SALIVARY NEUTROPHILS LEVEL AS AN INDICATOR OF BONE MARROW ENGRAFTMENT

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Aim: The aim of this study was to investigate the neutrophils level in saliva as an adequate alternative to other methods for evaluating the neutrophil engraftment after autologous stem cell transplantation (ASCT) in hemato-oncology.

Method: A total of 35 patients treated for non-Hodgkin's lymphoma or multiple myeloma were stomatologically examined before planned high-dose chemotherapy with ASCT. After removal of potential foci of odontogenic infection all the patients underwent transplantation and during the treatment they were monitored for the level of neutrophils in saliva as a possible early indicator of the neutrophil engraftment. Neutrophil levels in saliva were compared to the neutrophil level in blood and to the degree of oral mucositis (the nurses study).

Results: An increase of salivary neutrophils in the mouth rinse of $> 25 \times 10^6/l$ was identified as an early sign of successful neutrophil engraftment that occurred 1 to 2 days before the rise of neutrophils in peripheral blood ($> 0.5 \times 10^9/l$).

Conclusions: Follow-up of neutrophil levels in saliva might be an adequate alternative to other methods for evaluating the neutrophil engraftment after ASCT in hemato-oncology.

INTRODUCTION

High dose chemotherapy with the support of autologous peripheral stem cell grafts (ASCT) has been considered now as standard treatment for a number of hematological malignancies. However, aggressive chemotherapy, apart from having systemic side effects, is also followed by severe oral mucositis with significant impact on the psychological well-being of the patient. To avoid this development, preventive dental intervention is necessary and this should take place prior to the start of the oncological treatment: all potential infectious foci must be healed, devitalized teeth have to be removed and patients must be instructed in the principles of oral hygiene. Developing a state of oral health and following-up levels of granulocytes in saliva after ASCT might be significant indicators of neutrophil engraftment.

MATERIAL AND METHODOLOGY

The sample included a total of 35 patients treated at the Department of Hemato-Oncology of the University Hospital, Olomouc in 2006–2008. All the patients were treated on the basis of established treatment protocols for non-Hodgkin's lymphomas and multiple myeloma. Before inclusion in the study, they signed a written informed consent to the treatment protocol approved earlier by the local institutional Ethical Committee.

At least 3 weeks before myeloablative chemotherapy

was started, standard stomatological examination was carried out. This included OPG (Orthopantomogram) X-ray, as well as parodontological examination and removal of potential sources of mucous membrane mechanical irritation such as grinding of sharp edges of teeth, dental fillings and dentures. Patients preparation before the transplantation included also practice in the Stillman method of brushing teeth and instruction on the harmful effects of smoking, alcohol and consumption of irritating food during treatment. To remove potential foci of odontogenic infection, we extracted gangrenous teeth, teeth with X ray confirmed periostitis, partially erupted third molars with clinical symptoms of pericoronitis and teeth mobility of the 2nd, 3rd degrees with active periodontal pockets. We did not extract teeth imperfectly endodontically healed, which did not clinically respond to percussion and vitality tests and showed negative X ray findings. The start of the stomatological examination also included determination of PBI (Papilla Bleeding Index), CPITN (The Community Periodontal Index of Treatment Needs) and supragingival scaling. Before starting the chemotherapy, the patients included in the study went through one week treatment of a twice daily rinse with the oral antiseptic Listerine (Pfizer).

Patients with severe parodontitis who refused extraction and patients with poor oral hygiene who declined cooperation were excluded from the study.

From the hemato-oncological point of view all our patients were treated for non-Hodgkin's lymphomas and multiple myeloma according to established treat-

ment protocols. (Table 1). In the case of non-Hodgkin's lymphoma, high-dose chemotherapy BEAM was used for the conditioning (BCNU, cytarabine, etoposide, and melphalan). In the case of multiple myeloma the treatment was monotherapy with high-dose (HD) melphalan in one dose of 200 mg/m² infused over one hour. ASCT was performed in all patients with special isolation¹¹ and using selective decontamination of GIT (gastro-intestinal tract) 24 hours after the performed myeloablative chemotherapy. Other prophylactic measures included application of fluconazole 200 mg daily, co-trimoxazole 480 mg twice a day and acyclovir 400 mg three times a day. In patients unable of oral food intake the same antibiotics were given parenterally. In case of febrile neutropenia empiric broad spectrum antibiotics were applied according to the Institutional protocol. To accelerate the neutrophil engraftment, all patients with multiple myeloma received G-CSF (Granulocyte Colony Stimulating Factor) at a dose of 5 ug/kg starting on the day +7 after graft reinfusion until full neutrophil recovery. In patients with malignant lymphomas single dose of G-CSF was applied according to the protocol described earlier¹.

