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Morphological Investigation and Functional Aspect of the Skin of the Snout Region in Koi Fish (*Cyprinus carpio*)

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ABSTRACT: *Fish's skin serves a variety of functions that are essential for survival, including communication, respiration, sensory perception, excretion, ion regulation, and heat regulation. This research aimed to examine the snout skin of koi fish (Cyprinus carpio) to determine its structural characteristics. Using light microscopy, the histochemical elements of the skin were examined in a total of 20 adult, healthy koi fish. The skin of was made up of three layers: epidermis, dermis, and hypodermis. The epidermis consisted of epidermal cells, club cells, mucous goblet cells, rodlet cells, eosinophilic granular cells, serous goblet cells, and melanocytes. large amounts mucous of cells which reacted positive to Alcian blue (AB) and Periodic Acid–Schiff (PAS), few club cells which reacted positive to bromophenol blue and light green. many eosinophilic granular cells (EGCs) reacted positive to PAS and light green. Moreover, many taste buds also were demonstrated. The dermis was consisted of collagenous bundles, bundles of myelinated nerve fibers, blood vessels and immune cells. Many telocytes were detected in dermis in close relation to nerve fibers, blood vessels and immune cells. The lateral line system was well-developed in koi fish, where it was arranged into mechanoreceptive superficial and canal neuromasts and electroreceptive tuberous and ampullary organs. In conclusion, the epidermis of snout region in Koi fish consisted of a variety of cells with diverse functions.*

KEYWORDS: Koi, EGCs, club cells, Telocytes, neuromast

1. Introduction

The skin of fish is a significantly large organ that covers the fins and is continuous with the lining of all body openings. Besides the fish skin's protective functions, it is a multi-functional organ where its constituent parts are crucial for communication, locomotion, breathing, sensory perception, excretion, ion regulation, and thermal regulation [1, 2]. Pathogenic organisms are abundant in the aquatic environment [3]. Therefore, the skin of teleosts differs greatly from that of mammals in that it secretes mucus that aids in immunological function and it consider the first line of defense against environmental pathogens invasion [4, 5]. The skin is a self-secreting active organ whose cellular components produce a variety of useful products. Goblet cells produce mucus, which maintains the body surfaces moist and protects them from stressors. Also, club cells secrete alarm substances, which start the alarm reaction. Moreover melanocytes secrete pigments, which provide the fish with distinct colors [6, 7]. Fish skin differs between species because some have scales and others have special cells such as eosinophilic granular cells, rodlet cells, and sacciform cells [8]. The lateral line is divided into two subsystems: the mechanoreceptive neuromasts and the electroreceptive ampullary and tuberous organs [9]. All fish have lateral line systems, but their morphology and distribution vary [10]. Koi carp is an ornamental species of common carp, Cyprinus carpio L., which is a member of the Cyprinidae family with a high economic value [11]. Koi carp have large colorful cycloid scales covering their entire body except for the head, which is scaleless [12]. The snout is the fish's most anterior part (the forward end of the head). In the majority of cases, it is rounded or obtuse. The snout carries paired holes, or nares, that are used to detect odors in the water. Although several previous studies have described the variance of snout structure in many fish species [13, 14, 2, 15], koi fishes gained no attention. Therefore, the aim of this study was to describe the morphology of skin at the snout region

of koi fish (Cyprinus carpio), with a focus on patterns of distribution of sensory structures.

2. MATERIALS AND METHODS

The current study was carried out in accordance with Egyptian animals' laws and relevant "Institutional Review Board" of the Faculty of Medicine in Assiut university, Assiut, Egypt (IRB local Approval number 04-2023-200282).

2.1. Samples collection and preparation

In the present study, a total of 20 healthy mature koi fishes (Cyprinus carpio) were randomly purchased from ornamental fish commercial shops in New Valley governorate, Egypt, and brought to the laboratory. The fishes were anaesthetized deeply with benzocaine (4mg/L) and then were decapitated. To get rid of the mucous coating the surfaces, fish heads and trunks were rinsed with physiological saline (0.9% NaCl). Specimens from the snout skin were dissected and processed for further analyses.

