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# Histochemical Characteristics and Density of Mast cells in the Porcine Conjunctiva and Eyeball

*Ivaylo Stefanov[1,2*], Stefan Stefanov[3]*

*[1] Department of Anatomy, Faculty of Medicine, Trakia University, Stara Zagora, Bulgaria*
*[2]Department of Anatomy, Histology and Embryology, Pathology, Burgas University Prof. Dr. Asen Zlatarov, Burgas, Bulgaria*
*[3] Fifth year student at Medical Faculty, Trakia University, Stara Zagora, Bulgaria*

*Corresponding author e-mail: ivstefanov@abv.bg

The anatomic similarities to human eyes, along with comparable physiologic processes, enable swine to be suitable experimental model. The aim was to define the histochemical characteristics and number of mast cells in the porcine conjunctiva and eye ball using toluidine blue and alcian blue staining. It was found that in the sclera, cornea, choroid, retina, lens and optic nerve mast cells were absent. When compared the mast cell density in all layers of eyeball we found that their number was the highest in the ciliary muscle, followed by the iris, and the lowest – in the stroma of ciliary body. The number of mast cells in bulbar conjunctiva was similar to that in the posterior part of the iris.

The current study showed that the mast cells are resident cells in the ciliary stroma and muscle, iris and ciliary body participating in maintaining the homeostasis in the porcine eye.

*Key words:* mast cells, metachromasia, toluidine blue, alcian blue, eye, swine

## Introduction

The miniature swine is widely used as ophthalmology models [47]. The anatomic similarities to human eyes, along with comparable physiologic processes, enable miniature swine to be suitable model for surgical procedures and subsequent testing of potential therapeutic agents at treating multiple ophthalmic diseases such as uveitis, retinal detachment, cataracts, glaucoma and diabetic retinopathy [15, 26, 42, 47, 51].

Besides their well-known role in IgE-mediated hypersensitivity responses, mast cells have been implicated in a range of non-IgE-mediated immunological and pathological processes including responses to parasites and neoplasms, chronic inflammation, fibrosis, angiogenesis and wound healing [12, 17]. Leonardi [28] reported that mast-cell activation and release of the main mediator, histamine, has been described in all allergic ocular diseases: seasonal allergic conjunctivitis (SAC), perennial conjunctivitis (PAC),

vernal keratoconjunctivitis (VKC), atopic keratoconjunctivitis (AKC), and contact lens-associated giant papillary conjunctivitis (GPC). Although ocular allergic reactions involve many cell types and mediators, mast cells play a crucial role in the common pathogenesis of these different forms of ocular allergy.

In addition to preformed stored mediators (e.g. histamine, heparin) mast cells are known to be a potent source of many de novo synthesized proinflammatory cytokines and chemokines [17]. They also appear to regulate blood flow in some tissues and organs (Stead and Bienenstock 1990). It is known that mast cells constitute heterogeneous populations, and at least three types have been well characterized in common laboratory mammals and humans: bone marrow mast cells; mucosal mast cells; and connective tissue mast cells [13, 14].

Enerback [10] demonstrated in 1986 that rodent MC subpopulations could be differentially stained with alcian blue and berberine sulfate. The toluidine blue staining allows the visualization of metachromatic granules in the cytoplasm of mast cells. Enerback [10] also demonstrated that MCs from mucosal surfaces were sensitive to formaldehyde fixation in that they failed to stain metachromatically on additional exposure to dyes, whereas MCs from connective tissues were insensitive to formaldehyde fixation. This principle has now been extended to human MCs that have also been distinguished by their formaldehyde sensitivity [10, 19, 27, 48].

There have been a number of studies of the distribution of mast cells in the normal iris of various species (29, 16, 30-34, 45, 52]. These cells are absent or extremely rare in rabbits, rats and mice, but are present in some carnivores (e.g. dogs, cats), marsupials and humans, although no true estimates of density are available in the literature. In the human iris, they appear more randomly distributed than in the choroid where they are periarteriolar [33].

Mcmenamin and Poll [35] revealed that in bony fish, mast cells stained by toluidine blue were clearly identifiable and were present in all of the layers of the choroid, including the choriocapillaris. In the sharks or cartilaginous fish, no mast cells were identified in the choroid.

