Original Article

The Role of Inflammation in the Progression of Ocular Surface Damage in Children Following Allogeneic Haematopoietic Cell Transplantation

(impression cytology / stem cell allotransplantation / HLA-DR expression / conjunctiva)

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Abstract. The aim of the study was to evaluate HLA-DR expression and cellular morphology of the conjunctival epithelium cells in children who underwent haematopoietic cell transplantation, and to assess the relation between HLA-DR expression and cellular morphology. Impression cytology with staining was used to visualize epithelium cells, whereas immunohistochemistry was applied to assess HLA-DR expression. Elevated HLA-DR expression and increased cytological abnormalities were observed in the study group when compared to the controls. An increase in HLA-DR expression was accompanied by a decrease in the number of eyes with normal epithelium morphology together with the increase in squamous metaplasia features. We can conclude that inflammation of conjunctiva can follow stem cell allotransplantation. Ocular surface inflammation may lead to squamous metaplasia of the conjunctiva.

Introduction

The conjunctival epithelium plays an important role in ocular immunologic defence. The conjunctiva is a highly active and abundantly vascularized tissue, which is also linked to the lymphatic system (conjunctiva-associated lymphoid tissue, CALT), fully capable of antigen capturing, processing and presenting to the immunocompetent cells (Dua at al., 1994). Apart from the goblet cells, the conjunctival epithelium also contains Langerhans cells and lymphocytes (Records, 1998; Tsubota et al., 1999a, Zhan et al., 2003). The antigen-presenting cells (APCs) displaying the MHC II antigens, such as HLA-DR, are also present in the conjunctival epithelium. APCs can initiate inflammatory reactions by presenting foreign antigens and activating lymphocytes (Records, 1998; Tsubota et al., 1999b, Zhan et al., 2003). Therefore, the conjunctival epithelium cells may themselves become the T-cell targets, as observed in allogeneic haematopoietic cell recipients (Dua at al., 1994; Records, 1998). The allogeneic haematopoietic cell transplantation (allo-HCT), performed in the treatment of various neoplastic and non-neoplastic disorders, is associated with a high risk of toxicity due to the use of high-dose chemotherapy and/or radiotherapy in conditioning, as well as to certain infections and graft-versus-host disease (GvHD). In the course of GvHD the donor's immunocompetent T cells, with the aid of cytokines, mount an immunologic attack on the host's cells leading to cytotoxic damage (Billingham, 1959; Ogawa and Kuwana, 2003b; Vogelsang et al., 2003; Anderson and Regillo, 2004). The target tissues in GvHD are the skin, liver, gastrointestinal tract, as well as the eye tissues (mostly the conjunctiva and the lacrimal glands). The activation of the donor's T cells and their attack on the recipient's tissues are mediated by the above-mentioned MHC class II-presenting cells, among them HLA-DR-expressing cells (Xun et al., 1994; Remberger et al., 1995; Paczesny et al., 2010).

Chronic inflammation may induce ocular surface cell damage (Ogawa et al., 2001; Ogawa and Kuwana, 2003a). According to the literature, cytokines play a vital role in the epithelium keratinization process (Hogaboam et al., 1998; Douglas et al., 2002), both cytokines excreted from the damaged epithelial cells and

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Abbreviations: allo-HCT – allogeneic haematopoietic cell transplantation, APCs – antigen-presenting cells, CALT – conjunctiva-associated lymphoid tissue, DES – dry eye syndrome, GvHD – graft-versus-host disease, HE – haematoxylin eosin, IC – impression cytology, PAS – periodic acid-Schiff, TBI – total body irradiation.

lymphocytes or leukocytes migrating from the dilated conjunctival vessels, which further contributes to the epithelial cell damage.

The aims of our study were: (1) to evaluate HLA-DR expression on conjunctival epithelial cells and the cellular morphology of the conjunctival epithelium, and (2) to evaluate the relations between the HLA-DR expression and the conjunctival cellular morphology in children subjected to allo-HCT.

