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ORIGINAL ARTICLE

HISTOCHEMICAL EXPRESSION OF TRANSFORMING GROWTH FACTOR BETA AND TUMOR NECROSIS FACTOR ALPHA IN RABBITS (*Oryctolagus cuniculus*) (MAMMALIA: LAGOMORPHA: LEPORIDAE) INFECTED WITH *Sarcoptes scabiei* (ARACHNIDA: ACARI: SARCOPTIDAE)

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ABSTRACT

***Sarcoptes scabiei* infection causes type I and IV hypersensitivity reactions induced by cytokines TGF-β and TNF-α. This study was conducted to analyze the TGF-β and TNF-α expression in rabbits with severe scabies. Five mixed-bred rabbits (3 males and 2 females), with age 12-18 months, were obtained from farms in East Java. Rabbit ear skin samples that showed clinical symptoms of severe scabies (without medical therapy), such as crusts, pus, excessive hyperkeratosis on nose, muzzle, around the eyes, ears and legs were collected. Skin scraping was done to detect the presence of *S. scabiei* mites, and skin samples were stained with hematoxylin eosin (HE) and subjected to immunohistochemistry. Strong staining of TGF-β and moderate staining of TNF-α were evident in all samples. TGF-β was expressed on stratum granulosum to stratum basalis of epidermis layer, half of the dermis, sebaceous gland, and hair follicle, while TNF-α was expressed on half of the epidermis layer, stratum spinosum to stratum basalis, and half of the dermis. This study illustrates that severe scabies infection triggered increased expression of TGF-β and TNF-α in rabbit ear skin, where TGF-β expression was more pronounced than TNF-α.**

Key words: cytokine, immunohistochemistry, *Sarcoptes scabiei*, scabies, TGF-ß, TNF-α

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INTRODUCTION

Scabies is a zoonotic disease caused by the parasitic mite *Sarcoptes scabiei*. It is a highly contagious infection that affects humans, wild animals, domestic animals and livestock (Bandi and Saikumar, 2013). Damage incurred from this infection can result to economic loss for the livestock industry, including rabbit farming industry (Tarigan, 2003; Wardhana *et al.*, 2006; Desiandura *et al.*, 2017).

Sarcoptes scabiei var. *cuniculi*, a round/oval mite, specifically causes scabies in rabbits (Arlian and Morgan, 2017). Its life cycle spans several stages: egg, larva, deutonymph, tritonymph, and adult phase (Scott *et al.*, 2001; Arlian and Morgan, 2017). Mites mate on the host epidermis, and male mites explore the skin continuously for several days to find female mites (Orkin and Maibach, 1985). Female mites lay 2-4 eggs every day. Eggs are placed 1 cm inside the stratum corneum, and, sometimes, mites construct a tunnel to reach the stratum granulosum of the epidermis (Orion *et al.*, 2006). Scabies infection can be

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classified as mild, moderate and severe (Davis *et al.*, 2013). Severe stage is characterized by the presence of crusts, pus, and excessive hyperkeratosis on the nose, muzzle and around the eyes, ears and legs (Sofyan and Chrismanto, 2017).

Scabies transmission to humans happens because of direct contact with the infected animal, causing pruritus (itch) and irritation due to hypersensitivity reactions to mites (Bandi and Saikumar, 2013). Specifically, types I and IV hypersensitivity reactions are involved in this process (Rook *et al.*, 1972; Hick and Elston, 2009), which are mediated by the cytokine tumor necrosis alpha (TNF-α) (Rook *et al.*, 1972; Mullins *et al.*, 2009; Baratawidjaja and Rengganis, 2014; Bhat *et al.*, 2017) and transforming growth factor-beta (TGF-ß), respectively (Tizard, 2004).

Bhat *et al.* (2017) stated the important role of the activated mast cell in producing histamine and TNF-α as an early inflammatory response to scabies infection. Janeway *et al.* (2001) reviewed that in type I hypersensitivity reaction, Th2 cell is involved in immediate hypersensitivity reaction initiation by stimulating IgE production and promoting inflammation. It then produces a large amount of cytokines (IL-4, IL-5 and IL-13). Interleukine (IL) 4 stimulates B cell to produce IgE. Mast cell and basophil have a receptor called FcεR1 that specifically recognizes IgE. Once IgE is bound to FcεR1, this activates transduction signal to mast cell cytoplasm, causing degranulation of mast cell and release of the active mediator in cytokines (TNF, IL-1, IL-4, IL-5, and IL-6) and chemokines, which have an important role in type I hypersensitivity reaction.