In the context of the mammalian eye, mast cells are invariably absent from the neural retina in all species studied to date. In 1937, Jorpes et al. first observed mast cells at the limbus in human and bovine corneas [22]. Later, Smelser and Silver [45] described many mast cells in the limbus and choroid of guinea pigs, rats, and rabbits. Smelser and Silver [45] studied the distribution of mast cells in sclera, uvea (iris, choroid and ciliary body) and retina of guinea pigs, rat, ferret and rabbit, using formalin for fixation and toluidine blue staining. In guinea pigs, rat and rabbit, large number of mast cells were seen in the posterior half of the choroid [45]. The anterior portion of the choroid contains fewer cells. A dense accumulation of mast cells forms a ribbon-like band completely in the deeper part of the ciliary body. The processes themselves contain very few mast cells. In many guinea-pigs the iris was totally devoid of mast cells, but in some animals occasionally a few cells were found. In the guinea pig, rat, and rabbit, practically no mast cells were found except for a few which at times followed a large vessel or nerve as it penetrated through the sclera. None was present in the normal cornea, but a limbal concentration of such ceils in the loose episcleral tissue was found regularly. Whole mounts of the retina were free from mast cells in all species examined. The lens was not preserved.

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The distribution, morphology, and staining characteristics of choroidal mast cells were similar from teleost fish through to eutherian mammals resembling the mammalian connective tissue mast cells [35].

Since we have not found the information about the distribution of mast cells in porcine eyeball`s layers decided to perform this study.

The aim of the study was to define the histochemical characteristics and number of the mast cells in the porcine conjunctiva and eyeball using toluidine blue staining and alcian blue staining with pH 1.0 and 2.5.

## Materials and Methods

### *Material*

Twelve whole eyeballs were collected from 6 male, 6 month-old pigs, weighing 91-115 kg. The animals were slaughtered in a licensed abattoir for a meat consumption in accordance with the European Union's and Bulgarian legislations. Specimens from the whole eyeball together with bulbar conjunctiva were processed by the classical histological methods. Serial histological and longitudinal sections of 5 μm thickness from material fixed in 10% aqueous solution of formalin were obtained.

### *Histochemical staining*

Serial sections were stained by toluidine blue dye [50]and alcian blue dye with both pH 1.0 and 2.5 plus Safranin, then morphometric study was performed. Alcian blue staining at pH 2.5 visualizes all acid mucins: sulfated and carboxylated acid glycosaminoglycans, but at pH 1.0 – sulfated mucins only [18, 39, 40].

### *Micromorphometric study*

The number of mast cells per microscopic field (x 200with an area of 0.163 $mm^2$) were estimated by light microscope (LEIKA DM1000) equipped with a digital camera (LEIKA DFC 290) and software (LAS V4.10.0 2016).

### *Statistical analysis*

The morphometric data were processed by GraphPad Prism 6 for Windows (GraphPad Software, Inc., USA) via one-way analysis of variance (one-way ANOVA) followed by

Tukey-Kramer's post-hoc test and are presented as mean ± SD. P-values < 0.05 were considered statistically significant.

## Results

In the current histochemical study three stainings were used: toluidine blue staining and alcian blue staining with both pH 1.0 and 2.5 in order to define mast cell distribution in the three tunics of the eyeball (*bulbus oculi*) and bulbar conjunctiva (*tunica conjunctiva bulbi*). The toluidine blue staining allowed detecting β-metachromasia in granules of the toluidine blue positive mast cell type ($MCTB^+$), but alcian blue staining in pH 1.0 and 2.5 marked the granules in light and dark blue, respectively in the cytoplasm of both alcian blue positive mast cell types: $MCAB1^+$ in pH 1.0 and $MCAB2.5^+$ in pH 2.5.

The bulbar conjunctiva showed that $MCTB^+$ were two times less than both MCAB1+ and $MCAB2.5^+$ ($p < 0.001$). No significant difference was observed between the number of $MCAB1^+$ and $MCAB2.5^+$ (**Table 1**). All mast cell types were localized in close proximity to the microcirculatory bed under the epithelium but not into the epithelium (**Fig. 1).**

In the fibrous tunic (*tunica fibrosabulbi***)** of the eyeball represented by sclera and cornea, no mast cells were identified (**Table 1**).

