IMMUNOMORPHOLOGICAL FEATURES OF FEMALE PATIENT'S SKIN WITH ANDROGENETIC ALOPECIA IN THE TREATMENT OF PLATELET-RICH PLASMA IN COMBINATION WITH TOPICAL MINOXIDIL 2 % LOTION

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Abstract: The aim. The research was provided to obtain immunohistochemical changes in scalp biopsies of female patients with androgenetic alopecia (AGA) I-II stages according to the Ludwig scale and to detect possible changes after usage of platelet-rich plasma (PRP) injections in combination with topical minoxidil 2 % lotion.

Materials and methods. Skin biopsies of 30 patients with AGA were examined by immunohistochemistry before and after treatment, which lasted for 3 months and included 3 PRP sessions (once per month) and topical application of minoxidil 2 % lotion twice daily.

Results. It was found that AGA is accompanied by inflammatory perifollicular infiltration by T-lymphocytes CD3+, CD4+ and CD8+, macrophages (CD68+); imbalance of growth polypeptides VEGF, TGF-β1, EGFR; accumulation of oxidative stress enzymes eNOS and iNOS; accumulation of pathological fraction of Collagen IV. The use of platelet-rich plasma in combination with topical 2 % minoxidil as AGA therapy leads to the normalization of immunohistochemical parameters of the skin, which indicates the possibility of its use for long-term therapeutic effect.

Conclusions. This study supplemented the understanding of the pathogenesis of AGA and serves as the basis for improving treatment regimens for this pathology. But more research is required to further study the pathomorphology of androgenetic alopecia and to standardize the technique of using platelet-rich plasma in patients with this disease.

Keywords: androgenetic alopecia, female patients, platelet-rich plasma, topical minoxidil, immunohistochemical examination of skin biopsies.

1. Introduction

Research has opened up a huge potential for developing ways to correct and manage the hair growth cycle with biologically active substances, growth factors and hormones, and has actually revolutionized this. The aggressive effect of androgens on the hair follicle in the androgen-dependent areas of the scalp is considered as the main etiopathogenetic mechanism of AGA [1, 2]. However, recent scientific data show that oxidative stress, microinflammation, apoptosis and fibrosis can be considered as non-androgenic cofactors of its development [3, 4]. The targeted treatment, that must show effect on each link in the pathogenesis of AGA, is likely to lead to a more long-lasting and greater therapeutic result and to complete existing treatment regimens for this pathology.

Great therapeutic potential involves the use of autologous plasma, enriched with platelets, as a method that indirectly stimulates the cells of the dermal papilla [5]. The dermal papilla and connective tissue are formed from the same progenitor cells as the fibroblasts in the interfollicular dermis, but their gene expression profile and biological functions are radically different [6]. If interfollicular fibroblasts promote the growth and differentiation of upper epithelial cells (keratinocytes), the dermal papilla and connective tissue play a major role in regulating hair growth. The method is based on the concept of platelet-enriched plasma as a natural source of signalling molecules that have a paracrine effect on other cells. The main functions of platelets in the repair of damaged tissue are modulation of inflammation through the interaction of innate immune cells, regulation of angiogenesis and stimulation of cell migration and proliferation [7].

Taking into account the molecular-biological control elements of the system of physiological degeneration of the hair follicle, the effectiveness of the use of platelet-rich plasma in AGA has not been established yet.

The aim of the research was to study the immunohistochemical (IHC) characteristics of the skin in women with AGA before and after treatment with platelet-rich plasma in combination with topical minoxidil 2 %.

2. Materials and methods

Female patients with AGA I-II according to Ludwig scale were included in the study after signing the informed consent.

The material for the study were scalp biopsies of 30 women with AGA aged 22 to 40 years (mean age 32.1 years) who were monitored in the period from 2017 to 2020 at the clinical base of the Department of Infectious Diseases and Clinical Immunology of the Medical Faculty of V. N. Karazin Kharkiv National University – "Trichology Institute". Patients received injections of platelet-rich plasma in combination with topical minoxidil 2 % lotion.

In the control group there were 20 skin samples of women without signs of AGA and other diseases aged 25–40 years (mean age 34.7 years), who underwent autopsies in the pathology department of Clinical city hospital No. 17 in Kharkiv.