2.2. Histological and histochemical examination

First, small samples for histological techniques were dissected at 1x1x.05 cm and immediately fixed in 10% neutral buffered formalin and aqueous Bouin's fluid for 22 hours. Following this, the fixed samples were subsequently dehydrated in an ascending series of ethanol, cleared in methyl benzoate, and embedded then in paraffin wax. Transverse sections at 5-8µm thickness were cut using Richert Leica RM 2125 Microtome, Germany in the department of cell and tissues, faculty of veterinary medicine, Assiut University. The previously prepared sections were stained with the following stains; Harris Hematoxylin and Eosin stain (Hx and E) for general structure [16], Crossmon's trichrome stain was used to visualize collagenous fibers, cartilage and the muscles [17]. In addition, for carbohydrates histochemistry, representative sections were stained by Periodic Acid –Schiff (PAS) [18] and Alcian blue (AB) technique (pH 2.5) [19], for demonstration of neutral and acidic carbohydrates respectively [20]. For demonstrating protein contents, sections were stained by bromophenol blue [21]. Bancroft's theory and practice of histological techniques were used to prepare all staining [20]. Stained sections were examined using a Letiz Dialux 20 Microscope. Photos were taken using a Canon digital camera (Candison Powershot A95) in the department of cell and tissues, faculty of veterinary medicine, Assiut University.

2.3. Morphometrical analysis

Mean epidermis and dermis thickness (in µm) and mucous and club cell density (per 100 µm2 of epidermis) in the skin of snout region of the koi fish were performed on the images of the light microscopy using Image J processing software. The obtained data were subsequently compared and were presented as mean ± SEM.

3. RESULTS

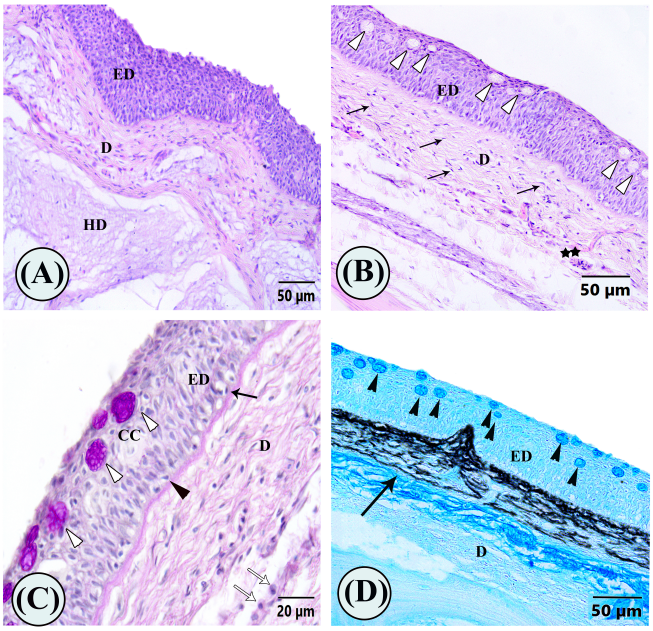

Figure 1: Histological and histochemical analysis of the skin of snout region of koi fish. (A, B): sections stained with hematoxylin and eosin (H&E) showing the organization of skin layers. Note, epidermis (ED) is followed by dermis (D) then hypodermis (HD). Mucous cells (arrowheads) in apical part of epidermis exhibiting negative-staining. Telocytes (arrows) and nerve bundle (asterisks) in dermis (D) were observed. (C) The skin of the snout stained by (PAS) demonstrating negative-stained club cells (CC), and positive-stained mucous cells (white arrowheads) and rodlet cell (arrows) at epidermis (ED). Wavy thick basement membrane (black arrowhead) above the dermal layer (D) was seen. (D) The skin of the snout was stained by alcian blue (AB) depicting positively stained mucous goblet cells (arrowheads) at the epidermis (ED) and continuous layer of melanocytes (arrows) at the dermis (D).