Mangan (2006) noted that type IV hypersensitivity reaction has two stages: the first stage is proliferation and differentiation of CD4 T cells and the second stage is CD8+ T cell reaction. Proliferation and differentiation of CD4+ T cells will recognize the antigen presented by the dendritic cell (APC). APC produces the cytokines IL-1, IL-6, IL-12 and IL-23. When these cytokines interact with TGF-ß, this will stimulate the T cell to differentiate into Th17 cell, and the activated Th17 cell will produce IL-17 (Mangan, 2006).

Research on immunohistochemical cytokine expression in animal skin infected with scabies, especially in rabbits, has never been done. Therefore, this research intended to determine the extent of TGF-ß and TNF-α expression in rabbit skin infected with severe scabies.

MATERIALS AND METHODS

Samples

This research was carried out from January to February 2018 using five, 12-18 month-old, mix-bred rabbits (3 males and 2 females) obtained from farms in East Java, Indonesia. Tissue histology was done on rabbit ear skin tissues that showed clinical symptoms of severe scabies but without medical therapy. Symptoms include presence of crusts, pus and excessive hyperkeratosis on nose, muzzle, around the eyes, ears and legs. This study was conducted at the Veterinary Pathology Laboratory and Veterinary Parasitology Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga.

Ethical approval

This research has been approved by the ethical committee of the Faculty of Veterinary Medicine, Universitas Airlangga, Number: 630-KE, in accordance with the rules on animal testing and usage.

Parasitological examination

Rabbit ear skin was scraped from *S. scabiei* mites. Scraped skin sample was purified using 10% KOH solution, placed on a glass object and examined under a microscope at 100× magnification. Identification of *S. scabiei* was based on Soulsby (1982).

Histopathological examination

Skin tissue was fixated in 10% formalin, and the histology slides were made by embedding rabbit skin tissue (1 cm × 1 cm) on paraffin block. Afterwards, skin tissue was cut 4 µm thick with a microtome, then samples were submerged in water bath at 55°C. Tissues were picked up with a glass object slide and dried in 40°C hot plate overnight, deparaffinated and rehydrated. A slide was placed on a slide holder, soaked gradually in

xylol solution twice, each for 5 min, then soaked incrementally in alcohol (96%, 90%, 80% and 70%) for 4 min. The slide was then washed (immersed) with water for 5 min and stained with hematoxylin eosin (Merck, Germany), and finally dehydrated, cleared and mounted.

Immunohistochemical examination

Immunohistochemistry was performed using LSAB kit from Dako® (Carpinteria, California, USA). The skin tissue in paraffin block was cut 4 µm thick, deparaffinated, rehydrated, incubated with primary polyclonal antibody TGF-ß or TNF-α (Santa Cruz Biotechnology Inc., Dallas, Texas, USA) and antibody titer at 1:100. Procedure was carried out following the manufacturer's protocol. Finally, tissue was counterstained with hematoxylin (Merck, Germany).

Histologic slides were observed under a light microscope (Nikon® E-100, Japan) at 100× and 400× magnification, and photos were taken with a camera (OptiLab® MTN001, Indonesia). Histology data of rabbit skin with immunohistochemical staining was presented according to Nassef *et al*. (2016). Positive expression of cytokines TGF-ß or TNF-α was denoted by brown colored cells in histologic tissue. Intensity of cytokine expression denoted by brown colored cells was categorized into strong, moderate, weak and negative, or, by percentage, corresponding to ≥50%, 20-50%, <20%, and absence of brown colored cells per field, respectively (Nassef *et al*., 2016).

All histologic data were presented descriptively.

RESULTS AND DISCUSSION

Scabies is a common skin disease that can be easily transmitted in environments with poor sanitation, especially in less developed countries. Common symptoms of scabies are pruritus and scabs, where scratching could result in secondary infection (Feldmeier *et al*., 2009). Severe cases of scabies are marked by crusts, pus and excessive hyperkeratosis on nose, muzzle, around the eyes, ears and legs (Sofyan and Chrismanto, 2017).