During the study, the provisions of the Helsinki Declaration of the World Medical Association, the ethical code of the doctor of Ukraine and informing the patient about the nature of the study were strictly observed. All patients involved in the study signed informed consent to participate in the study. Protocol No. 2 of the Commission on Biomedical Ethics of V. N. Karazin Kharkiv National University of the Ministry of Science of Ukraine dated 13.05.2021.

Topically, patients used Minoxidil Inteli 2 % dermal solution 60 ml, manufacturer: Indastrial Farm. Cantabria (Spain), registration certificate: UA/14771/01/02, Inteli Generics Nord (Lithuania). Applied every morning and evening to androgen-dependent areas of the scalp (frontal and parietal), kept the exposure for at least 4 hours.

RegenBCT kits (Regenlab PRP Kit-RegenACR®, Le Mont-sur-Lausanne Switzerland) were used for PRP preparations and further injections. From 8 ml of blood by a single centrifugation for 5 minutes received 4-5 ml of plasma, in which the concentration of platelets was ×1.6 from the initial.

Injections were performed into the thinning areas of the hair in the technique of injection-by-injection with a 30G needle. At each injection, approximately 0.15–0.20 ml of the obtained PRP was administered. The distance between the injection points was 0.5–1 cm. Patients were advised not to wash his head for 4 hours after the procedure.

The biopsy was taken from the center of the hair loss area. The technique consisted of making deep cylindrical incisions covering all layers of the skin, using a 4-mm punch. 1 punch biopsy with a diameter of 4 mm was taken. Vertical and horizontal (transverse) sections were made from one biopsy, which was divided into two parts by a vertical section.

Tissue fragments were performed by standard histological methods, stained with hematoxylin and eosin.

IHC study was conducted on the basis of the laboratory of the Department of Pathological Anatomy of the Kharkiv Medical Academy of Postgraduate Education using antibodies and imaging system of Thermo scientific, Germany. The qualitative composition of the inflammatory infiltrate was studied using monoclonal antibodies (MCAB) to CD 3, CD 4 Clone 4B12, CD8 (SP16) (different fractions of T lymphocytes, Ready-to-Use), CD68 KP1 (macrophage marker, Ready-to-Use). To detect oxidative disorders, the expression of nitric oxide metabolism markers was investigated: endothelial nitric oxide synthase (eNOS, Nitric Oxide Synthase, Rabbit Polyclonal Antibody at a dilution of 1:50) and inducible synthase of Nitric oxide (iNOS), Rabbit Polyclonal Antibody at a dilution of 1:100). The nature of angiogenesis was assessed by the expression of vascular endothelial growth factor (VEGF (VG1)). Epidermal growth factor expression was determined using EGFR Polyclonal Antibody (titer 1:100). As a factor in the induction of the inflammatory process, transforming growth factor (PCAT to TGF-β1 (V) Antibody, Ready-to-Use) was studied. Bcl 2 (124), Ready-to-Use, was used as a marker of apoptosis. Collagen IV (CIV22) with a titer of 1:50 was detected in the areas of fibrosis.

Unmasking heat treatment was performed by boiling the sections in citrate buffer (pH 6.0–7.0). UltraVision Quanto Detection Systems HRP Polymer (Thermo scientific, Germany) was used to visualize the primary antibodies. DAB (diaminobenzidine) was used as the chromogen.

The results were calculated using the Avtandilov eyepiece in 10 randomly selected fields of view at a magnification of ×400 [8]. The IHC label was evaluated on two parameters: the degree of distribution and intensity of staining, taking into account the severity of the reaction and its location.

The degree of spread of the label was taken into account by the percentage of positively stained brown organelle cells from the total number of cells in the field of view. To assess the degree of color intensity used a semi-quantitative scale: negative (0) was the reaction in the absence of staining of specific cell structures, weak positive (1+) was the reaction with weak or focal staining from 0 to 30 % of cells, moderate positive (2+) – when sufficient or focal staining of 30–60 % of cells, pronounced or diffuse (3+) was considered a reaction when staining 60–90 % of cells.

