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Vitiligo in Arabian Horses: Histopathological, Immunohistochemistry and Electron Microscope Investigations

Research Article

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Abstract

Vitiligo is a skin disorder associated with the loss of melanocytes and the development of depigmented patches appearing as white spots. Little information about vitiligo within the Arabian horse population is available. In this study we try to present a pathological and hematological approach for vitiligo status within the Arabian grey horses in a Private Arabian horse farm with a population of one hundred Arabian horses (80 mares and 20 stallions). Six gray Arabian mares (4-8 years old) were developed small multifocal de-pigmented areas around the facial and neck regions. Skin biopsies were taken from the neck skin of the vitiligo affected mares (lesional and adjacent non-lesional) for histopathology, immunohistochemistry (IHC) and electron microscopy (EM) investigation. The studies revealed normal occurrences of melanin pigment granules in the non-lesional skin with gradual loss in the direction of adjacent lesional skin areas. IHC investigation revealed down-regulation of Bcl-2 and HMB-45 in the vitiligo-lesional area, while TNF-α revealed up-regulation in comparison with the adjacent non-lesional area.

EM investigation detected an abundance of Langerhans cells (LC) cells in the deeper layer of st. spinosum. Blood investigation showed no variation regarding the total leukocyte counts, nor the absolute differential counts between vitiligo and healthy mares. Flow-cytometry revealed no difference in CD4+, CD8+ lymphocytes and CD4/CD8 ratios between vitiligo and healthy mares. A treatment trial using topical daily application of Tacrolimus ointment, immune-modulator, on vitiligo area of affected mares was carried out for sixteen weeks, showed gradual improvement of the affected facial areas and re-pigmentation was detected through the treatment course. Observation of those horses for 8 months after stopping the treatment showed continuing improvement of the lesions.

Keywords: Arabian horses; TNF-α; Bcl-2; HMB-45; Ultrastructure; T cell lymphocytes

Introduction

Vitiligo is an autoimmune disease of the skin which affects man and animals [1] Vitiligo occurs in horses, dogs, cats and pigs as an autoimmune condition that destroys the skin melanocytes [2]. It has affected several breeds of horses: Gelderlands, Spanish, Welsh ponies, Arabians, Belgians, Oldenburg, Mecklenburg, gray horses and quarter horses [3-6]. There is no available data regarding the incidence of vitiligo in horses worldwide. Vitiligo was reported to account for 0.7% of equine dermatoses examined at New York Cornell University [7]. In Egypt, there was no published work on vitiligo among the Arabian horse population.

Vitiligo is a multifactorial dermatologic disorder characterized clinically by white patches on the skin, either segmental or generalized, with different patterns of distribution. Several theories try to investigate vitiligo pathophysiology. There are accepted theories for vitiligo pathogenesis including biochemical and autoimmune, in addition to adhesion defect and oxidative stress effect [8] but autoimmune theory has been the leading one [9-11] as either a primary or secondary factor. Vitiligo is usually diagnosed clinically as patches of depigmentation appearing as white spots. Histopathology and EM studies are used as confirmatory diagnostic techniques. There is controversy over whether melanocytes in vitiligo lesions are actually lost or are still present but inactive. IHC assay demonstrates decreased melanocytes

within lesional skin and the presence of immune cells in the upper dermis and epidermis [12].

The aim of this study is to investigate the appearance of vitiligo among Arabian grey horses in Egypt for the first time, covering clinical signs and possible pathogenesis using histopathology, IHC and EM studies; in addition to analysis of CD4 & CD8 in peripheral blood. A treatment trial of vitiligo using topical application of Tacrolimus 0.03% ointment was carried out, as well as evaluation of its effectiveness to arrest progression and restore skin re-pigmentation.