As seen in Fig. 1, all samples manifested cytokine expression: *S. scabiei* mites were found in the stratum corneum layer up to the stratum granulosum of the epidermis (Fig. 1b, 1c), characterized by hypergranulosis (Fig. 1b), spongiosis (Fig. 1b, 1d), acanthosis (Fig. 1b, 1c), epidermal tunnel (Fig. 1c), hyperkeratosis (Fig. 1b), abscess, elongation of rete ridges, dermal infiltrate and vascular proliferation. It can be said then that rabbits in this study were infected with severe scabies. Similar findings have also been reported by Mittal *et al*. (2004), Orion *et al*. (2006), Falk and Eide, (2008), Bhattacharjee and Glusac (2010), Nassef *et al*. (2016) and Salvadori *et al*. (2016). These studies found *S. scabiei* mites on the epidermis, and histologic changes seen were hyperkeratosis, hypergranulosis, spongiosis, acanthosis, epidermal tunnel, elongation of rate ridges, dermal infiltrate, vascular proliferation and parakeratosis.

Immunohistochemical images of rabbits infected with severe scabies revealed strong staining on TGF-ß and moderate staining on TNF-α. Cytokine TGF-ß expression is illustrated in Fig. 2. Strongly stained TGF-ß was evident in the epidermis layer (Fig. 2b, 2c, 2d), stratum granulosum to stratum basalis (Fig. 2c, 2d), part of the dermis (Fig. 2b, 2d), part of the sebaceous gland (Fig. 2c, 2d) and hair follicles (Fig. 2b).

Strong expression of TGF-ß in this study is consistent with the research conducted in humans (Walton *et al*., 2008) and dogs (Singh *et al*., 2014). According to Bhat *et al*. (2017), TGF-ß expression triggers inflammation response in scabies infection.

Fig. 3 shows the moderate expression of cytokine TNF-α in rabbits with severe scabies. Cytokine TNF-α was found in the epidermis (Fig. 3b, 3c, 3d), stratum spinosum to stratum basalis (Fig. 3b, 3c), and part of the dermis (Fig. 3b, 3c, 3d), but none was found in the hair follicle and sebaceous gland. This result differs with the research done by Bandi and Saikumar (2013), wherein they reported strong expression of TNF-α during the early stage of infection. Notably, Walton *et al*. (2008) did not find cytokine TNF-α in severely infected patients, implying that TNF-α expression depends on the progression of the disease and the infected host.

