on a sagittal incision, the course of the needle tract formed after acupuncture is observed, which passes through the epithelium, lamina propria, fascia, epimysium of the muscles of the tongue and after this goes deep, and in some cases, there is a breakthrough on the other side of the tongue. The tissue folds in the direction of the needle tract. We observe that large, deep-lying structures near the point, such as vessels and nerves, are not affected. This showed that the acupuncture needle placed at this research point did not cause severe damage to the surrounding tissues and did not affect large vessels and nerves in the depth of the tongue.

Histological examination of the tissues in the vicinity of the acupuncture needle tract reveals a violation of the integrity of the epithelium, the subcutaneous loose connective tissue with the elastic and collagen fibers located in it, the fascia and the muscles or their deformation (**Figs. 1B, D, F**). The integrity of the epithelium was disrupted and it folds in the direction of the needle tract. We observed compaction and displacement of the connective tissue and the cells contained in it near the needle

tract (**Fig. 1**). The canal formed by the acupuncture needle (the needle tract) is very clearly visualized in the muscles. There is a partial destruction of the transverse striated muscle fibers in the needle tract, in the depth of the tongue. Detached pieces of skeletal muscle fall into the needle tract formed after acupuncture (**Fig. 1D**). Collagen and elastic fibers are deformed, and some of them are torn. Parts of the collagen and elastic fibers that are entrained by the acupuncture needle fall among the transversely striated skeletal muscles. (**Figs. 1B, D, F**).

We observed nerve fibers in the vicinity of the needle tract formed after acupuncture. Some of these nerve fibers were unaffected by the needle (**Fig. 1E, F**).

Compression and displacement of the tissue near the needle tract is observed, together with the mast cells (MCs) contained in it. In the field of the needle, some of the MCs showed signs of degranulation with released granules found a considerable distance from the cell (**Fig. 1C, Figs. 2C, 2D**). Acupuncture causes a reaction in the mast cells in the tissues surrounding the needle tract. This reaction is expressed in their clustering near the needle tract and their degranulation.

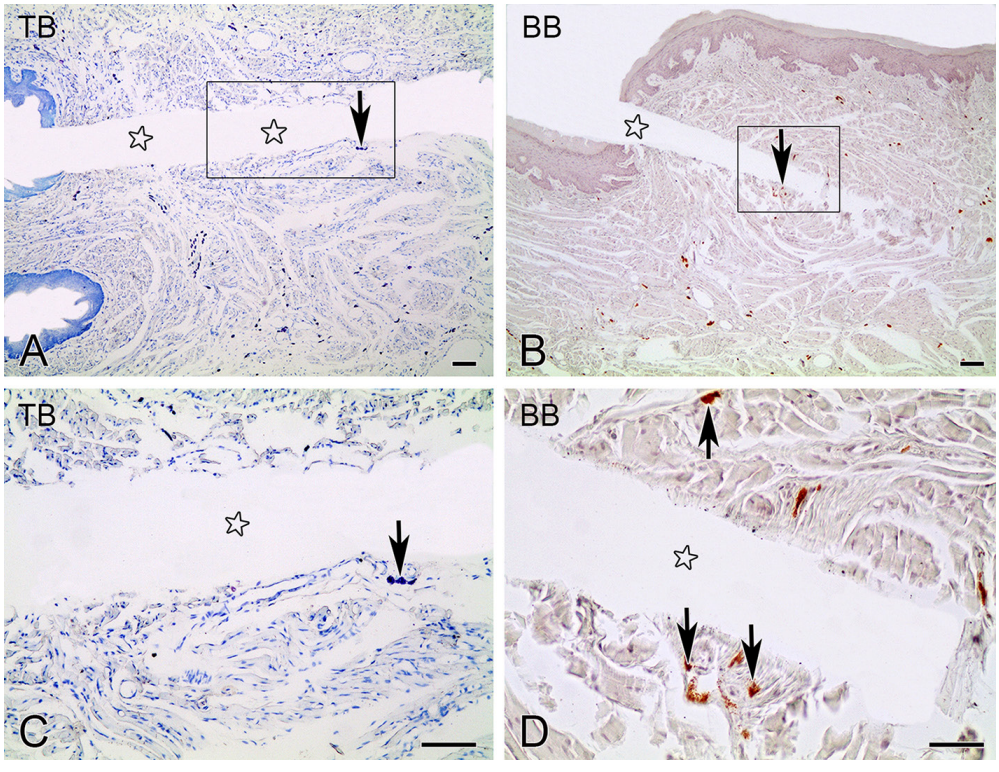

Fig. 2. Toluidine blue (A, C) and Bismarck Brown (B, D) stained micrographs visualizing mast cells in the vicinity of the needle tract formed after acupuncture. Needle tract (star), mast cells (arrows). (C, D) Mast cells showed signs of degranulation with released granules found at a considerable distance from the cells. Scale bar = 50 μm.

