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*Research Article*

# The Histochemical Characterization of the Glycoconjugates in the Epidermal Mucous Cells of the Red Californian Earthworm, *Eisenia foetida*

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The aim of this study was to characterize the nature and regional distribution of the glycoconjugates secreted by epidermal mucous cells in *Eisenia foetida* (Annelida). Specimens were divided into six regions from anterior to posterior. The histochemistry was carried out by using standard histochemical methods. Histochemical staining properties of glycoconjugates in epidermal mucous cells were determined regionally. The epidermis of all regions contained strong to stronger PAS (+) cells in various degrees. The epidermis of the first, fourth, fifth, and sixth regions had strong to stronger AB pH 2.5 (+) cells. On the contrary, all regions contained weak to moderate AB pH 0.5 and AB pH 1.0 (+) cells. Most of mucous cells in epidermis of the first region contained both PAS (+) and AB (+) mucosubstances. All regions included weaker to weak AF (+) cells. All regions featured KOH/PAS (+) cells, with a slight reduction in reaction intensity in the epidermis of the last three regions. In this context, the different staining patterns observed in epidermal mucous cells hinted at their functional roles with respect to production of mucus with different physical properties. This study provided comprehensive information about the regional distribution patterns of the glycoconjugates and an opportunity to compare their distributional patterns in other annelids.

## 1. Introduction

Histological, histochemical, and ultrastructural studies have unveiled the existence of a variable and complex structure of the epidermis in annelids [1–3]. The epidermis of the annelids consists of a monolayered epithelium, which contains glandular, supporting, ciliated, and sensory cells. The epidermis is covered by a cuticle composed of collagen fibers embedded in a matrix [4]. The glandular cells in the surface epithelia secrete mucus that is rich in glycans, glycoproteins, and sialic acid residues [5–7]. In the annelids, this mucous secretion also plays a fundamental role in the formation of the ground substance of tubes, protection from dehydration, absorption of metabolites, and protection against parasites [4].

The glycoconjugates, which are essential components of the glycocalyx of many cell types, take part in many functions, including osmoregulation, cell to cell recognition, binding of hormones, protection of cells from phagocytosis and dehydration, differentiation, defense, and ion transport [8]. In some epithelial cells, which morphologically and functionally polarize in relation to different behaviours in ion transport [9], it is likely that a functional specialization of the glycoconjugates is observed on the basolateral surface. While the glycoconjugates on the basolateral surface are of acid characteristic, glycoconjugates on the apical surface are neutral [10, 11]. In many groups of animals, the surface acidic mucosubstances are considered to act as an ion barrier owing to a selective or buffering ability. Moreover, the mucous film absorbs heavy metals and delays their entry into the integument [12]. In addition to the aforementioned, acidic polysaccharides—specifically sulfated glycosaminoglycans—whose presence has been shown by using electrophoretic and chromatographic methods in *Eisenia andrei* [13] also fulfil important biological functions.

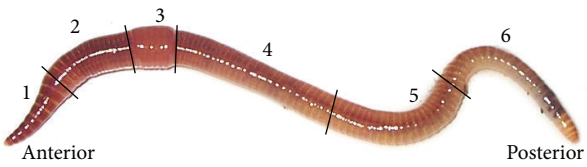


Figure 1: Sampling regions for histochemical characterization of glycoconjugates.

Many studies have been carried out regarding the histology and morphology [1], lectin histochemistry [8], immunohistochemistry [14–16], and histochemistry [2, 3, 5, 7, 17–23] of the epidermal glandular cells of oligochaetes. These studies, however, mostly focus on the species belonging to the genus *Lumbricus* and some other oligochaetes species. Little is known about the histochemistry of the glycoconjugates present in the epidermis of the species *Eisenia foetida*.

In recent years, new insights into the management of organic wastes, removal of heavy metals from soil, and detoxification of xenobiotics have been gained, and much attention has been focused on the earthworms in many countries. Worm farms have been established in Burdur and Giresun (Turkey) for the purpose of utilizing organic wastes of animal origin. In this regard, the Red Californian Earthworm, *Eisenia foetida* (Annelida, Oligochaeta), is one of the most commonly used worms for the management of organic wastes by vermicompost [24]. There are several reasons why this species is preferred for the vermicompost production. *E. foetida* is ubiquitous with a worldwide distribution and many organic wastes become naturally colonized by this species. It is a rugged worm with a wide temperature tolerance range and can live in organic wastes with a range of moisture contents. Moreover, it is an iteroparous earthworm species with continuous and high reproduction rates, and it is resistant to adverse environmental conditions [25].