Material and Methods

We enrolled 55 children and adolescents (27 girls and 31 boys) aged 7–20 years (mean age 13.55 ± 5.71), who had undergone allogeneic haematopoietic cell transplantation due to acute lymphoblastic leukaemia, chronic myeloid leukaemia, myelodysplastic syndrome, severe aplastic anaemia, rhabdomyosarcoma, neuroblastoma, X-linked adrenoleukodystrophy, Hodgkin's disease, Nijmegen syndrome, Duncan's syndrome, or Griscelli's syndrome. The patients were recruited from the Department of Paediatric Oncology, Haematology and Bone Marrow Transplantation of Wrocław Medical University. The control group consisted of 57 healthy children and adolescents (25 girls and 32 boys) aged 10–20 years (mean age 15.09 ± 1.85) with unremarkable medical and ophthalmic history, recruited from a regional public high school. The follow-up time after allo-HCT ranged from six months to five years (median 26.54 months ± 19.53).

Patients received specific conditioning regimens according to the diagnosis. The protocols included high-dose chemotherapy and total body irradiation (TBI), depending on the graft type. The GvHD prophylaxis also depended on the graft type:

– 20 patients with HLA-matched sibling donors were administered cyclosporine alone;
– 37 patients with unrelated donors were administered anti-thymocyte globulin, cyclosporine and methotrexate.

The exclusion criteria were as follows:

– a history of ocular surface inflammatory and/or infectious diseases
– a history of ocular surface injuries
– previous anterior segment surgery
– diabetes or neurologic, dermatologic and allergic disorders that might have influenced the anterior segment homeostasis.

We performed a complete anterior and posterior segment ophthalmic examination. Additionally, impression cytology with pathology assessment and evaluation of HLA-DR expression in conjunctival epithelium cells were also carried out.

Impression cytology (IC)

Conjunctival cytologic specimens were collected by impression. The samples were taken after topical anaesthetic agent administration (1% proparacaine), from each eye separately, from an area located 3–5 mm from the limbus in the superior, superotemporal, and temporal part of the bulbar conjunctiva. Millipore VSWP filters (Millipore, Warszawa, Poland) with pore diameter of 0.025 μm cut into triangular-shaped pieces (10 × 5 × 10 mm) were used. The base of each triangle was pressed against the conjunctiva. Epithelium specimens obtained in the way described above were subsequently applied onto gelatin-coated slides in a manner ensuring that as many cells as possible were transferred. The slides were then sprayed from a distance of 30–50 cm with CYTOFIX (Samko, Warszawa, Poland) aerosol preparation used to fix cytologic samples. The preparations were subsequently stained with standard haematoxylin and eosin (HE) (Dako, Gdynia, Poland) dye to visualize cell morphology, and with periodic acid-Schiff (PAS) reagent (Dako, Poland) for detection of the mucus containing goblet cells.

Moreover, the preparations were also subjected to immunohistochemical staining in order to determine HLA-DR antigen expression with the use of monoclonal rodent anti-HLA-DR antibodies (Dako, Poland). Cells were incubated on ice with monoclonal antibodies conjugated with fluorochromed antibodies against class II antigen HLA-DR for 30 min, then rinsed with cold phosphate-buffered saline three times, and subsequently incubated with DAB-chromogen, submerged in DPX medium and covered with a coverglass.

The histopathology assessment of the samples was performed with a light microscope (BX-50 Olympus) (Olympus, Warszawa, Poland) in three randomly selected power fields (magnification 40×–400×). Grading according to the 4-grade Nelson (1983) classification was implemented (Nelson et al., 1983).

The grading was as follows:

Grade 0 (normal appearance) – normal conjunctival epithelium, small, round, tightly adherent epithelial cells with eosinophilic cytoplasm. Nucleus/cytoplasm (N/C) ratio around 1/2. Goblet cells numerous, with PAS (+) cytoplasm.

Grade 1 (slightly abnormal) – epithelial cells slightly larger and polygonal, with slightly smaller nuclei, N/C ratio 1/3. Goblet cells lower in number, with PAS (+) cytoplasm.