Mast cells were found to be localized mainly in ciliary part and iridial part of the vascular tunica (*tunica vasculosa*) (**Fig. 2)**. In the choroid no mast cells were observed (**Table 1**).

In the ciliary body (corpus ciliare) $MCTB^+$, $MCAB1^+$ and $MCAB2.5^+$ were detected near the capillaries and smooth muscle cells of the ciliary muscle (*musculusciliaris*). In the *orbiculusciliaris* mast cells were also

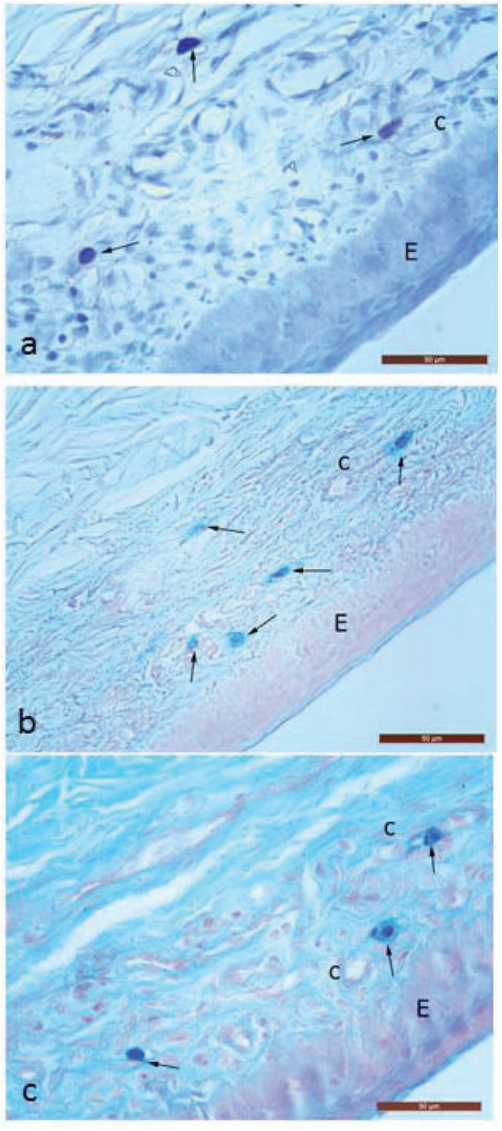


**Fig. 1**. Serial sections with toluidine blue positive mast cells (a) and alcian blue positive mast cells in pH 1.0 (b) and pH 2.5 (c) in the *lamina propria* of conjunctiva near the capillaries (c) and under the conjunctival epithelium (E). Arrows – mast cells. Bar = 50 μm.

observed close to pigment cells of its stroma. In the *corona ciliaris* the three types of mast cells were predominantly situated in the stroma of the anterior part near the capillaries and less in the central part near the pigment cells. The density of MCTB+ was lower than MCAB1+ and MCAB2.5$^{+}$ density ($p < 0.001$) (**Table 1**). No significant difference was established between the density of MCAB1$^{+}$ and MCAB2.5$^{+}$.

In the iris the number of MCTB$^{+}$, MCAB1$^{+}$ and MCAB2.5 was higher in its posterior part than in its anterior part. In both parts of the iris the mast cells were localized in close proximity to the blood vessels and pigment cells of the iridal stroma. The number of MCTB+ was lower than that of MCAB1$^{+}$ and MCAB2.5$^{+}$ ($p < 0.001$) (**Table 1**). No significant difference was established between the density of MCAB1$^{+}$ and MCAB2.5$^{+}$.