The skin of the snout consisted of epidermis, dermis and hypodermis. The epidermis of the snout formed of

stratified squamous epithelium non-cornified, which was 76.43 µm in thickness. It consisted of basal layer contained simple columnar epithelial cells with oval to rounded nucleus rest on wavey thick basement membrane. Followed by middle layer contained 7-9 layers polyhedral epithelial epidermal cells with central oval to rounded nucleus and between these cells, there were club cells, mucous cells, eosinophilic granular cells and rodlet cells (Fig. 1 A, B & C). Apical layer consisted of several layer of squamous cells with deep basophilic nucleus and contained mucous cells arranged in rows or groups (Fig. 1 B, C & D). Many

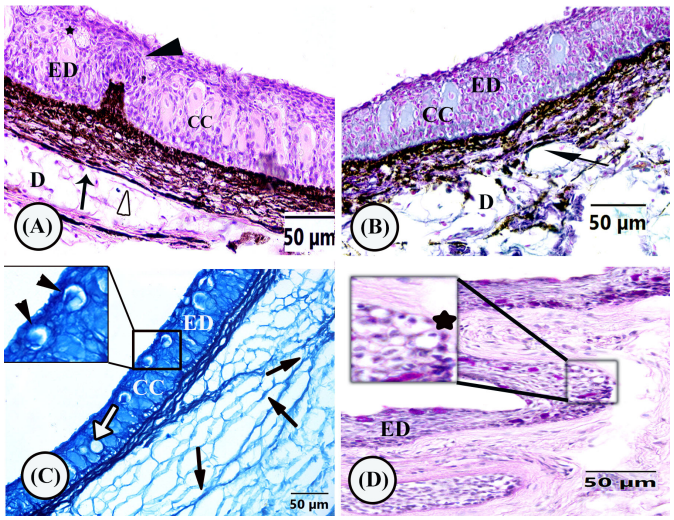

Figure 2: Histological and histochemical analysis of the skin of snout region of koi fish. (A) The skin of the snout stained by H&E showing negatively stained club cells (CC) and mucous cells (asterisk) and tuberous organ (arrowhead) in the epidermis (ED). Melanocytes (arrow) layer in deeper layers of epidermis and dermis (D) was observed. Note, (white arrowhead) indicates telocyte (B) The skin of the snout stained by Crossmon's trichrome exhibiting club cells (CC) positive-stained in the epidermis (ED) and melanocytes (arrow) distributed in the dermis (D). (C) The skin of the snout is stained by bromophenol blue clarifying positive-stained club cells (CC) and sacciform cells (arrowheads) in inserted figure, and negative-stained mucous cells (arrow) in the epidermis (ED). Telocytes (black arrow) make network and react positive to bromophenol blue (D) The skin of the snout was stained by PAS demonstrating rodlet cells (asterisk) in the inserted figure in the epidermis (ED).

mucous cells situated at middle and superficial layers. It appeared as empty space surrounded by thin rim of cytoplasm and had peripheral flattened nucleus by H&E (Fig. 1 B). It was also PAS positive and AB positive (Figure 1B & C). The density of mucous cells was about 11/100 µm^2. Eosinophilic club cells (CC) appeared rounded, oval to elongated in shape with acidophilic cytoplasm and have been found among the intermediate cells by H & E (Figure 2A). They reacted negative to PAS (Fig. 1 C), while reacted positive to light green and bromophenol blue (Fig. 2 B & C) The density of club cells was about 3/100 µm^2. Melanocytes were demonstrated as continuous layer at upper part of dermis (Fig. 2 A, B & 1D), and sometimes at border line between dermis and hypodermis (Fig. 2A). Protein secreting cells (sacciform cells) positively reacted to bromophenol blue (Fig. 2 C). Rodlet cells (RCs) were found in the epidermis, which were distinguished by its ovoid shape, single ovoid to flattened basal nucleus and the presence of cytoplasmic rodlet inclusions (Fig. 2 D & 1C). There were many eosinophilic granular cells (EGCs) identified between club cells and intermediate cells characterized by peripheral nucleus and acidophilic cytoplasmic granules (Fig. 3 A). They reacted positive to PAS and light