Grade 2 (abnormal) – epithelial cells larger and polygonal with eosinophilic or basophilic cytoplasm. N/C ratio 1/4–1/5. Goblet cells significantly lower in number, with weakly PAS (+) cytoplasm.

Grade 3 (significantly abnormal) – epithelial cells larger and polygonal, with small, pycnotic nuclei and basophilic cytoplasm. N/C ratio 1/6. Goblet cells not present.

Grade 0 represents normal conjunctival epithelium, whereas grades 1, 2 and 3 represent squamous metaplasia.

Goblet cell density

The goblet cell density was determined after PAS staining. The goblet cell count per 1000 visible cells was

expressed as a mean number obtained from three randomly selected, representative, non-superimposing power fields (x400). The inflammation profile was evaluated by assessing the HLA-DR expression and by determining the percentage of immunoreactive cells in the specimens obtained by IC.

The intensity of HLA-DR expression

The intensity of HLA-DR expression (membrane reaction) was analysed based on the brown staining intensity in three representative power fields (x450). The intensity of chromogen reaction was graded according to the following subjective scale:

Grade 0 – no staining (negative)
Grade 0.5 – weak positive reaction
Grade 1 – moderately positive reaction
Grade 2 – quite strong reaction
Grade 3 – strong reaction

Grades 2 and 3 implied significant expression of inflammation markers.

The percentage of cells reactive to anti-HLA-DR Ab

The percentage of cells reactive to anti-HLA-DR Ab was evaluated in three representative high-power fields (x400). The grading was subjective and based on the reactive cell count as follows:

Grade 0 – less than 20 % cells reactive – negative
Grade 1 – 20–39 % cells reactive – very weakly positive
Grade 2 – 40–59 % cells reactive – weakly positive
Grade 3 – 60–79 % cells reactive – moderately positive
Grade 4 – 80–100 % cells reactive – strongly positive

All the analysed samples showed 80–100 % reactivity to anti-HLA-DR Ab and were therefore not taken into account for the purposes of statistical analysis.

The statistical analysis was performed using the STATISTICA 8.0 software package for Windows, StatSoft, Inc. (Tulsa, OK, 2008).

Results

Comparative analysis of the HLA-DR expression between the study and control groups

Expression of HLA-DR was studied in 228 cases. Grade 3 expression of HLA-DR was found in 14 cases (6.1 %; 8 patients), grade 2 in 28 (12.3 %; 24 patients) and grade 1 in 76 cases (33.3 %; 50 patients), whereas grade 0.5 and 0 in 56 (24.6 %; 44 controls) and in 54 cases (23.7 %; 34 controls), respectively (Fig 1.3). High expression of HLA-DR (grade 1–3) was significantly more frequently observed in the study group when compared to the control group (72.0 % vs. 31.6 %, respectively; χ^2 test, $P < 0.0001$; Fig 1.1). Elevated expression of HLA-DR was observed in patients, whereas low expression was more common in controls (Fig 1.2).

Comparative analysis of conjunctival epithelial cell morphology between the control and study groups

A statistically significant increase in cytologic abnormalities was observed in the study group (grade 1–3 according to Nelson's (1983) scale in 84 patients, 73.7 % vs. 30 patients, 26.3 %), whereas grade 0 (absence of abnormalities) was more frequently found in the control group (78 controls, 68.4 % vs. 36 controls, 31.6%; χ^2 test; $P < 0.0001$; Fig 2.1 and Fig 2.3). Abnormal epithelium morphology (grade 1–3) was found to be predominantly associated with the study group (Fig 2.2).