The aims of the present study are to characterize histochemically the nature and regional distribution of the glycoconjugates secreted specifically by secretory cells on the surface epithelia of *Eisenia foetida* (Annelida, Oligochaeta) and to compare them to various glycoconjugates demonstrated in other annelids.

## 2. Materials and Methods

*2.1. Source of Worms.* This study was carried out on 20 adult specimens of *Eisenia foetida* (Annelida, Oligochaeta) provided from a local supplier (Burdur, Turkey).

*2.2. Histochemistry.* After killing, the specimens were divided into six regions from anterior to posterior as shown in Figure 1, immediately immersed in Bouin's fluid, and fixed for 18–24 hours at room temperature. After fixation and washing three times in 50% of alcohol, the tissues were dehydrated in series of ascending ethanol, cleared in xylene, and embedded in paraffin. Serial sections, 5–7 $\mu$m in thickness, were collected on albumin-coated slides. For the histochemical study, the methods in Table 1 were followed. The sections were then examined under light microscope (Olympus, CX 41) and photographed using a digital camera mounted on the microscope.

Table 1: Conventional histochemical staining procedures for visualizing and identifying glycoconjugates in epidermal mucous cells of *Eisenia foetida*.

| Procedures | Glycoconjugates unveiled | References |
|---|---|---|
| PAS | Neutral Glycoconjugates | [35] |
| PAS/AB pH 2.5 | To distinguish between neutral and acidic glycoconjugates | [36] |
| AB pH 0.5 | Very sulfated glycoconjugates | [37] |
| AB pH 1.0 | Glycoconjugates with O-sulfate esters | [37] |
| AB pH 2.5 | Acidic glycoconjugates with carboxylated and sulfated esters | [37] |
| AF | Glycoconjugates with sulfate | [38] |
| AF/AB pH 2.5 | To distinguish between sulfated glycoconjugates and acidic glycoconjugates | [39] |
| KOH/PAS | Glycoconjugates with sialic acid residues | [40] |

AB: alcian blue; PAS: periodic acid Schiff reagent; AF: aldehyde fuchsin; KOH: potassium hydroxide.

## 3. Results

Histological and histochemical methods revealed that the epidermis covered by cuticle consists of a monostratified epithelium, which contains many glandular mucous cells that have a variable morphology, and that the vast majority of cells in the epidermis are also composed of these glandular mucous cells. It was observed that mucous cells are predominant in ventral body side and they were also discerned at various stages of mucous release. However, we have not made any morphological discrimination of cell types which may have caused a confusion in the terminology of earthworm histology. On the basis of these findings, the intensity of histochemical reactions is summarized in Table 2.

PAS histochemical staining showed that the epidermis of the first, second, and third body regions contains many strong to stronger PAS (+) cells (Figure 2(a)). In the epidermis of the last three body regions (not shown here), we observed a decrease in the number of stronger PAS (+) cells and an increase in the number of strong PAS (+) cells (Table 2). We determined that strong to stronger KOH/PAS (+) cells appear in epidermal mucous cells of the former three regions (Figure 2(b)) rather than in epidermal mucous cells of the latter three regions (Table 2).

We observed that weaker to weak reactions exist in AF (+) cells of all regions (Figure 2(c)). The PAS/AB pH 2.5