As stated earlier, cytokines TGF-ß and

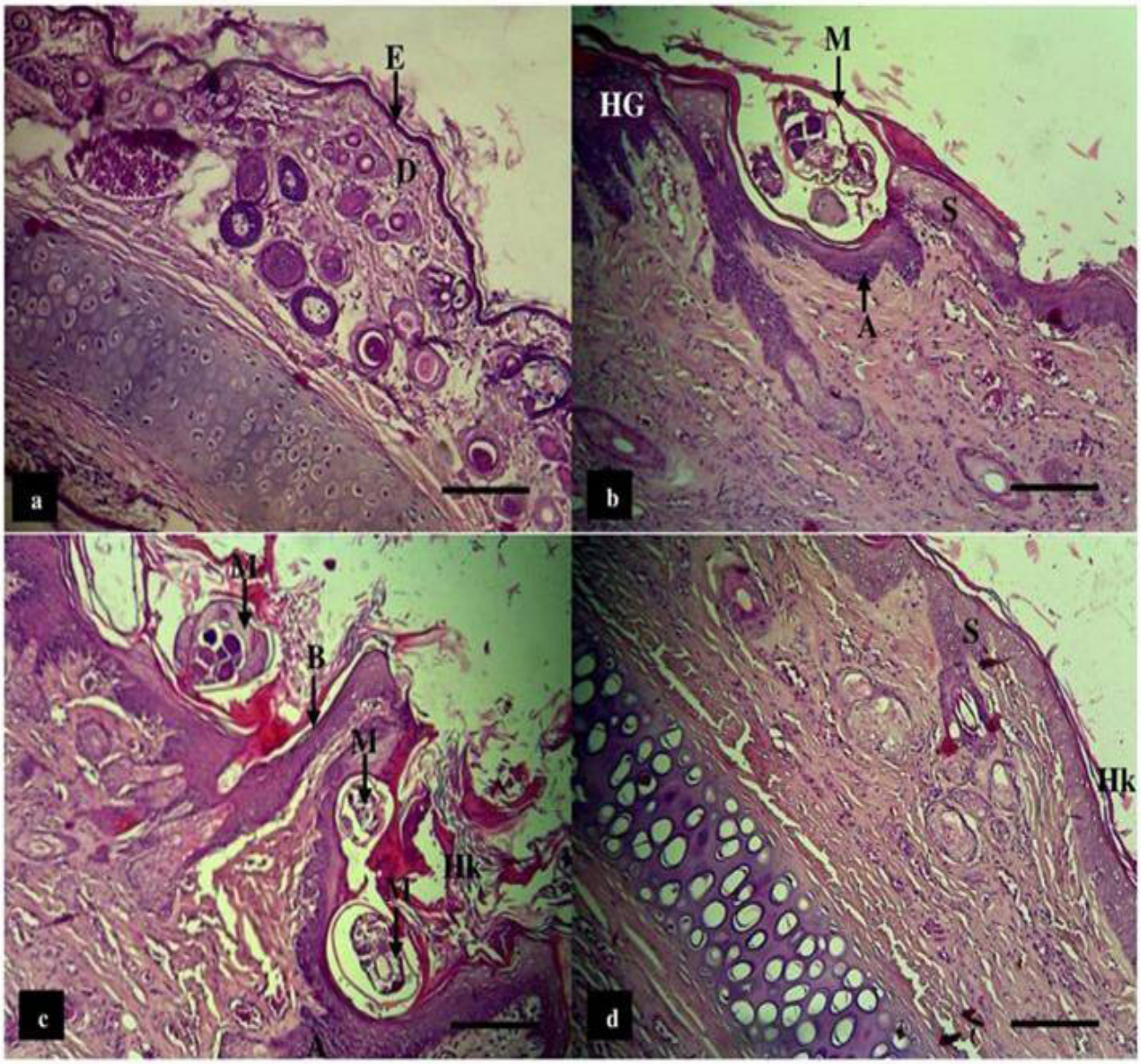

Fig. 1. Histology of rabbit skin stained with hematoxylin and eosin. a: control; b-d: *S. scabiei* infected skin. A: acanthosis, B: epidermal tunnel, D: dermis, E: epidermis, HG: hypergranulosis, HK: hyperkeratosis, M: *S. scabiei* mites, S: spongiosis (bar = 100 µm).

TNF-α play important roles in type I and IV hypersensitivity reactions, respectively (Tizard, 2004; Bhat *et al.*, 2017). Thereby, their increased expression in rabbits with scabies can potentially trigger these hypersensitivity reactions. With type I reaction, mites antigen and immunoglobulin E on mast cell in the epidermis cause the degranulated mast cell (Rook *et al.*, 1972) to release TNF-α, along with other cytokines (Baratawidjaja and Rengganis, 2014). Meanwhile, type IV hypersensitivity reaction is mediated by the activation of Th1 or Th17 cells, where Th1 cells link with IFN-γ as a result of TGF-β activity (Tizard, 2004). Considering the findings of Mounsey *et al.* (2015), the researchers hypothesized that high level of TGF-β in this study activated the Th17 cell to release IL-17, causing type IV hypersensitivity reaction. Mounsey *et al.* (2015) found increasing amount of cytokine IL-17 in pigs with severe scabies. Based on Bhat *et al.* (2017), secretions of IL-6, TGF-β