Discussion

The results obtained should be considered in the light of the importance of tongue acupuncture in the treatment of diseases in humans and animals. These rat studies will expand our understanding of tongue acupuncture in humans. From the incisions made in the frontal, horizontal, and sagittal planes of the tongue, sagittal sections provided the largest area for single-section study and were, in our opinion, more suitable than other sections for tracing the needle tract formed after acupuncture of the tongue. Longitudinal (sagittal) sections of the needle tract give a better idea of the changes that occur in the tongue as a result of experimental acupuncture because we can examine the epithelium, underlying connective tissue, and musculature in a single section. In control transverse (horizontal) sections, only the changes occurring in the relevant tissue and in particular in the musculature could be examined. The tract of the needles is very well visualized on the sagittal section, as well as the surrounding structures. To study the normal structure of the tongue in rats, other authors have preferred sagittal sections of the tongue [2]. Due to the innovation of our research, we were unable to find data on morphological studies of the tongue by other authors before us. Therefore, we compare our results with the described changes in acupuncture points located on the body. Although there is a difference in the structures, the principles of influence of the acupuncture needle are similar. Some authors have described the changes caused by acupuncture in acupuncture points located on the body. Acupuncture in acupuncture points located on the body causes destruction in the vicinity of the needle tract formed after acupuncture caused by the needle and deformation in the structures adjacent to the needle tract [3]. Structures such as sebaceous glands, hair follicles, blood and lymph vessels are deformed and flattened, and intercellular distances are reduced. Local irritation and release of neuropeptides from sensory nerves as a result of acupuncture has been described [10]. In the immediate vicinity of the needle tract formed after acupuncture, partial destruction has been observed and described by us in research on acupuncture points located on the body, and deformation of the collagen and elastic fibers and folding of the epithelium in the direction of the needle tract [3]. The acupuncture needle displaces the tissues in its course and the deep blood vessels and nerves near the needle tract are not affected [3]. This showed that tongue acupuncture at point Ex-HN-10 is a relatively safe treatment method that does not cause severe damage to deep vessels and nerves. This determines the possibility of its safe useage for treatment in humans and animals. Acupuncture has a strong mechanical effect. We observe destruction of collagen fibers in the connective tissue by the acupuncture needle, which also affects the mast cells located there and MCs in connective tissue have a role in transmitting the mechanical signal from the acupuncture stimulation. The results obtained in this study confirm our previous studies. The observed accumulation of MCs around nerve bundles and blood vessels coincides with the described normal distribution of MCs for the respective age. In 3-month-old rats, more MCs were observed in the deep muscle layer of the tongue than the lamina propria [12]. Feng and Jinglan [6] believe that the reactivity of MCs in rat skin to serotonin is high. The role of MCs in analgesia in acupuncture and increased degranulation of MCs after acupuncture has been described by many authors [5, 16]. Accumulation of histamine and serotonin positive MCs near the needle tract formed after acupuncture has been observed after acupuncture into the body‘s acupuncture points in our previous studies

[4, 5]. Serotonin secretion from MCs was observed as a result of corporal acupuncture in rats [5]. We assume that serotonin and histamine are released by mast cells during tongue acupuncture, and this could be a subject of additional immunohistochemical studies. But there are also authors who believe that the number and degranulation of MCs are not affected by acupuncture [17]. Our previous research confirms the thesis of the influence of acupuncture on MCs of the tongue [13]. Studies have shown that the acupuncture needle affects certain structures in the tissues or passes close to them without directly affecting them [3]. These results are consistent with the results obtained in this study

In conclusion, acupuncture causes morphological changes in the tissues of the tongue and degranulation of MCs.

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