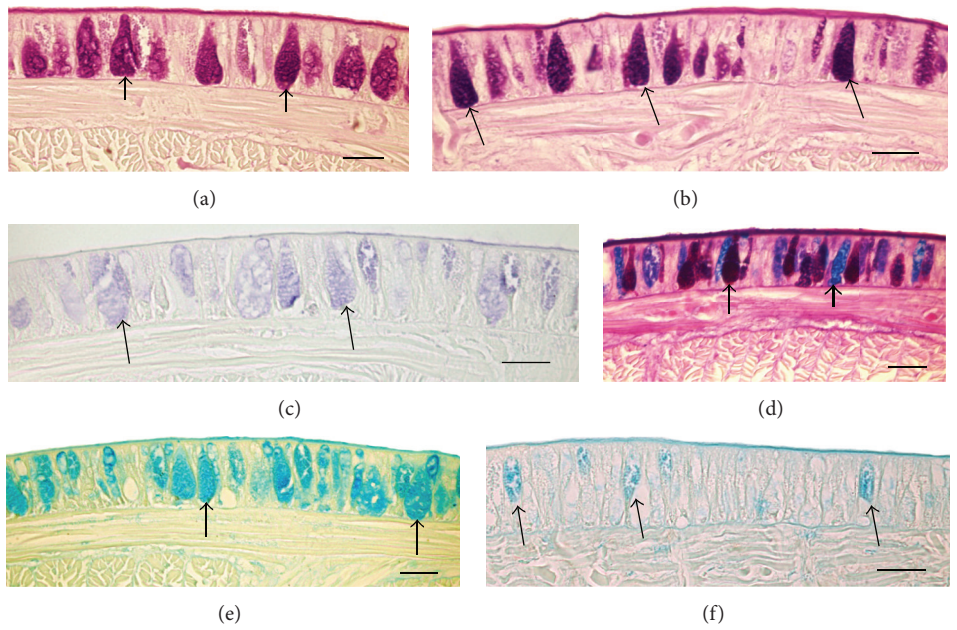


Figure 2: Photomicrographs of glycoconjugates in the epidermal mucous cells of *Eisenia foetida*. (a) PAS staining: neutral glycoconjugates in epidermal mucous cells of second region (arrows). (b) KOH/PAS staining: glycoconjugates with sialic acid residues in epidermal mucous cells of first region (arrows). (c) AF staining: glycoconjugates with sulfate in epidermal mucous cells of second region (arrows). (d) PAS/AB pH 2.5 staining: neutral and acidic glycoconjugates, first region. Thin arrow: PAS (+); thick arrow: AB pH 2.5 (+). (e) AB pH 2.5 staining: acidic glycoconjugates with carboxylated and sulfated esters in epidermal mucous cells of second region (arrows). (f) AB pH 0.5 staining: very sulfated glycoconjugates in epidermal mucous cells of first region (arrows). Scale bar: 50 $\mu$.

Table 2: Histochemical staining properties of glycoconjugates in epidermal mucous cells of *Eisenia foetida*.

| | Body regions illustrated in Figure 1 | | | | | |
|---|---|---|---|---|---|---|
| Technique | First | Second | Third | Fourth | Fifth | Sixth |
| PAS | 4-5[†] | 4-5 | 4-5 | 3-4 | 3-4 | 3-4 |
| AB pH 0.5 | 2-3 | 2-3 | 2-3 | 4 | 4 | 4 |
| AB pH 1.0 | 2-3 | 2-3 | 2-3 | 2-3 | 2-3 | 2-3 |
| AB pH 2.5 | 4-5 | 3 | 3 | 4-5 | 4-5 | 4-5 |
| PAS/AB pH 2.5 | AB[*] PAS[**] | AB[*] PAS[**] | AB[*] PAS[**] | AB[*] PAS[**] | AB[*] PAS[**] | AB[*] PAS[**] |
| AF | 1-2 | 1-2 | 1-2 | 1-2 | 1-2 | 1-2 |
| AF/AB pH 2.5 | AB[+] | AB[+] | AB[+] | AB[+] | AB[+] | AB[+] |
| KOH/PAS | 4-5 | 4-5 | 4-5 | 3 | 3 | 3 |

[†]Reaction intensity: 0: negative; 1: weaker; 2: weak; 3: moderate; 4: strong; 5: stronger; [*]alcian blue dominant (in PAS/AB pH 2.5 staining, it refers mostly to AB (+) cells); [**]PAS dominant; [+]alcian blue dominant (in AF/AB pH 2.5 staining, it refers mostly to AB (+) cells).

application showed that most of mucous cells in epidermis of the first region include both PAS (+) and AB (+) mucosubstances (Figure 2(d)). We also noticed that AB (+) cells and PAS (+) cells individually exist in this region. It was determined that AB (+) cells are increased in the epidermis of the second and fifth body regions. It was found that the cells which contain approximately equal amounts of AB and PAS (+) mucosubstances still predominate in other body regions (Table 2). We observed AB dominance in the mucous cells of all these regions (Table 2).