Analysis of the relationship between HLA-DR expression and conjunctival epithelium cell morphology

In the study group (N = 114) a statistically significant drop in the number of cells with normal morphology (grade 0 according to Nelson, 1983) was noted along with the increased expression of HLA-DR (Fig 3.1) – from 70 % with grade 0 HLA-DR expression to 0 % with grade 3 HLA-DR expression. Also, along with the increase in HLA-DR expression, a significantly higher number of cells with grade 1 squamous metaplasia features (according to Nelson) was observed – from 20 % with grade 0 HLA-DR expression to 58.33 % with grade 2 expression and 50 % with grade 3 (χ^2 test; $P < 0.0001$). A paradoxical drop in the number of eyes with grade 3

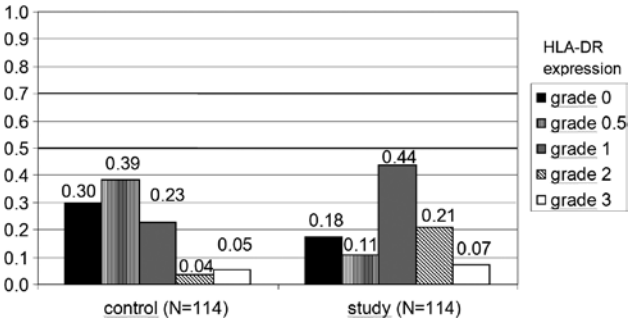

Fig.1.1. Percentage of particular HLA-DR expression grades in the control and study groups

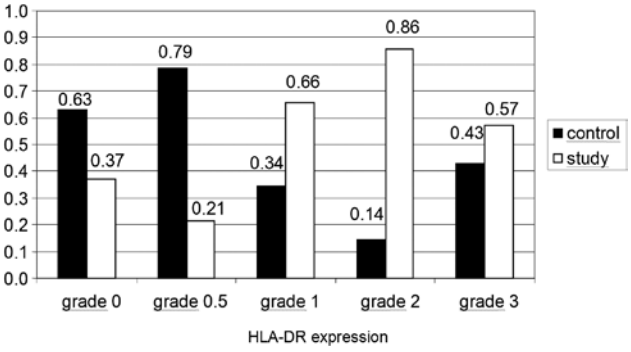

Fig 1.2. Percentage of controls and studied subjects in each of the HLA-DR expression grades

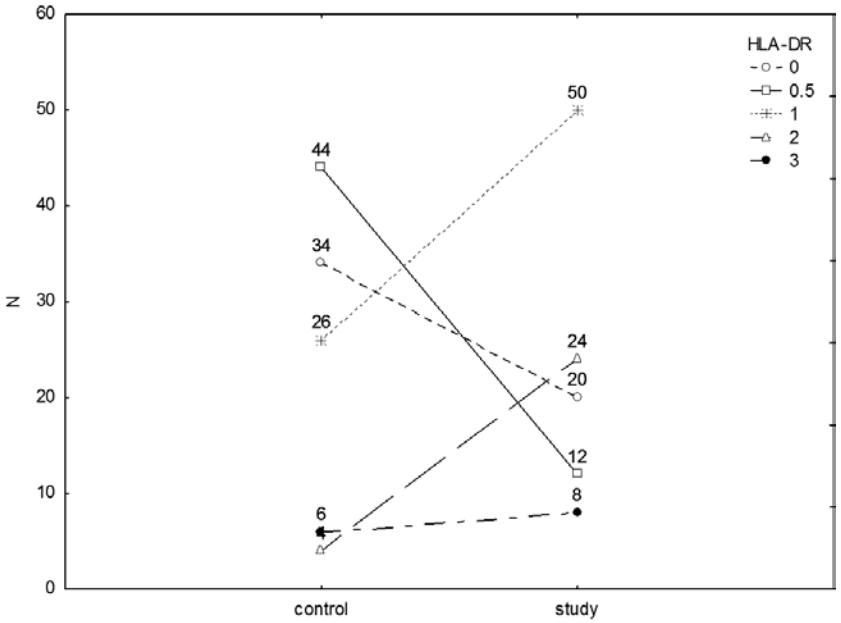

Fig 1.3. Interaction plot of HLA-DR expression in the studied groups

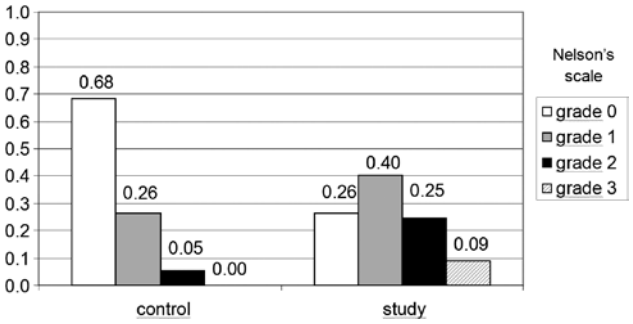

Fig 2.1. Percentage of particular Nelson's grades in the control and study groups