Along with the examination of blood neutrophil levels, we quantitated the neutrophils present in saliva. Salivary sampling was started on day 0 the day of graft transfer. The patients rinsed their oral cavities with 10 ml saline solution for a period of 30 sec each morning before breakfast and before oral cavity hygiene. The sample was then centrifuged for at 200g for 10 min and the supernatant fluid was carefully sucked away to leave 1 ml sediment. The sediment was stained with methylviolet, showing neutrophils with a characteristic multi-lobulated shape and typical kernel nuclei that could be easily distinguished from the other cells and epithelial cells. The samples were observed on a light microscope and neutrophils were counted in 16 large squares of a Fuchs-Rosenthal chamber at a magnification of 50x. Over weekends and bank holidays the material was preserved in 2 ml 39% formaldehyde, stored at 4°C and processed the first day following the weekend or bank holidays to ensure the continuity of evaluation². Salivary samples were examined every day until successful neutrophil engraftment was confirmed.

RESULTS

A generally accepted indicator of successful engraftment is the achievement of the $0.5 \times 10^9/l$ level of granulocytes in blood on 3 successive days. Determination of the granulocyte level in saliva is an alternative indicator. The granulocyte level marking a successful engraftment is $25 \times 10^6/l$ on 2 successive days, counted in 16 fields of Fuchs-Rosenthal chamber³. Typical was a somewhat variable curve of neutrophil levels in saliva after autologous transplantation with a rapid decrease in neutrophils towards zero values, followed by a subsequent rapid rise as demonstrated in Graph 1.

In fourteen patients (patient 1, 3, 7, 14, 16, 17, 18, 22, 25, 28, 29, 31, 32 and 33) registered in our study the level of neutrophils in saliva indicating successful engraftment was achieved approximately 1–2 days sooner than in peripheral blood (Graph 2), (Table 3). In seven patients (patient 2, 8, 9, 13, 27, 34 and 35) the required increase was noticed both in saliva and peripheral blood on the same day (Graph 2, Table 3). Seven patients (patient 4, 12, 19, 21, 23, 24 and 30) engrafted the bone marrow in peripheral blood sooner than in saliva (Graph 2, Table 3).

According to median leucocyte levels (WBC > $0.5 \times 10^9/l$) all patients engrafted on day 12 (9–18), patients with myeloma on day 12 (10–13), patients with lymphoma on day 11 (9–18). In saliva according to the level of neutrophils (> $25 \times 10^6/l$) all patients engrafted on day 11 (9–17), patients with myeloma on day 11 (9–14), patients with lymphoma on day 11 (9–17), (Table 2).

In six cases (patient 5,10,11,15,20 and 26) we did not assess neutrophil levels in saliva, since the required values of $25 \times 10^6/l$ (patient 10,11 and 26) were not achieved or to the contrary, because the persisting neutrophil levels (patient 5,15 and 20) had not reached zero or close to zero levels (Graph 2). We noticed that the required level of neutrophils in saliva had not been achieved mostly in patients without teeth, where the primary entering level of neutrophils is lower compared to patients with teeth. In one case (patient 6) neutrophil levels indicating successful engraftment of the bone marrow had not been achieved in saliva and blood (Graph 2).

Table 1. An examined collection of patients.

Diagnosis	Number of patients	Women	Men	Average age (years)	Chemotherapy regimen
Non-Hodgkin's lymphoma	20	9	11	50	BEAM
Multiple Myeloma	11	5	6	57	HD melphalan
Hodgkin lymphoma	4	3	1	36	BEAM
Total	35	17	18	50

Graph 1. Variability in neutrophil levels in saliva and blood in a group of hemato-oncological patients after autologous bone marrow transplantation and immunosuppressive treatment.

Graph 2. Time difference between reaching the required levels of neutrophils in saliva and blood.

To assess the success of the hemato-oncologic treatment it was very important to determine the entering neutrophil level in saliva before starting chemotherapy. The group of patients with teeth showed higher entering neutrophil levels in saliva compared to patients without teeth. (Graph 3 and 4)⁴. The average neutrophil level in patients with teeth was $171 \times 10^6/l$, while in those without teeth it was $24 \times 10^6/l$.

In 9 patients the neutrophil levels did not decrease to zero or even close to zero (zero level represented the lowest achieved level). Of these, 3 patients treated for myeloma (patients 6,7,8) and 6 patients treated for NHL (patients 10, 14, 15, 16, 17 and 18) - (Graph 5 and 6)

To determine the physiological neutrophil level in saliva, saliva samples were obtained from 22 healthy volunteers by rinsing the oral cavity by the method mentioned above. The average neutrophil level in saliva was $174 \times 10^6/l$, Graph 5.