Materials and Methods

Our study was conducted on a private Arabian horse farm with a total number of one hundred Arabian horses (80 mares and 20 stallions) in which six vitiligo gray mares (age 4-8 years) had clinically developed small multifocal depigmented areas around the facial and neck regions. The history of vitiligo appearance in Egypt was associated with the importation of European gray stallions for breeding selection in some Egyptian private Arabian horse farms beginning in the year 2000 (personal communication). The appearances of vitiligo in some offspring of the following generation were detected. Skin biopsies were taken from both lesional and adjacent non-lesional vitiligo skin from the neck of the six vitiligo mares using a five millimeter punch under local anesthesia. The biopsy was divided into two parts: one half was kept in 10% neutral formalin for histopathology and IHC studies; the other half was kept in 3% buffered glutaraldehyde for EM investigation.

Pathological & IHC Investigation

Tissue samples fixed in neutral formalin were processed for hematoxylin and eosin (H&E) and Fontana-Masson's stains [13]. Other tissue sections were cut on coated positive charged slides for IHC. Tissue sections were investigated using primary antibodies of Bcl2, TNF-α, HMB-45, and Avidin-biotin-peroxidase complex (Vectastain ABC Kit Standard, VectorLaboratories) was used according to the manufacturer's instruction protocol.

EM Investigation

Samples for transmission electron microscopy (TEM) preserved in 3% glutaldehyde were prepared and processed [14].

Hematological Examinations

Blood samples were collected on EDTA from the six vitiligo mares and from three with healthy skin appearance (control group) for determination of total leukocytes and differential leukocytes using BC-3000 plus auto hematology analyzer. The flow cytometry analysis of CD4 and CD8 T cells was performed on fresh blood collected on heparin from vitiligo mares and from three healthy mares using BD FACSCanto II Flow Cytometry System. Flow cytometry CD4 and CD8 monoclonal T Cell Kit were obtained from Merek KGaA, Germany.

Treatment Trial

Daily topical application of Tacrolimus 0.03% ointment (Glenmark©) on the depigmented areas of the six vitiligo affected mares was carried out for 16 weeks with follow up for one year after the start of treatment.

- Statistical analysis: All data was analyzed using the T test by following the statistical methods [15].

Results

Developed Gross investigation of affected mares revealed multifocal un- depigmented with irregular boundaries mainly on muzzle, around eyes and even all over the face (Figure 1). Regarding the histopathological examination of vitiligo-depigmented skin in comparison with adjacent normal pigmented areas in all investigated cases, the characteristic histopathological finding of vitiligo skin was hyperkeratosis and focal epidermal hyperplasia (Figure 2a). These changes were associated with partial to complete loss of melanin pigment granules in the epidermis and corresponding skin adnexa (Figure 2b). Vitiligo skin revealed numerous numbers of dendritic Langerhans cells (LC) throughout the supra-basal epidermal layer (Figure 2c). The dermo-epidermal junction showed mild edema and round cell infiltration (Figure 2d). By application of Fontana- Masson stain, melanin granules were detected in the melanocytes of normal non-lesional skin while melanin granules were absent in adjacent Vit. lesional skin (Figure 3a & b).

Figure 1: Gross Investigation demonstrated un-pigmented areas with irregular boundaries mainly on muzzle, around eyes and even all over the face.

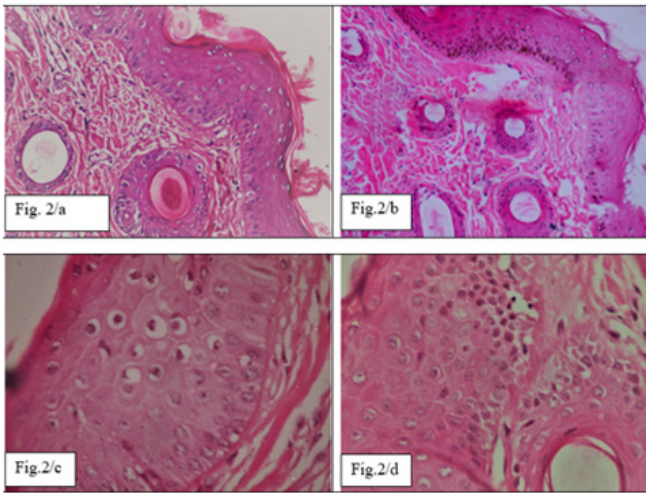

Figure 2: Epidermal layer at the junctional area between vitiligo and non-vitiligo area showing

a. Hyperkeratosis and focal epidermal hyperplasia (H&EX400).

b. Gradual partial till complete loss of melanin pigment in epidermis and corresponding dermal skin adnexa (H&EX200).

c. Numerous dendritic Langerhans cells within the supra-basal epidermal layer (H&EX1000).

d. Mild edema with round cells infiltration at the epidermal-dermal junction (H&EX400).