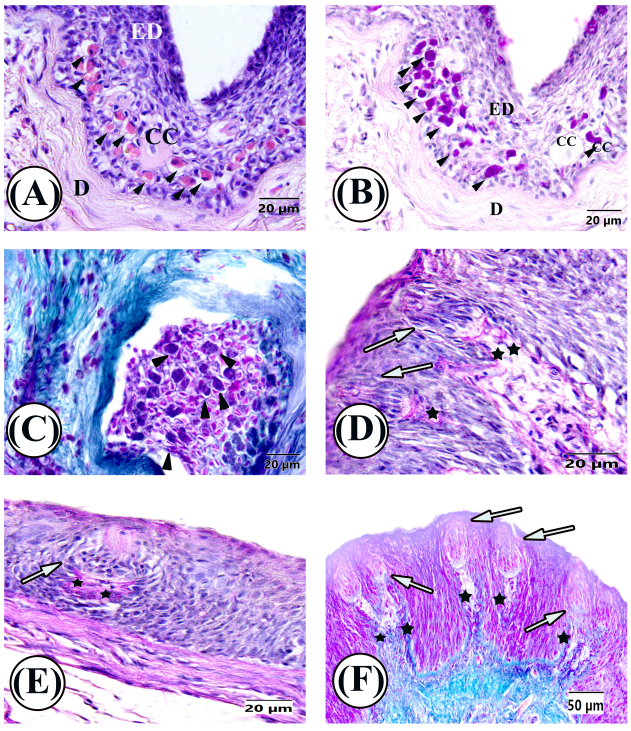

Figure 3: Histological and histochemical analysis of the skin of snout region of koi fish. (A, B) The skin of the snout was stained by H&E and PAS respectively exhibiting club cells (CC) and several eosinophilic granular cells (arrowheads) in the epidermis (ED). (C)The skin of the snout is stained by Crossmon's trichrome depicting positive-stained eosinophilic granular cells (arrowheads). (D, E, F) The skin of the snout was stained with PAS and Crossmon's trichrome, respectively, displaying numerous taste buds (arrows). Note the presence of nerve fibers (asterisks) at the base.

green (Fig. 3 B & C). Several taste buds were found as

pale stained pear-shaped structures. They consisted of columnar supporting cells and had deep basophilic nucleus alternately with fusiform sensory cells had lightly basophilic nucleus and surrounded by marginal cells, and connected to basal pyramidal small cells. There were bundles of nerve fibers connected to taste buds basally (Fig. 3 D, E & F). The lateral line system divided into two subsystems: mechanoreceptive neuromasts and electroreceptive ampullary and tuberous organs. There were 2 types of neuromast: superficial and canal (deep) neuromasts. Several superficial neuromasts (SN) was detected in epidermis of the skin. It consisted of 4 main types of cells: supporting cells, sensory hair cells, basal cells and surrounded by mantle cells of crescent shape. The cupula (gelatinous material dome extending into the surface) covered the superficial neuromast. There were nerve fibers supporting SN basally (Fig. 4 A, B & C) Canal neuromast (CN) was embedded within the dermis forming tunnel-like canals. They were lined by stratified cuboidal epithelium and sensory cells. It was supported by dermal bone (DB). Large canal neuromasts were found in the dermis of the snout (Fig. 4 D). The ampullary organ (AO) was made up of supporting and sensory cells and was covered by a cupula Fig. 5 A). It opened to the surface by ampullary pore (Fig. 5 B). While, the tuberous organ appeared as a spherical mass of sensory cells surrounded by cellular capsule (Fig. 5 C & D). The epidermis was followed by the dermis, which was 364.34 µm in thickness. It composed of two distinct layers; the outer stratum spongiosum (s.laxum) and the inner stratum compactum. Dense regular connective tissue containing undulated compactly arranged collagenous bundles, bundles of myelinated nerve fibers, blood vessels, immune cells and telocytes were observed in dermis (Fig. 4 E & F). Telocytes (TCs) were distinct dermal cells composed of cell bodies and extremely long, branched thin cell processes (telopodes, Tps). TCs was observed in a close relation to nerve fibers, blood vessels and immune cells (Fig. 1 B, 2C, 4A, E & F). The hypodermis layer was made up of loose connective tissue including white adipose tissue.