**Table 1.** Distribution of mast cells stained with toluidine blue (MCTB+), alcian blue pH 2.5 (MCAB2.5+) and alcian blue pH 1 (MCAB1+) in conjunctiva bulbi (Con B), cornea (Cor), sclera (Scl), corpus ciliaris (CCil), iris, choroidea (Ch), retina (Re) and nervusopticus (N).

| **Parameters** | **Con B** | **Cor** | **Scl** | **CCil stroma** | **CCil Musculus Ciliaris** | **Iris Anterior Part** | **Iris Posterior part** | **Ch** | **Re** | **N** |
|---|---|---|---|---|---|---|---|---|---|---|
| Number of: MCTB+ | 4.50±0.51 **A4, B4** | - | - | 1.44±0.51 | 8.05±0.80 **A4, B4** | 2.22±0.42 | 3.22±0.42 **A4,B4** | - | - | - |
| MCAB2.5+ | 7.50±1.09 | - | - | 2.05±0.63 | 9.88±0.83 | 3.00±0.48 | 8.55±0.51 | - | - | - |
| MCAB1+ | 8.05±0.80 | - | - | 2.16±0.71 | 10.17±1.04 | 3.00±0.59 | 8.61±0.50 | - | - | - |

Legend: (-) absence of staining
A4 – statistical significant difference between MCTB+ and MCAB2.5+
B4 – statistical significant difference between MCTB+ and MCAB1+

In the retina, lens and optic nerve mast cells were not detected.

When compared the mast cell density in all layers of eyeball we found that their number was the highest in the ciliary muscle, followed by posterior part of the iris, smaller – in the anterior part of the iris and the lowest - in the stroma of ciliary body.

The number of mast cells in bulbar conjunctiva was similar to that in the posterior part of the iris.

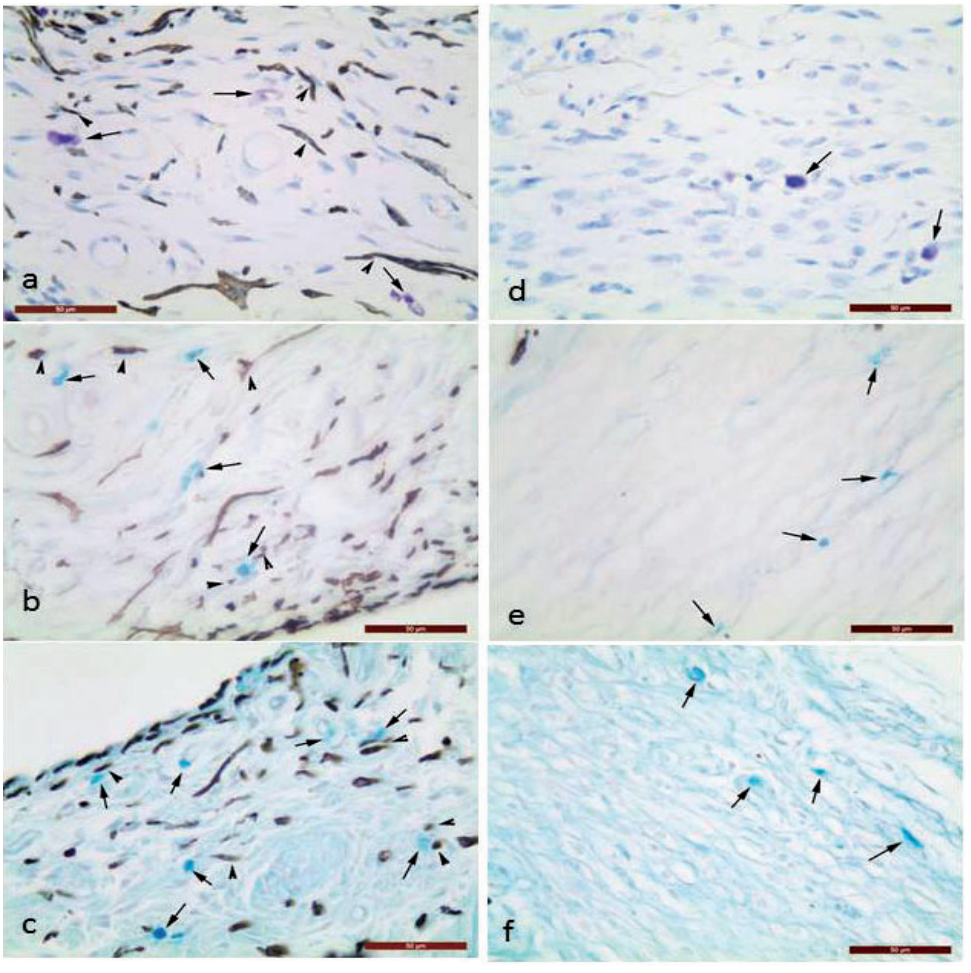


**Fig. 2.**Toluidine blue positive mast cells (a, d) and alcian blue positive mast cells in pH 1.0 (b, e) and pH 2.5 (c, f) in the stroma of the anterior part of the iris (a, b, c) and of the ciliary body (d, e, f) near the blood vessels (c) and pigment epithelium (arrowheads). Arrows – mast cells. Bar = 100 μm.