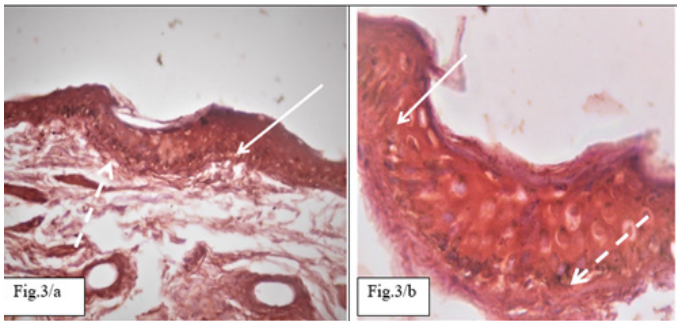

Figure 3: Epidermal layer showing melanin granules within the melanocytes of normal non-lesional skin area (dashed arrows), while melanin granules were absent in adjacent vitiligo lesional skin (thin arrows), (a &b) (Fontana-Masson, a X 200 & b X1000).

The epidermal changes were associated with degeneration and/

or necrosis of corresponding hair follicles in the dermal layer (Figure 4a). In addition, there was inflammatory cell infiltration with the predominance of round cells and few numbers of polymorphic cells; some cases demonstrated a perivascular inflammatory cell infiltration pattern (Figure 4b).

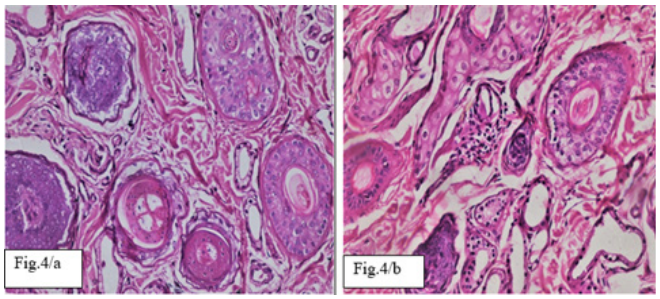

Figure 4: Dermal layer of vitiligo skin showing

a. Degeneration and necrosis of hair (H&EX400).

b. Peri-vascular inflammatory round cells infiltration (H&EX400).

IHC investigations revealed strong expression of Bcl-2 in non-lesional skin areas and a marked loss in lesional vitiligo skin (Figure 5a) and corresponding dermal skin adnexa (Figure 5b). Referring to TNF-α, our IHC results demonstrated partial to complete absence of TNF-α within the melanocytes of the epidermal basal cell layer of lesional skin areas compared with non-lesional skin areas (Figure 5c). Expression of HMB-45 was detected in the non-lesional epidermal cell layer and its corresponding skin adnexa of the dermal layer, but partial to complete loss of its expression was noticeable within vitiligo lesional areas (Figure 5d). EM study of the non-lesional skin area revealed keratinocytes laden with melanin pigment granules (Figure 6a) while there was partial loss of melanin granules within the keratinocytes in the vitiligo skin (Figure 6b). Vitiligo Skin showing keratinocytes free from melanin granules (Figure 6c).