We observed that the epidermis of the first body region contains many strong to stronger AB (+) cells (Figure 2(e)). A similar staining pattern was also noticed in the epidermis of the fourth, fifth, and sixth body regions (not shown here) (Table 2). On the contrary, a weaker reaction was discerned in the same cells of the second and third body regions (Table 2). AB pH 0.5 application demonstrated that while the epidermis of the former three regions has weak or moderate AB (+) cells (Figure 2(f)) with a granular appearance, this reaction is strong in the latter three regions (Table 2). We also observed

a sharp increase in the number of alcianophilic cells in the latter three regions (not shown here). In AB pH 1.0 application, we observed that weak to moderate AB (+) reactions are in mucous cells of all regions (Table 2).

## 4. Discussion

In this study, we discerned the secretory epidermal mucous cells at different secretion stages in *Eisenia foetida* unlike those reported in epidermis of various oligochaetes in previous studies [18, 19, 22, 26]. Early stages of mucous secretion may have resulted from the initial contact of the worm with the fixative. However, the late stages of release at which the cells are almost entirely void of mucus were also observed and, apparently, the cells released mucus prior to fixation.

In parallel with the studies of Richards [17, 18], Aguirre et al. [2], Dall Pai et al. [23], and Gorgees and Rashan [5], we also clearly noticed that PAS/AB pH 2.5 staining reveals at least three different types of glycoconjugates intermingled in the same cell. As reported by Herlant-Meewis [27], this can be consistent with the fact that the neutral mucous material converts into acidic mucous material.

Unlike the findings of Sutuzani, who studied the histochemical nature of the clitellar mucous cells in a freshwater oligochaete, *Tubifex hattai* [22], we discerned that AB, PAS, and AB + PAS (+) cells are evenly prominent in the epidermis of all regions. This finding can point to the defensive importance of neutral, acid, and mixing glycoconjugates, since Smith [28] suggested that acid glycoconjugates are more defensive than neutral glycoconjugates against intestinal lumen contents. Perhaps they may play a key role in the protection against ectoparasitic agents and xenobiotics exposed to the epidermis of earthworms.

Aguirre et al. [2] demonstrated the presence of neutral and acid mucins in normal skin glands of *Allolobophora chlorotica*. Likewise, we observed that all regions contain moderate to stronger PAS (+) cells. We also discerned that all regions show weak to moderate alcianophilia at pH 0.5 and 1.0 and moderate to stronger alcianophilia at pH 2.5, indicating the presence of both sulfated and carboxylated acid mucopolysaccharides and sulfated and carboxylated sialomucins. In respect to this, Richards [18] suggested that carboxylated and slightly sulfated mucus provides a respiratory film adsorbed to the surface of the cuticle by epicuticular projections. He had also put forward that a secretion rich in proteins could aid water retention in the acidic mucous film or modify the viscosity of the lubricating mucus. On the side, the presence of sulfated and carboxylated sialomucins in secretion of epidermal mucous cells may be significant because these substances are considered to have a special importance in the glycocalyx that is an essential component of most cell membranes [29]. In a sense, sialomucins may undertake an important protective task in oligochaete epidermis. In addition to their protective roles, sialomucins could also have a regulatory function in the oligochaete epidermis.

Dall Pai et al. [23] showed the presence of small amounts of acidic mucopolysaccharides and neutral polysaccharides. Accordingly, we observed a variable degrees of reactions in histochemical staining patterns of acidic mucopolysaccharides and neutral polysaccharides present in epidermal mucous cells. Gorgees and Rashan [5] studied histomorphologically and histochemically the epidermis of the skin from the most anterior, the middle, and the most posterior regions of the body in *Dendrobaena atheca* Cernosvitov. They indicated that mucous cells in the epidermis are quite different from histochemical and histomorphological aspects. They also demonstrated the presence of the acid mucopolysaccharides, which exhibit intense AB-positivity, and of the neutral mucopolysaccharides or glycoproteins, which show weak to moderate PAS-positivity. In partial contrary to the results of Gorgees and Rashan [5], our study showed that acid glycoconjugates with carboxylated and sulfated esters are more dominant than other mucosubstances. Morris [26] indicated the existence of neutral, sulfated, and acidic mucosubstances in the clitellar epithelium of *Eisenia foetida*. We obtained similar findings in epidermis covering the clitellar region corresponding to third region. We also observed that, as well as neutral and acidic glycoconjugates, sialic acid residues predominate in the epidermis of the third region. The presence of sialic acid residues together with sulfated groups is responsible for the negative charge of the glycoconjugates and may conceal receptor sites for viruses and mycoplasma species [30].