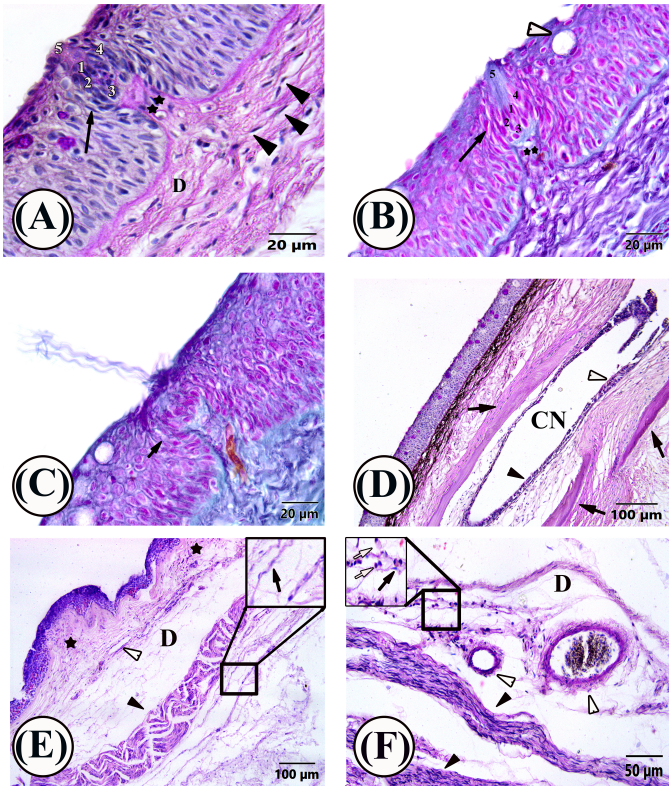

Figure 4: Histological analysis of the sensory structures and the dermis of the skin of snout region of koi fish. (A, B) superficial neuromast (arrow) stained by PAS and Crossmon's trichrome respectively, consisted of (1) sensory cells, (2) supporting cells, (3) basal cells, and surrounded by (4) mantle cells, with attached cupula (5). Note, it was innervated basally by nerve fibers (asterisks). In the dermis, arrowheads represent telocytes. Mucous cell (arrowheads) negative-stained. (C) Superficial neuromast stained with Crossmon's trichrome without attached cupula (arrow). (D)canal neuromast (CN) stained by PAS formed of sensory cells (white arrowheads) and supporting cells (black arrowheads) and surrounded by osteoid tissues (arrows). (E, f) The skin of the snout stained with H&E demonstrating dermis (D) formed of densely packed bundles of collagen fibers (asterisk), a high amount of nerve bundle (black arrowheads), and blood vessels (white arrowheads). Note, telocytes (black arrow) in inserted figures in close relation to nerve fibers, blood vessels and immune cells (white arrow).