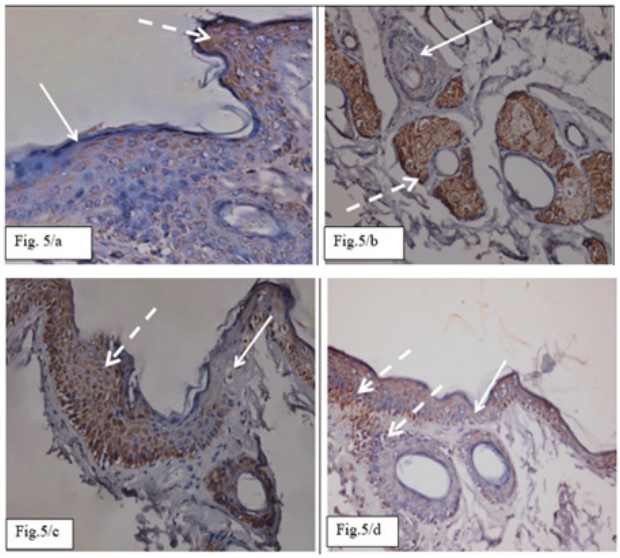

Figure 5: IHC showing

a. Strong expression of Bcl-2 within the epidermal melanocytes of non-vitiligo skin (dashed arrow) and partial loss in adjacent vitiligo area (arrow), X400.

b. Same reaction in corresponding dermal hair follicles and sebaceous glands (dashed arrow) (IHC/peroxidase X 400).

c. TNF-α showing strong expression within melanocytes of non- vitiligo skin (dashed arrow), while lost in adjacent vitiligo area (arrow) (IHC/peroxidaseX200).

d. HMB-45 showing expression within the non-vitiligo epidermal cell layer and corresponding dermal adnexa (dashed arrows), and partial loss of expression in vitiligo epidermal and dermal melanocytes (arrows) (IHC/peroxidase X 200).

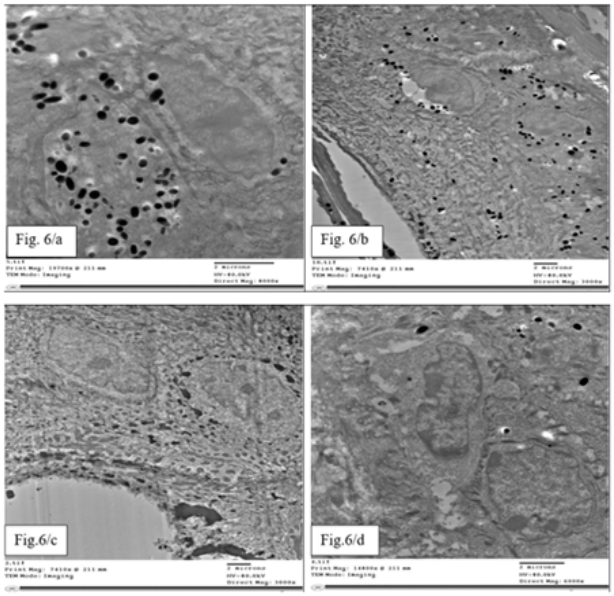

Figure 6: EM

a. Normal skin area showing numerous keratinocytes laden with melanin pigment granules.

b. Vitiligo skin showing partial loss of melanin granules within the keratinocytes.

c. Vitiligo Skin showing keratinocytes free from melanin granules.

d. Vitiligo Skin showing presence of Langerhans cells in the deeper layer of st. spinousum, these cells characterized by translucent cytoplasm and lobulated nucleus, scare melanin granules within adjacent keratinocytes.

In lesional areas LC were prominent in the deeper layer of st. spinousum, membrane, with characteristic translucent cytoplasm and lobulated nucleus (Figure 6d). Blood investigation showed no variation of the total leukocyte counts, nor the absolute differential counts between vitiligo and healthy mares with the exception of a slight increase of monocytes. Flow cytometry analysis of CD4+ and CD8 + T cell subtypes also revealed no difference in CD4+ and CD8+ T cell lymphocytes and CD4/CD8 ratios between vitiligo and healthy mares (Tables 1 & 2).

Treatment Trial: Gradual improvement of affected areas as re-pigmentation was detected along the treatment course for sixteen weeks using topical application of 0.03% Tacrolimus ointment as presented in (Figure 7). Observation of those horses for 8 months after stopping the treatment showed continuing improvement of the lesions.

Table 1: Leukocytes and differential absolute counts of vitiligo and control mares.