The complex of morphological studies was performed on a Primo Star microscope (Carl Zeiss) using the program AxioCam (ERc 5 s).

Statistical processing of the obtained results was carried out using the Statistica 6.0 software. When interpreting the significance of the difference in the results, the Student's test was used, the critical value of the significance level was considered $p<0.05$.

3. Results

In all cases of the main study group before treatment, the dermis was thinned with signs of hair loss. Hair of various diameters and vellus hair grew from miniaturized follicles. Between the preserved hair follicles, in the area of empty hair bags in the area of the upper part of the hair follicles, areas of dermal fibrosis were found, they consisted of concentrically located bundles of connective tissue fibers. The number of sebaceous glands was increased compared to the skin of healthy women. In 28 cases out of 30 perivascularly, perifollicularly and between the acinuses of the sebaceous glands, we observed lympho-histiocytic infiltrate of varying severity.

About ¾ of inflammatory infiltrate cells in the main group were represented by CD3+ T-lymphocytes; 40 % of them were CD4+ T-lymphocytes, and they were observed mainly in the perifollicular area outside the foci of fibrosis, between the acinuses of the sebaceous and sweat glands. CD8+ T lymphocytes accounted for one third of all T lymphocytes and were found in the connective tissue layers and perivascularly in the dermis and hypodermis. 11 % of inflammatory infiltrate cells accounted for CD68+ macrophages, which were located mainly in the fibrosis fields and deeper parts of the dermis.

Expression of eNOS in the skin of healthy women was found only in single macrophages, fibroblasts and vascular endotheliocytes, while in the main group it was observed in numerous macrophages, fibroblasts, endothelial insole and vascular layer, as well as in epitheliocytes. Inducible nitric oxide synthase (iNOS) was not determined in the control group, in biopsies with AGA it was expressed in macrophages, vascular endothelial cells, single epitheliocytes of follicles and sebaceous glands and in fibroblasts of the perifollicular fibrosis zone.

The content of VEGF, as well as collagen type 4 was reduced: the reaction with vascular growth factor in the vascular endothelium was weak (1+), especially in areas of concentric fibrosis with depleted vascularization. The basement membranes of the epidermis, glands, hair follicles and blood vessels lost specialized type 4 collagen, and, conversely, its accumulation was recorded in atypical areas of perifollicular fibrosis.

The content of TGF-β1 and EGFR was increased in comparison with the control group. A pronounced positive reaction (3+) with PCAB to transforming growth factor was observed above all in numerous macrophages around the vessels and among the connective tissue strands of fibrosis. Epidermal growth factor marked both the thinned epidermis and the cells of the epithelial sheaths of the hair.

The distribution of the antiapoptotic protein Bcl-2 was moderate and, more often, weakly expressed (1+), its expression was detected in inflammatory infiltrate cells and correlated with increased activity of nitric oxide synthase, especially inducible.

After treating patients with platelet-rich plasma in combination with minoxidil 2 % lotion, we observed the following. The thickness of the dermis ranged from 1.19 to 1.58 mm and averaged 1.40 mm, which was 0.04 mm less compared to the control group, and 0.3 mm more than before treatment ($p\leq0.05$). Hair of the same diameter grew from follicles of normal size. The hair follicles of the hair follicles, as in the control group, were evenly distributed in the reticular layer of the dermis and in the hypodermis. The reticular layer differed in a network of connective tissue fibers with a free location of sebaceous and sweat glands, as observed in the skin of healthy women. In all 30 cases, mainly perivascular, diffuse lympho-histiocytic infiltrate occurred, perifollicular and between the acinuses of the sebaceous glands' infiltration was not observed.

CD3+T-lymphocytes accounted for 17.6 % of the total pool of the inflammatory component, 6.8 % and 5.9 % were CD4+ and CD8+ T-lymphocytes, respectively. 3.4 % of inflammatory infiltrate cells accounted for CD68+ macrophages, which were located mainly around the vessels and deeper parts of the dermis (**Table 1**).

The expression of nitrogen synthases of eNOS and iNOS in the skin of patients of the main group after treatment, as in the control group, was unidirectional, was detected at the level of 1+ mainly in the cells of the inner insole, single macrophages, fibroblasts.