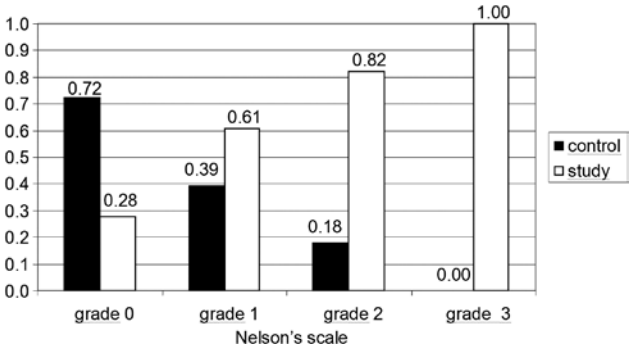

Fig 2.2. Percentage of controls and studied subjects in each of the Nelson's grades

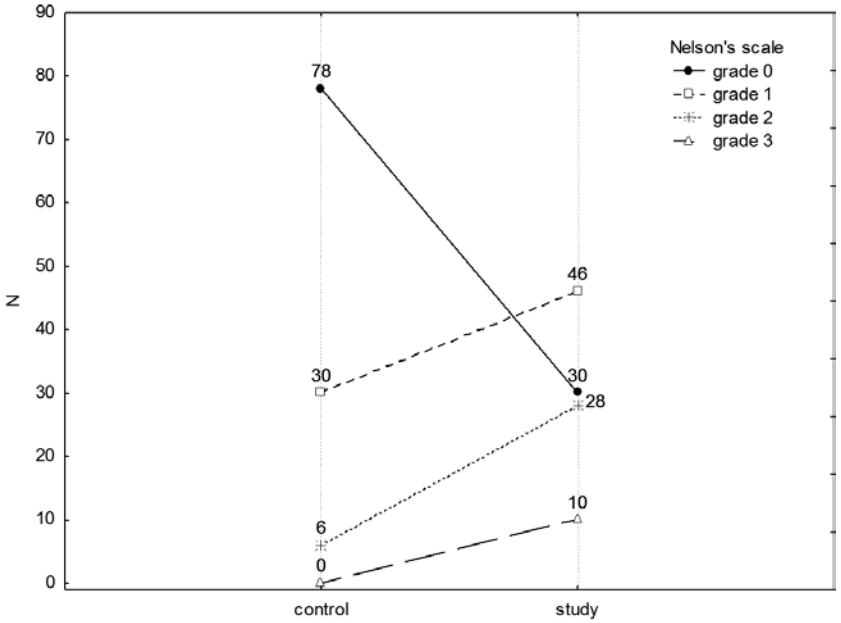

Fig 2.3. Interaction plot of the Nelson's scale in the study and control groups

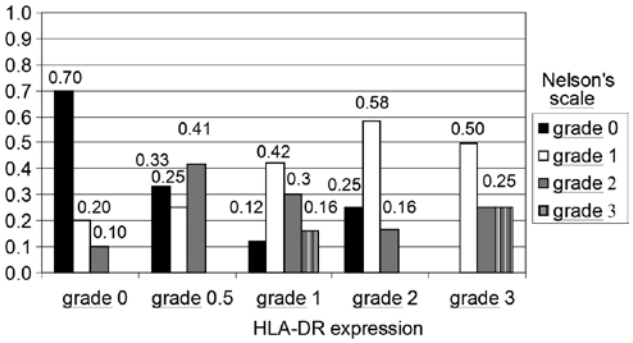

Fig 3.1. HLA-DR expression and cell morphology (Nelson's scale) analysis in the study group