	Control Cases (n3)	Vitiligo Cases (n6)
White blood cell count	8900 +/- 57.74	8950 +/- 76.38
Neutrophil	6292 +/- 60.38	6281 +/- 70.31
Lymphocytes (103/ml)	1745 +/- 47.36	1724 +/- 22.96
Monocyte (103/ml)	459 +/- 12.00	570 +/- 47.36 *
Eosinophil (103/ml)	376 +/- 33.65	399 +/- 30.32
Basophil (103/ml)	0.00	0.00

Values represent the mean +/- St. Er

* Significant difference of vitiligo against control cases by t-test at P< 0.05.

Table 2: CD4 and CD percentage, absolute value and ratio of Vitiligo and control Mares.

	Control Cases		Vitiligo Cases	
	Percentage	**Number**	**Percentage**	**Number**
CD4	45.6 +/- 0.88	4064 +/- 74.00	44.5 +/- 1.40	3988.8 +/- 120.19
CD8	23.3 +/- 1.33	2078 +/- 131.15	22.3 +/- 0.71	2002 +/- 62.89
CD4/ CD8 ratio	1.96 +/- 0.10	1.94 +/- 0.032		

Values represent the mean +/- St. Er

No significant difference of vitiligo against control cases by t-test at P< 0.05.

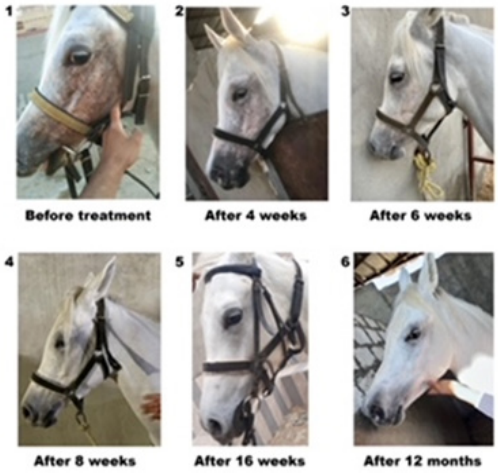

Figure 7: Showing the progress in re-pigmentation throughout the treatment period with complete re-pigmentation at 16 week and after 12 months

Discussion

The current investigation detected the presence of six vitiligo mares in a private farm of one hundred Arabian horses. The history of vitiligo appearance in Egypt was associated with the importation of European gray stallions for breeding selection in some Egyptian private Arabian horse farms beginning in the year 2000 (personal communication). The appearance of vitiligo in some offspring of the following generation supports the genetic hypothesis. Human familial risk of vitiligo among first degree relatives has been previously documented [16,17]. Clinical signs are associated with the appearance of localized white patches which corresponded with the loss of functional epidermal and dermal melanocytes [18]. The pathogenesis of vitiligo in humans and animals was suggested to have the same pathophysiology and reported that dogs, cats and horses with vitiligo have antibodies against melanocytes [3]. In this respect, an increase in auto antibodies against melanin tyrosinase was detected [5]. An overview of our results was in accordance with previous series of research in horses and humans which reported vitiligo as a disorder with epidermal and hair de-pigmentation caused by melanocyte loss and characterized histologically by infiltration of T-lymphocytes (active or generalized form), LC proliferation, vacuolation of epidermis and degeneration of cutaneous nerve fibrils [5,19]. Lesional skin infiltration by inflammatory cells, dendritic cells, macrophages and natural killer cells was previously described [20]. Cytotoxic CD8+ T cells have been detected in the lesional dermis and epidermis, with close adjustment to the degenerated melanocytes; they infiltrate the perilesional margin and lesional skin and in turn attack, destroy and eradicate pigment cells [21] the histological features of vitiligo skin were illustrated by loss of melanocytes in the epidermis and/or hair follicles [2].

Our investigation using Fontana-Masson's stain revealed normal occurrences of melanin pigment granules in the non-lesional skin with gradual to complete loss in the direction of adjacent lesional skin areas which were reflected clinically by white patches. White patches were explained as a result of Melanoma Inhibitory Activity (MIA) factors secreted by melanocytes which attack and weaken the melanocytes' attachment to the basement membrane, leading to their loss and exfoliation within the adjacent keratinocytes and hence de-pigmented macules formation [22]. This form of vitiligo is non-inflammatory and characterized by the presence of functional melanocytes that are silenced by MIA [23]. These previous explanations are supported by the present EM findings. Meanwhile, melanocyte movement and loss of melanocyte adhesion to the basement membrane was attributed to the effect of friction or oxidative stress, [17] as dysregulation of melanogenesis results in high production of free radicals and toxic products which damage melanocytes [24].