The content at the location of growth peptides was also close to the indicators of the control group: vascular-endothelial growth factor VEGF, transforming factor TGF-β1 marked the endothelium of the numerous capillaries of the reticular layer

(2+ and 1+, respectively). Epidermal growth factor EGFR was determined as a weak reaction (1+) in the epidermis and epithelial cells of the vaginal hair.

Table 1
The ratio of immunocompetent cells in the dermis infiltrate in AGA by study groups

Immunocompetent cell	Control group *n*=20	Main group *n*=30	
		before treatment	after treatment
CD 3	14.2±7.8 %	76.6±7.7 %	17.6±6.9 %
CD 4	3.3±4.0 %	40.0±1.05 %	6.8±4.6 %
CD8	4.6±4.7 %	26.7±8.1 %	5.9±4.3 %
CD 68	2.1±3.2 %	11.0±5.7 %	3.4±3.3 %*

*Note: * – p≤0.05*

Type 4 collagen was found only in the basement membranes of the epidermis, sebaceous and sweat glands, hair follicles and blood vessels. The expression of the antiapoptotic protein Bcl-2 was moderate (2+), was determined in single inflammatory infiltrate cells, both in cases after treatment and in the control group (**Table 2**).

Table 2
Expression of IGH markers in AGA by study groups

IHC marker	Control group *n*=20	Main group *n*=30	
		Before treatment	After treatment
eNOS	1+	3+	1+
iNOS	–	3+	–
VEGF	3+	1+	2+
EGFR	1+	3+	1+
TGF-β1	1+	3+	1+
Bcl 2	2+	1+	2+
Collagen IV	1+	3+	1+

4. Discussion

Diagnosis of AGA includes assessment of clinical manifestations of the disease, detection of pathology of the thyroid gland, pituitary gland, ovarian and adrenal gland diseases, liver, without detailed consideration of the molecular and biological characteristics of the skin of patients. It is known that the main number of regulatory proteins and growth factors, such as VEGF, TGF-β1, EGFR, is synthesized in the dermal papilla. Fibroblasts, epitheliocytes, endothelial cells of its capillary in the inflammatory process, which we found in 93.3 % of observations of the main study group, as well as T-lymphocytes and macrophages of inflammatory infiltrate lose the ability to fully produce these control substances. In turn, the matrix of the hair follicle and the adjacent dermis begin to accumulate the products of enzymatic decomposition of nitric oxide eNOS and iNOS, which can be regarded as a manifestation of oxidative stress [9]. Type 4 collagen deposition is observed in the connective tissue fibers of the dermis and bundles of concentric perifollicular fibrosis. At each of these stages' apoptosis-mediated cell death has been detected.

The search for the most effective protocols for the treatment of AGA continues. Techniques with pronounced antioxidant, anti-inflammatory and immunocorrective effects are discussed as promising areas of etiopathogenetic therapy of AGA. The use of finasteride, minoxidil, other topical and oral drugs, instrumental methods does not lead to a lasting effect [10]. The use of platelet-enriched plasma in combination with topical minoxidil is quite common in trichological clinics around the world, so we tried to find the immunomophological basis for the treatment of AGA with this combination.

Study limitations. In accordance with the limitations of this study, the results can be determined as preliminary and requires further research to standardize the PRP method for female patients with AGA.

Prospects for further research. For further detailed analysis, we plan to continue this study with a larger number of patients.

5. Conclusions

The results of the IHC reaction in the dermis of patients with AGA in the form of increased expression of nitric oxide synthase eNOS and iNOS indicate the presence of oxidative disorders with an imbalance of immunocompetent T-lymphocytes CD3+, CD4+ and CD8+, macrophages (CD68+) and growth factors VEGF, TGF-β1, EGFR. The basis of progressive alopecia is the production by fibroblasts of hair follicles of the pathological fraction of collagen type 4 with the development of perifollicular fibrosis and increased apoptosis. The use of platelet-rich plasma in combination with 2 % minoxidil as therapy for AGA leads to the restoration of functional skin morphology, which is manifested by changes in the expression of IHC markers, with levels close to normal skin. Further study of the signalling pathways involved in hair growth should open up new medicinal targets in the future.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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