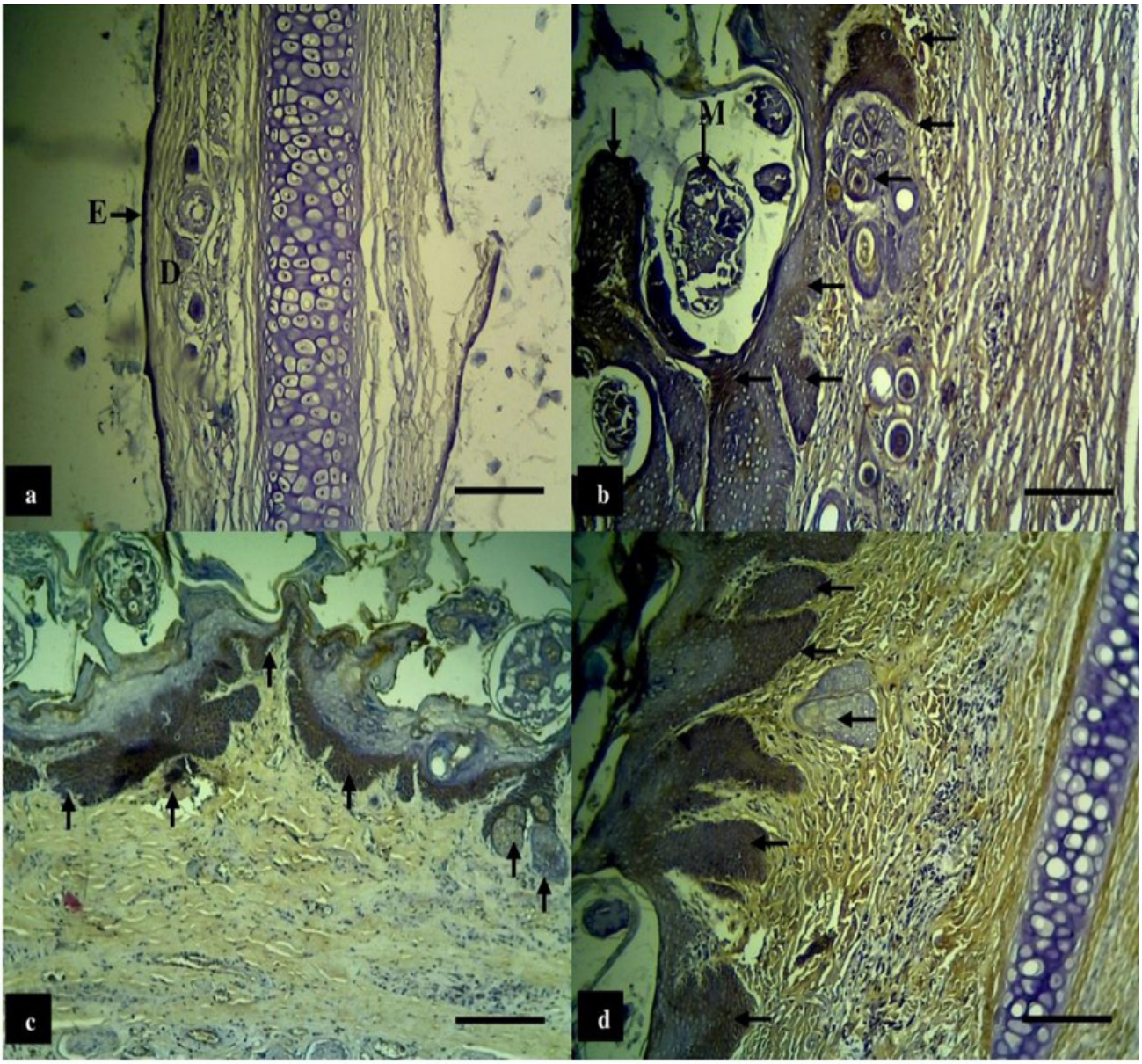

Fig. 2. Histology of rabbit skin stained with anti TGF-ß antibody. a: control, b-d: *S. scabiei* infected skin. Arrow pertains to cells that expressed TGF-ß (bar = 100 µm).

and IL-23 trigger Th17 or Tc17 differentiation and production of IL-17 (McGeachy and Cua, 2008). In detail, TGF-ß and IL-2 induce Tregs cell. Tregs produces TGF-ß and IL-10, possibly contributing to delayed inflammatory response in scabies and thus curbing inflammation (Bhat *et al*., 2017). Ohno *et al*. (1996) reported that eosinophils express TGF-ß that can depress local inflammation response and regulate the activity and growth of T cell (Tregs). Eosinophils that produce TGF-ß can inhibit the differentiation from naive T lymphocytes to Th1 or Th2 (Jacobsen *et al*., 2007). If the number of eosinophils on scabies patient is high, as seen in the study of Sluzevich and Lucky (2007), this could translate to a concomitant increase in IL-17 produced by Th17 (Dias and Banerjee, 2013), causing type IV hypersensitivity reaction.