## Discussion

In the current study the histochemical properties and distribution of mast cells in the porcine conjunctiva and eyeball were established for the first time. For this purpose toluidine blue and alcian blue dyes were used as specific markers of these cells [40, 50].

In this study we support the finding of Morgan et al. [37] about the existence of mast cells exhibiting staining that was resistant to formalin and suggest that they were connective-tissue type MCs. Morgan et al. [37, 38] used toluidine blue staining to demonstrate the presence of human conjunctival mast cells in normal tarsal conjunctiva and increased numbers in subjects with allergic conjunctivitis because of pollenosis. The

authors also revealed that the number of mast cells does not depend on the fixatives such as Camoy's fixative and formalin. These findings showed that MCs in the conjunctiva are not a static population but are involved in the pathogenesis of allergic eye disease.

We found that in all porcine conjunctiva specimens the presence of mast cell is obligatory. These results support the finding of other authors studying the distribution of mast cells in the human conjunctiva [6,21, 36, 37, 38]. In normal individuals, mast cells are abundant in the conjunctival stroma, particularly at the limbus, but are not present in the epithelium. In the conjunctival stroma, mast cells are localised in the subepithelial layer and vicinity of the blood vessels. The number of conjunctival mast cells increases in all chronic ocular allergic diseases, PAC, VKC, GPC, AKC and SAC [5].

The pro-inflammatory mediators released by mast cells include histamine, leukotriene (LTC4), prostaglandin (PGD2), tryptase, chymase, carboxypeptidase A, cathepsin G, platelet activating factor (PAF, a powerful eosinophil chemotactic agent), and other eosinophil and neutrophil chemoattractants [9]. The acute inflammatory response may also induce the influx of neutrophils and eosinophils into the conjunctiva.

It has been calculated that a single conjunctival mast cell contains 4.6 pg of histamine [36], signifying that the total potential amount of histamine that can be released with massive mast-cell degranulation is 23 ng/mm$^3$. In the tear film of normal subjects histamine was found at concentrations of 5 to 10 ng/ml, whereas tear samples of patients with active VKC contain significantly higher levels [1, 3]. Itching, hyperemia, tearing, and chemosis are the classic ocular manifestations of histamine release. SAC, acute conjunctival reactions, and acute palpebral edema are the clinical features of typical IgE-mediated massive mast-cell degranulation. A similar reaction can be induced using the standardized conjunctival allergen challenge model [2], with which an immediate response, or early phase reaction (EPR), and a late-phase reaction (LPR) have been widely studied. Either in nonactive allergic patients or in normal subjects, tear histamine levels are very low at baseline. Inducing an EPR by challenging allergic patients with specific allergen results in tear histamine levels that are significantly increased compared with baseline [23].

It has been shown that mast cells store and release the proinflammatory cytokines including interleukins (IL-4, IL-5, IL-6, IL-8, IL-13) and tumour necrosis factor α (TNFα) which are involved in the ocular allergic response [9].

Anti-allergic strategies for ocular diseases must remain focused on conjunctival mast cell activation and degranulation. A better knowledge of conjunctival mast-cell biology and functions will help in development of new antiallergic drugs and, consequently, in the management of ocular allergic diseases [28].

Leonardi [28] reported that mast-cell activation and release of the main mediator, histamine, has been described in all allergic ocular diseases: seasonal allergic conjunctivitis (SAC), perennial conjunctivitis (PAC), vernal keratoconjunctivitis (VKC), atopic keratoconjunctivitis (AKC), and contact lens-associated giant papillary conjunctivitis (GPC). Although ocular allergic reactions involve many cell types and mediators, mast cells play a crucial role in the common pathogenesis of these different forms of ocular allergy.