Regarding the IHC results, our study revealed a high level of Bcl-2 expression in normal skin areas with gradual loss, to a moderate level, in perilesional areas and mild expression in lesional areas that were in accordance with previous findings [25]. Inhibition of Bcl-2 indicates apoptosis induction due to oxidative stress [26]. The accumulation of reactive oxygen species (ROS) results in degeneration of melanocytes, the release of auto-antigens and initiation of eat-me signals associated with induction of apoptosis and phagocytosis [27,28]. In the present work, TNF-α was detected within the melanocytes of the epidermal basal cell layer of lesional skin areas compared with non-lesional skin areas and sebaceous glands; the authors suggested the supportive effect of TNF-α on T-lymphocyte growth and survival, which react to melanocyte antigen and lead to vitiligo progress [29]. Our findings indicate the occurrence of HMB-45 in an intense and diffuse pattern in normal skin areas and a mild, focal pattern in vitiligo areas. HMB-45 could be detected in vitiligo lesion melanocytes and suggested that epidermal fetal melanocytes attempt to maintain normal pigmentation through maintenance of melanogenesis process [30]. Abundance of LC in vitiligo lesional skin indicates its progressive state as reported with an increase in the number of dendrites and an increase in their lengths and branches when compared to the control. These alterations could draw attention to the possible role of LC in vitiligo pathogenesis (LC-mediated cellular immunity) [31]. Increase in LC at vitiligo lesion edges, mainly in the lower half of the epidermis, indicated its role in auto-antigens processing and cellular immune response against attacked melanocytes [32].

The present investigation of the leukocyte profile revealed no difference in the total leukocyte counts, or the percentage and absolute differential counts between vitiligo and control cases. These results agree with previous research, [5,6] in which systemic changes are generally not observed in horses with vitiligo; this is likely due to the localized type of vitiligo in the present study. Generalized vitiligo seemed to be associated with increased systemic inflammation [33]. A slight increase in monocytes was detected in vitiligo cases compared to the healthy mares; this was likely due to the small sample size of

the control mares. Flow cytometry studies revealed no difference in CD4+ and CD8+ T cell lymphocytes and CD4/CD8 ratios between vitiligo and healthy mares. In human vitiligo, regulatory T cells were normal and unaffected in segmented vitiligo patients and decreased in non-segmental vitiligo patients [34]. As vitiligo is a multifactorial disorder, its effective treatment in animals is still unclear [2]. Some treatments are based on reduction of ROS and hence activate re-pigmentation, while others target autoimmunity, based on restoring regulation of T cells [28]. Tacrolimus is an immunosuppressant antibiotic which inhibits several inflammatory T cell cytokines [35], it offers an alternative to the application of corticosteroid which have localized side-effects and it is recommended to be applied twice daily for 6 months in human [36]. There are few reports on treatment of vitiligo in horse, mainly with dietary supplementation [2]. Our findings showed gradual improvement of the affected facial areas and re-pigmentation was detected along the treatment course (16 weeks) using localized daily application of 0.03% Tacrolimus ointment.

Conclusion

We can conclude that vitiligo appearance in Egyptian (gray) Arabian horses was associated with the importation of European gray stallions for breeding selection. There was no previous data regarding equine vitiligo in Egypt and this is the first report. Across our results we could state that oxidative stress leads to apoptosis of melanocytes (TNF-α & Bcl-2), with induction of cellular immunity and dysregulation of melanocyte localization and function (EM & HMB-45 investigations). It is highly recommended the use of topical daily application of 0.03% Tacrolimus ointment for a minimum of 4 months as it improve the affected facial areas and re-pigmentation was detected.

Recommandations

The results of our study represent a small sample size. Further studies are needed to investigate the acute phase of vitiligo as well as treatment of a large number of vitiligo cases.

Conflict of interest

The authors declare that they have no conflict of interest.

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