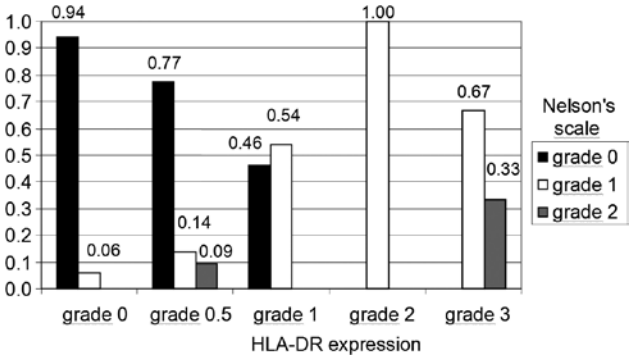

Fig 3.3. HLA-DR expression and cell morphology (Nelson's scale) analysis in the control group

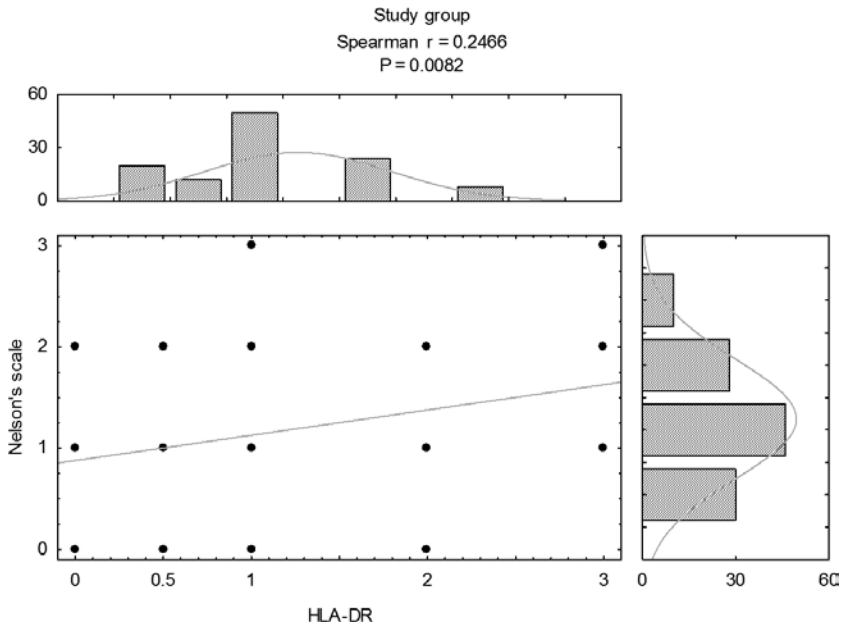

Fig 3.2. Correlation between HLA-DR expression and Nelson's scale in the study group

squamous metaplasia features when compared to grade 2 (according to Nelson) may be due to a small number of eyes with grade 3 HLA-DR expression (8 eyes), which can bias the interpretation. The correlation between the studied parameters (Fig 3.2.) was weak but statistically relevant (Spearman r = 0.2466, P = 0.0082). The same tendency was observed in the control group (Fig 3.3 and Fig 3.4).

Discussion

The pathological mechanism underlying the ocular surface abnormalities in children subjected to allo-HCT has been widely debated; nonetheless, the treatment results are still unsatisfactory.

Inflammation has been proposed as an underlying cause of squamous metaplasia by numerous authors (Chen et al., 2009). They imply that conjunctival epithelium, rather than merely being a target of the inflammatory processes, may also play an intermediary role through the expression of various adhesion molecules and other surface antigens such as HLA-DR (Rojas et al., 2005; Rolando et al., 2005; De Paiva et al., 2007; de Salamanca et al., 2008). The immunologically activated conjunctiva can in turn also become a target of the cytotoxic lymphocytes (Fujihara et al., 1999; Tsubota et al., 1999).