This study showed that severe scabies in rabbits enhanced the expression of cytokines TGF-ß and TNF-α, wherein TGF-ß expression

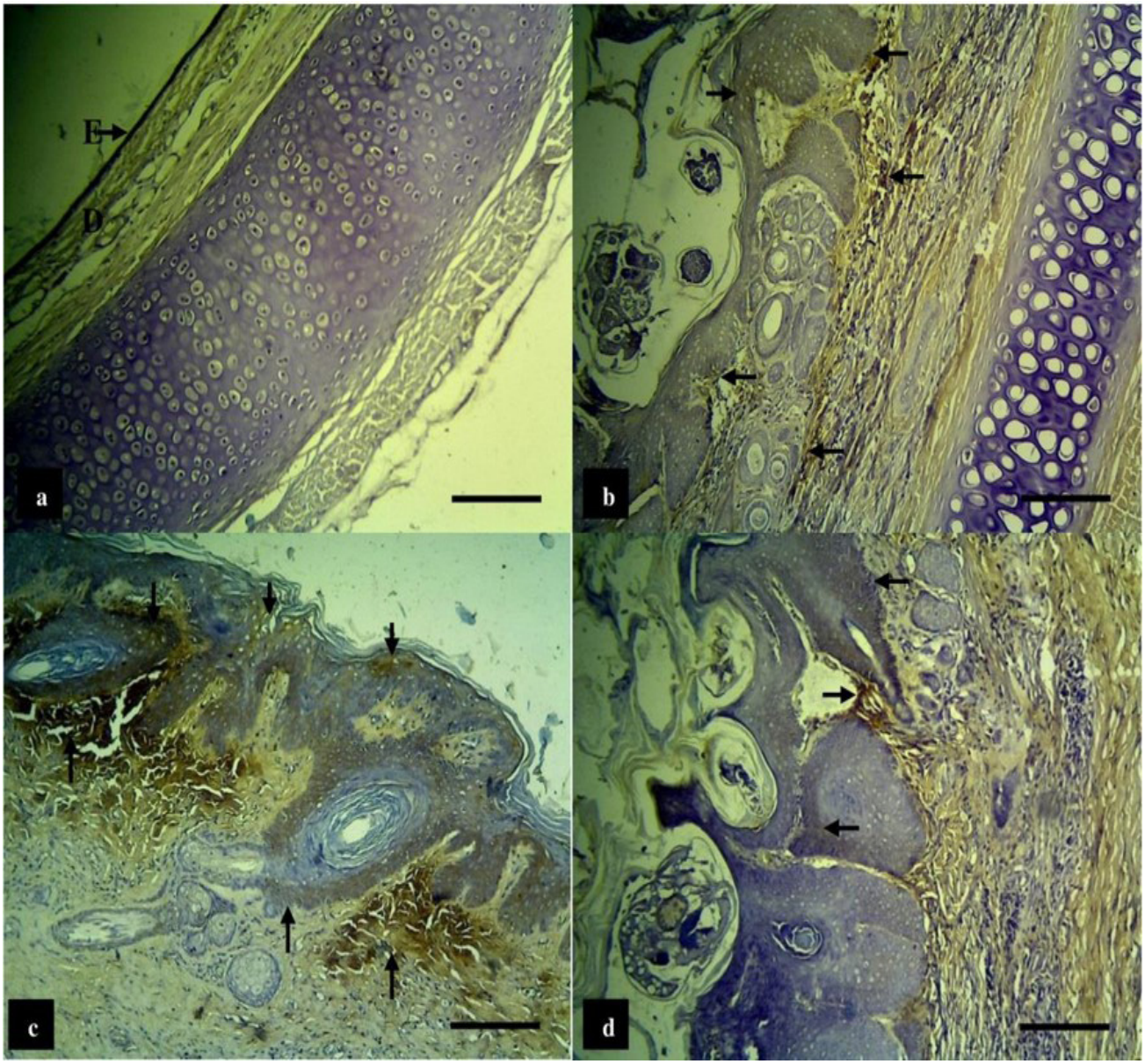

Fig. 3. Histology of rabbit skin stained with anti TNF-α antibody. a: control, b-d: *S. scabiei* infected skin. Arrow pertains to cells that expressed TNF-α (bar = 100 µm).

was more pronounced. Since this expression is tied to hypersensitivity reaction types I and IV, it is necessary to consider treatment in rabbits by suppressing cytokine expression, especially TGF-ß, as a means to control scabies.

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