Subjects and Methods

Type of the study

Quasi interventional study.

Study population

This study included twenty subjects of both sexes suffering from verruca vulgaris recruited from the Outpatient Clinic of Dermatology, Venereology and Andrology Department of Benha University Hospital in the period between January 2018 to January 2019. In addition, ten apparently healthy individuals who were attending Plastic Surgery Outpatient Clinic, Benha University were included as a control group.

Ethical considerations

A written informed consent was obtained from all participants. This study was approved by the local Ethics Committee on Research involving human subjects of Benha Faculty of Medicine.

Inclusion criteria

- Patients who were suffering from multiple verruca vulgaris (common warts) more than two lesions.
- Above the age of 18 years.
- No topical or systemic treatments for warts had been applied 4 weeks before injection of vitamin D3.
- The size of the wart was sufficient for the injection and for taking the biopsy (more than 4 mm).
Exclusion criteria

- Patients with a past history of allergic response to vitamin D.
- Patients with a past history of allergic skin disorder.
- Patients with bleeding disorders.
- Pregnant and lactating females.
- Patients with vascular diseases.
- Hepatic and renal patients.
- Patients with calcium metabolism disorders.

Methods:

All patients in this study were subjected to the following:

I-Full history taking

Personal history

- Name, age, sex, residence, occupation and special habits.

Present history:

- Duration of the existing warts.

Past history:

- Similar lesions (number, site, duration and how it was treated).
- Other skin lesions.

II-Clinical examination

- Complete general examination.
- Complete dermatological examination.
- All patients have been examined and photographed before each injection noting the number and surface area of warts.
Subjects and Methods

- Patients with multiple warts were selected for immunotherapy.

III-Technique

- Four mm Punch biopsy was taken before and after treatment with intrarolesional injection of vitamin D3 under local anesthesia.

- Vitamin D3: 0.6 ml of Devarol-S of Memphis ® (200,000 IU, 5 mg/2ml) was injected to the base of each wart using a 31-gauge syringe. The injections were repeated 2 weeks apart for a maximum of 4 sessions or until clearance of the lesion was achieved. A maximum of 2 warts had been treated per session (the biggest and oldest warts) and patients were followed up for 6 months after the last injection according to Kavya et al., 2017.

- The degrees of clinical response to treatment were divided into (‘‘no response,’’ ‘‘partial response,’’ and ‘‘complete response’’). Of note, ‘‘no response’’ was defined as absolutely no improvement with injections, ‘‘partial response’’ as a noticeable improvement but not full clearance, and ‘‘complete response’’ as total clearance of wart (Alikhan et al., 2016).

- Another biopsy was taken from apparently healthy individual as control group under local anesthesia.

- The biopsies had been sent to Pathology department, Faculty of Medicine, Menoufia University fixed in neutral formalin 10%, and submitted to routine tissue processing ending with paraffin embedded blocks formation. Two 5 micron cut sections had been done. One section was stained with hematoxylin and eosin for evaluation of histopathological changes. Other section was cut on poly L lysine coated slides for immunohistochemical staining using monoclonal antibody raised against LL 37.
IV-Histopathological evaluation

Haematoxylin and eosin stained sections were examined by light microscope to:

- Confirm the clinical diagnosis of warts which included: acanthosis, papillomatosis, hyperkeratosis, parakeratosis, elongated rete ridges and presence of koliocytopathic changes which are characterized by large keratinocytes with an eccentric, pyknotic nucleus surrounded by a perinuclear halo.

- Evaluation of histopathological changes after injection of vitamin D3 in warts as epidermal thickness and density of inflammatory cells.

V-Immunohistochemical staining

The method used for immunostaining was a streptavidin-biotin–amplified system. The antibody used was mouse monoclonal antibody raised against amino acids 131-170 mapping at C-terminal of LL-37 proteolytic fragment of CAP-18 of human origin. Each vial contains 200μg IgG kappa light chain in, 1 ml of FBS with < .1 % sodium azide and .1 % gelatin.

LL-37 is available conjugated to HRP (sc-166770AC), 200μg/ml (Santa Cruz Biotechnology, Inc, Europe). The dilution was 1:5 according to supplied data sheet.

Slides were subjected to deparaffinization and rehydration. Antigen retrieval was performed by boiling in citrate buffer saline (pH 6), followed by cooling at room temperature. Endogenous peroxidase was blocked by incubation with H2O2, 3%. The primary antibody was incubated overnight at room temperature, and then the secondary antibody (ready-to-use, Ultravision detection system anti-polyvalent
HRP/DAB, Thermoscientific, Labvision Corp., Fremont, CA, USA) was applied with DAB as a chromogenic substrate and Mayer’s hematoxylin as a counter stain. Human bone marrow was used as a positive control for LL37. Replacement of the primary antibody in the staining procedure with a blocking buffer was included as a negative control.

**Interpretation of LL37 expression**

- Positive expression was assigned when cytoplasmic or membranous expression was seen in any numbers of cells.
- Staining pattern of distribution: diffuse (when stained all epidermal layers) or focal (when stained some but not all layers of epidermis).

- **Intensity of expression was assessed subjectively according to depth of immunostaining into:**
  - Mild (+)
  - Moderate (++)
  - Strong (+++)
Statistical methods

The collected data were presented in suitable tables and illustrated in a suitable form. Quantitative data were summarized in the form of mean and standard deviation, while qualitative data were summarized in the form of frequency and percentage.

Comparisons between the different study groups were carried out using the Chi-square test ($\chi^2$) and Fisher’s Exact Test (FET) to compare proportions as appropriate. The independent t-test (t) was used to detect mean difference between two groups regarding parametric data and the Mann-Whitney test (z) was used to compare two non-parametric data.

Expression of LL37 before and after treatment was compared using Stuart-Maxwell test. Comparisons between different levels of expression and different degrees of response were done using Kruskal Wallis test for numerical data and Fisher's exact test for categorical data.

The corresponding P-values were obtained. A P-value $< 0.05$ was considered statistically significant, while a P-value $> 0.05$ was considered statistically non-significant.

The statistical analysis was conducted using Statistical Package for Social Sciences (SPSS), version 25 (IBM, Armonk, New York, United states) (Kareem et al., 2019).