In this study a statistically significantly higher HLA-DR expression on the conjunctival epithelium in children post allo-HCT when compared to the control group was found. This finding supports the conjunctival involvement in the inflammation.

Multiple factors may contribute to the HLA-DR overexpression by the conjunctival epithelium cells in children subjected to allo-HCT, such as the underlying disease itself, the pre- and post-transplant procedures (conditioning, radio- and chemotherapy) implemented in all children, as well as the concomitant GvHD.

Moreover, the peri-transplant infections as well as the dry eye syndrome (DES), frequently found in allo-HCT patients, should also be taken into account as contributing factors (Apostol et al., 2003; Sheppard, 2003).

Along with the increase in inflammation, an increase in the number of eyes with epithelium abnormalities

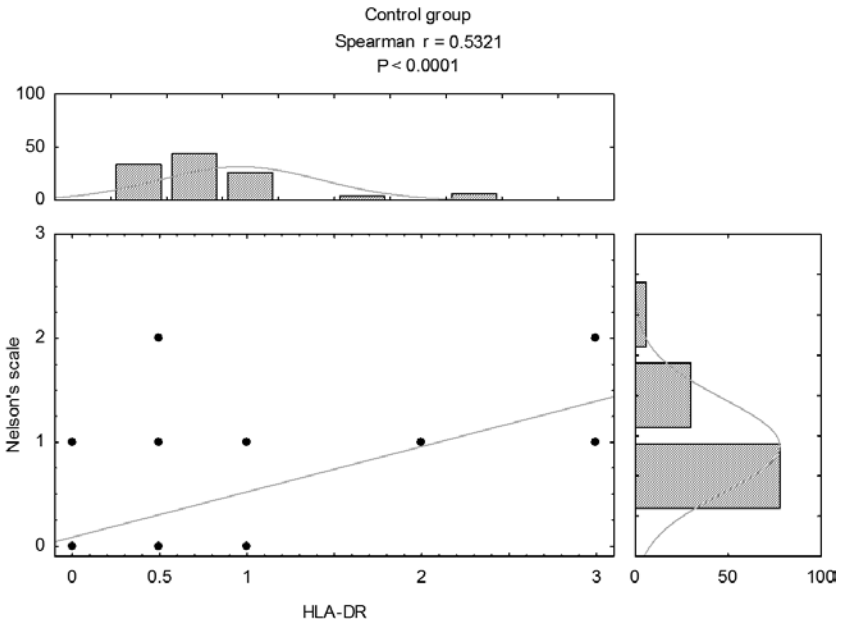

Fig 3.4. Correlation between HLA-DR expression and Nelson's scale in the control group

was also noted, which suggests that inflammation may lead to squamous metaplasia, as previously described by other authors (Rolando et al., 2005; De Paiva et al., 2007). Our results indeed imply a central role of immunological processes in the pathogenesis of cytological abnormalities in children subjected to allo-HCT.

On the other hand, we also demonstrated that the eyes with 0.5 grade HLA expression in the largest part presented cells with rather advanced cytological changes (grade 2 according to Nelson, 1983), whereas the eyes with grade 1 expression mainly presented only slightly abnormal cells (grade 2 according to Nelson, 1983). The above-mentioned results might be caused by the fact that the extent of conjunctival cytological changes is not solely due to the inflammation. Such factors as concomitant DES and the theory of mechanical epithelial damage due to tear insufficiency may also play a role.

In summary, the ocular surface inflammation should always be taken into account in children subjected to bone marrow transplantation, not only the ones with DES, but also the seemingly healthy ones on biomicroscopic examination. Therefore, apart from the wide range of lubricating drops, an anti-inflammatory agent should also be used in all children with HLA-DR expression on the ocular surface, both the ones with and without DES. Despite the lack of a systemic effect of such agents, they nonetheless inhibit local inflammation, which may be beneficial in the case of systemic treatment intolerance due to the side effects. An additional close link between inflammation and cell apoptosis, including conjunctival epithelium cells, also supports the need for use of immunomodulating agents.

Acknowledgement

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