We did not find any mast cells in porcine choroid. In contrast, Smelser and Silver [45]reported that in human and animal mast cells are frequently found to be associated with vessels of the choroid. In addition, the choroid of the ferret is highly vascular, yet in this species very few mast cells were found, although large numbers were seen in the other membranes. Smith and Trokel [44] revealed that histamine released from the mast

cells of the choroid is believed a likely explanation for large vessel dilatation. These experiments are an *in vivo* demonstration of the reactions of the ocular mast cells, and suggest that these cells may be significant in ocular inflammation.

Our finding about the distribution of $MCTB^+$, $MCAB1^+$ and $MCAB2.5^+$ near the pigment cells and microcirculatory bed in the porcine iris contradicted the results of Smelser and Silver [45] who claimed that although the iris also is a vascular structure, no mast cells were found in that tissue. The same localization was observed in the ciliary body. The mast cell localization near the pigment cells can be explained by the study of Yoshito et al. [2000] who examined the effects of histamine on cultured human melanocytes. The increases in melanin content of the histamine-treated melanocytes indicated an elevation of melanin synthesis by tyrosinase activation. It was observed an increase in the intracellular cAMP contents of human melanocytes induced by histamine via the H2 receptors. Therefore, histamine induces melanogenesis of human cultured melanocytes by protein kinase A activation via H2 receptors. Recently, Arai et al. [7] reported that mast cells might influence retinal pigment epithelial (RPE) cells via secreted mediators rather than cell contact-dependent mechanisms, because only a few mast cells are observed around the choroidal capillaries near Bruch's membrane despite the high number of these cells in the choroid. The authors evaluated interactions between RPE cells and mast cells via secreted mediators. They found that H1R was expressed by RPE cells, suggesting that mast cell degranulation in the choroid could have a strong impact on RPE cells via histamine release, but the role of histamine in this setting is not well known. The histamine enhances IL-8 production by RPE cells, raising the possibility that histamine released from mast cells may contribute to RPE inflammation.

It is known that the iris pigment epithelium is the layer of pigmented cells forming the posterior layer of the iris [11]. There is a remarkable resemblance between IPE and retinal pigment epithelial cells (RPE) due to their shared embryonic development [24, 43]. *In vitro*, IPE and RPE share functional properties such as phagocytosis and synthesis of cytokines and growth factors [41,49].Rezai et al. [41] showed that IPE elicited phagocytic activity similar to RPE. Non-immune cells, such as IPE and RPE, form an interface between the eye and the environment that is not readily accessible to myeloid cells. By virtue of their ability to detect signals via innate immune receptors, such as toll-like receptors, they are able to recruit myeloid cells, such as neutrophils and macrophages to the site of injury and induce inflammation.

Expression of TLRs has been reported in a number of ocular tissues such as cornea, conjunctiva, sclera and retina [8, 25]. Studies have emphasized the importance of the LPS receptor complex (TLR4 and co-receptors CD14 and MD2) expression in ocular tissues and cells such as corneal epithelial cells, cornea stroma fibroblasts, human ciliary body, human iris endothelial cells (TLR4 only), RPE and resident antigen presenting cells in human uvea [8]. It has been shown that human RPE express TLRs and are considered to play an important role in posterior ocular inflammation due to their ability to secrete several inflammatory mediators [20].

Mast cell modulation is a fundamental target for antiallergic components. In fact, most of the ocular antiallergic drugs have been designed as mast cell stabilizers. The mechanism of the most widely used ocular mast-cell stabilizers such as sodium cromoglycate, lodoxamide, nedocromil, and pemirolast involves a decrease of calcium influx into the cytoplasm. An advance in the treatment of ocular allergy comes from newly designed ocular anti-allergic compounds, such as olopatadine and ketotifen

[54]. These drugs have a dual activity as antihistamines and mast-cell stabilizers [4], probably due to their effect on calcium mobilization or on phospholipid cellular membrane. However, the most successful effects in terms of therapeutic response in chronic allergic diseases are obtained by topical corticosteroids. For example, cyclosporine may act on various mast-cell protein kinases, thus reducing calcium influx, degranulation, and cytokine gene expression.

## Conclusion

The current study showed that the mast cells are resident cells in the ciliarystroma and muscle, iris and ciliary body participating in maintaining the homeostasis in the porcine eye.

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