4. DISCUSSION

In general fish skin consists of two layers: an outer layer called the epidermis and an internal layer called the dermis, also known as corium layer as observed in Otolithes ruber and Pangasius catfish [22], olive flounder (Paralichthys olivaceus) and black rockfish (Sebastes schlegeli) [23]. Where some fish has skin consisted of three layers: an outer layer called epidermis, middle layer called dermis, and internal layer called hypodermis as observed in African Catfish (Clarias gariepinus) [24, 25, 26], and some hill-stream fishes [27].The current work showed

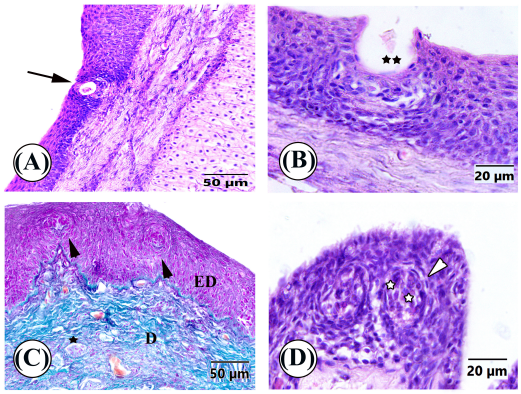

Figure 5: Histological analysis of the sensory structures of the skin of snout region of koi fish. (A, B) The skin of the snout was stained by H & E exhibiting ampullary organ (arrow) and ampullary pore (asterisk). (C) The skin of the snout was stained by Crossmon's trichrome depicting tuberous organs (arrowheads) in the epidermis (ED) and nerve fibers (asterisk) in the dermis (D). (D) Tuberous organs stained by H & E formed of sensory cells (asterisk) and supporting cells (arrowhead).

that skin of the snout region in koi fish (Cyprinus carpio) consisted of epidermis, dermis, and hypodermis. Epidermis was composed of three layers: inner layer, middle layer, and outer layer. The epidermis of snout region in Koi fish contained variety of cells as club cells, mucous cells, eosinophilic granular cells and rodlet cells similar to that demonstrated by [28, 5, 29]. The present study showed that Mucous cells (Ms) of the snout region of the koi were several, and spherical shape. They were situated in the middle and outer layers of the epidermis [26]. They were PAS positive and AB positive suggesting the presence of acidic and neutral mucopolysaccharides. The sulphate groups in glycoproteins gave acidification, which was effective in preventing bacterial and viral invasions as reported by [30]. Fish skin mucus acts as a biochemical, natural, dynamic, physical, and semipermeable barrier which allows nutrients, gases, water, hormones, odorants, and gametes to exchange [31, 29]. Moreover, the present study showed results revealed presence of the club cells, which were large voluminous cells with one or two nuclei that are found in the middle layer of the epidermis [32, 33, 7]. Their cytoplasm reacted negatively to the PAS and AB methods, indicating the absence of glycoproteins in its composition, as observed in Siluriformes [34, 35] and in Cypriniformes [36, 33, 37]. The club cells' cytoplasm reacted positively to the Bromophenol blue procedure, demonstrating a high protein content, as found in Siluriformes [34, 35] and in Cypriniformes [38]. Epidermal club cells (ECCs) or alarm substance cells are thought to be an essential component of the skin of many teleosts [7, 39]. The most important functional role of ECCs is the secretion of alarm substance during a predator attack; which results in intra or interspecific alarm reactions in phylogenetically related species [40, 41, 42].The present investigation revealed that rodlet cells were located basally in epidermis. Rodlet cells are unique cells found only in teleosts and are thought to be part of a generalized host reaction to a variety of stressors, particularly parasitic infection, as well as to toxins, neoplasia, and general tissue damage [43, 44, 45]. The accurate functions of these cells are unknown. They may be included in water or electrolyte transportation, or they may have functions similar to mucous cells, such as lubrication, pH control, and antibiotic effects. They could be modified goblet cells, or they could play vital roles in these cells' ion transport and osmoregulation [46, 47]. Notably, in our work, melanocytes, which are highly branched cells, were randomly distributed in the deep epidermal layers as well as in dermis of the snout region as mentioned by [48], who clarified that Fishes' coloration provides adaptive functions and is crucial for camouflage, aggressive purpose, and courting patterns. Other biological functions that melanin performs include thermoregulation, photoprotection, antibacterial, antiviral, cytotoxicity, anti-inflammatory, radioprotection, and immunomodulation [49, 46, 50, 51, 52]. Moreover, the skin of snout region in koi held protein secreting cells (serous goblet cells or Sacciform cells) which were similar to ordinary goblet cells. They react positive to bromophenol blue the same results was investigated by [53, 54, 2] who prove that Sacciform cells react positive to protein stains. These cells' secretions are serving a variety of functions, including those associated to predation [4]. Sacciform and club cells have recently been interoperated as a storehouse of biologically active substances as well as being linked to specific functions such as defense [55]. On the other hand,