In accordance with the findings in the epidermis of a nontubicolous polychaete, *Timarete filigera* [31], the epidermal secretory cells of a tubicolous polychaete, *Branchiomma* [6], and the secretory cells of *Ophryotrocha* sp. [32], we also observed PAS (+) and AB (+) cells in the epidermal secretory cells of *Eisenia foetida* in various degrees. Unlike their findings, however, we did not notice any AB and PAS (−) cells.

Amaral et al. [7] performed metachromatic staining with methylene blue to evaluate distribution of sulfated glycosaminoglycans in internal and external sites of embryonic, newborn, juvenile, and adult specimens of *Eisenia andrei*. They implied that the metachromatic staining shows the presence of sulfated compounds in both clitellar or postclitellar body sections of adult earthworms and that similar metachromatic staining patterns are observed in the juvenile and newborn earthworms. We performed the histochemical staining of mucous cells with AF to demonstrate sulfated glycoconjugates. Weaker to weak reactions are present in all body regions examined. Besides, AF/AB pH 2.5 histochemical staining revealed that alcianophilic cells are dominant in all regions, indicating more acid glycoconjugates than sulfated glycoconjugates. The presence of sulfate esters, however, deserves attention in the epidermis of the boar [33] and fishes [34]. It seems likely that such high acidity of the secretory glycoproteins is essential for the preservation of skin health. It has been demonstrated that the sulfated glycoconjugates can prevent the proliferation of pathogenic microorganisms on the fish and boar scrotal skin surface [33]. A similar function can be attributed to the mucus, which is produced by the epidermal mucous cells in the epidermis of the earthworm. Furthermore, the sulfated groups in the epidermis of the earthworm can be thought of as an indicative of irritation [17]. Thus, these substances may be a part of

the defensive system that has been expected to contribute to protective mechanisms in the epithelial barriers of many invertebrates, including annelids.

## 5. Conclusions

We noticed many types of secretory cells that cannot be easily discriminated especially due to various morphological appearances in the epidermis of *Eisenia foetida* (Annelida, Oligochaeta). The reaction intensity of these secretory cells ranged from 0 to 5. The type, content, and distribution of the glycoconjugates present in epidermal mucous cells varied significantly depending on histochemical methods employed. The observed diversity in the glycoconjugate composition and the types of secretory cells can be an important cue for a variety of vital physiological functions. The different staining patterns of secretory cells also hint at their functional roles with respect to production of mucus with different physical properties. On the one hand, on the basis of histochemical, histological, ultrastructural, and morphological observations of gland cells present in the epidermis of various annelids, especially of oligochaetes, it can be inferred that the types of these secretory cells are different stages of secretory activity of the same cell type or fewer cell types than those described before. For that reason, future immunohistochemical and glycohistochemical studies need to support such a reasonable inference. On the other hand, earthworms have been extensively used as biomarkers or model organisms for assessing the contamination of soil with metals and for addressing the unknown other questions regarding their decomposer functions and sensitive reactions towards environmental effects. With respect to the contamination of soil with heavy metals, the glycoconjugates, particularly sulfated glycosaminoglycans, are possible candidates that may be involved in the processes of uptake of heavy metal ions in earthworms. Large-scale histochemical and glycohistochemical researches on earthworms exposed to different environmental conditions and factors (pollution, parasitic infections, heavy metals, pesticides, herbicides, and xenobiotics) may therefore provide comprehensive information about what effect habitat and living conditions may have on the structure, function, and mucous secretion of the epidermal gland cells, in particular of mucous cells and hence on glycoconjugate composition in the mucous products of these cells.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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