skin of the snout contained large number of EGC. Their nuclei are located eccentrically [56]. The cytoplasm has rounded granules that are stained bright red by H&E due to presence of basic proteins. Furthermore, EGC granules are stained deep magenta with PAS, indicating that they contain sulfated glycosaminoglycans [57, 58, 59]. EGCs are common immune cells found in a variety of tissues in most teleost species, including salmonids and cyprinids [60, 57, 56]. EGCs are thought to be a component of the teleost innate immune system [61]. Many authors have noted the striking histological and biochemical similarities between fish eosinophils and mammalian mast cells including the response to known mast cell degranulating agents, researchers have proposed that these cells may play a role in the induction of an inflammatory response [62, 63, 64, 30, 56]. The stratum compactum of the dermis is responsible for the strength of the skin [65]. Additionally, there were some blood vessels, Melanocytes, and nerve fibers in addition to network of telocytes (TCs) also could be seen the dermis. Canal neuromast is discovered in the dermis recorded by [65, 22, 58]. Telocytes made relations to blood vessels, nerve fibers, immune cells, non-cellular elements, such as collagen and elastic fibers other stromal constituents [66]. The lateral line system in fishes shows physiological and morphological adaptations for perception of hydrodynamic stimuli, and as a result, its anatomy differs greatly between and within species. [67]. Additionally, the fish's skin contains sense organs that allow them to detect predators and foods. Taste buds (TBs) and the lateral line system, which includes electroreceptive organs and neuromasts, are examples of sensory structures [68]. Among the results of histochemical examination, were the presence of TBs in the skin of snout region. TBs are intraepithelial sensory organs that are made up of aggregates of modified epithelial sensory cells, tubular supporting cells, and non-specialized Merkel-like basal cells. Similar results showed by [69, 70, 71, 72, 11, 73, 58]. In current work the two types of neuromasts, superficial neuromasts and canal neuromasts, were demonstrated. Superficial one is located on the skin's surface while the canal or deep neuromast occur in dermis the same results exhibited by[9, 74]. The superficial neuromast comes into direct contact with water, while the canal or deep neuromast occur beneath the skin in canals that connect to the surrounding water through a series of minor pores in the skin. The current study showed the distribution of ampullary organs in the snout region of koi fish. Ampullary organs are made up of an electroreceptor sensory epithelium and supporting cells that are located at the base of a duct full with conductive jelly that leads to a surface pore. Ampullary organs are thought to have a variety of biological functions, including the detection of prey and predators, social communication, and mate detection [75]. Tuberous organs were also investigated in this study. They consisted of receptor sensory cells within a cellular capsule [2]. They play an important role in weak electrical stimuli sensation. They were made up of sensory cells and were surrounded by flattened cells to prevent current from passing to the organ [2]. In conclusion, the epidermis of snout region in Koi fish consisted of a variety of cells with diverse functions.

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