DISCUSSION

Preventive dental intervention is necessary and this should take place prior to the start of the oncological treatment. A standard part of preparing for ASCT is also using oral antiseptics. A number of publications focused

Table 2. Median values of engraftment according to levels of neutrophils in saliva and blood.

Engraftment (days)	WBC > $0.5 \times 10^9/l$	Saliva > $25 \times 10^6/l$
all	12 (9–18)	11 (9–17)
myeloma	12 (10–13)	11 (9–14)
lymphoma	11 (9–18)	11 (9–17)

Note:

All patients: 3 patients (No. 5, 15, 20) without decrease in saliva, 3 patients (No. 10, 11, 26) without engraftment in saliva, 1 patient (No. 6) engraftment neither in saliva nor in blood

MM: 2 patients (No. 5, 15) without decrease in saliva, 2 patients (No. 10, 11) without engraftment in saliva

NHL+MH: 1 patient (No. 26) without engraftment in saliva, 1 patient (No. 6) engraftment neither in saliva nor in blood, 1 patient (No. 20) without decrease in saliva

Table 3. Engraftment in time difference according to neutrophil level in saliva and blood.

	Total	MM	NHL+MH
No difference in engraftment	7 patients	2 patients	5 patients
Neutrophils sooner in saliva	14 patients	3 patients	11 patients
Neutrophils sooner in blood	7 patients	2 patients	5 patients

Graph 3. Entering neutrophil levels in saliva from patients without teeth.

on oral antiseptics but their conclusions are equivocal. For example Vokurka et al.⁵ found no difference between the use of iodide preparations as oral antiseptics and saline. The product, Listerine (Pfizer) we used is an antiseptic agent containing essential oils (thymol, eucalyptol, menthol, methyl salicylate) and alcohol. Its antibacterial effect is based on blockade of cellular metabolism⁶. In addition it has an anti-mycotic and anti-viral effect (Miller

et al)⁷. Listerine is very good at preventing plaque and removing existing plaque as well. Moreover it does not disturb the balance of bacterial flora in the oral cavity and does not stain dental enamel even after long-term use^{8, 9}. Its therapeutic effect on inflammatory parodontal disease is also noteworthy (Graph 8).

A saliva neutrophil level is nowadays already frequent used method to determine severity of periodontal disease-

Graph 4. Entering neutrophil levels in saliva from patients with teeth.

Graph 5. Check group: Neutrophil level in saliva from healthy people.

es. Recently, some studies, focused on detection saliva neutrophils count as a grade of severity of periodontal disease and treatment response were published^{2, 10-12}.

To avoid misinterpretation of some results, the responsible treatment of periodontal diseases must precede ASCT.

This was the reason why patients suffering from severe grade of periodontal disease (see Graph No.6) were excluded of our study.

In our sample of 35 patients treated for non-Hodgkin's lymphomas and multiple myeloma by high-dose chemotherapy with ASCT, successful engraftment was marked

by an increase in salivary neutrophil levels an average of 1–2 days earlier than in peripheral blood¹³.

Nowadays there are no studies, explaining earlier neutrophil penetration to the oral cavity in comparison to their manifestation in peripheral blood. Within the context of this should be referred the diversity of saliva and oral fluid which contains the saliva by itself, gingival cervical fluid, sloughed oral epithelial cells, food debris, and bacteria and their product^{14–18}.

In our study we found a higher level of salivary neutrophils in patients with teeth than in edentulous. We support the conclusions of Wright et al.⁴ who found very low salivary neutrophil level in four edentulous patients. Just the presence of dental plaque and oral bacteria (over 500 bacterial species are present in the mouth) could explain the chemotaxis of the neutrophils into the oral cavity.

The time interval of successful engraftment was considerably influenced by the myeloablative chemotherapy of non-Hodgkin's lymphomas³. A time difference between reaching favourable levels of neutrophils in saliva and in blood might correspond to the risks of infection and septic conditions occurring after graft reinfusion³. The longer this time interval is, the smaller the risk of infectious complications. This view is based on an assumption of gradual "preferential" feeding of tissues with neutrophils. The higher the neutrophil level in a tissue, the greater the self-defensive ability of the organism. This theory, however, would rather support reaching of neutrophils into the oral cavity through tissue penetration.

There are still doubts over the methodology of determining the physiological level of neutrophils in saliva. Wright et al.⁴ did rinses of the oral cavity in the forementioned manner using a number of volunteers with good oral health. The neutrophil level in saliva oscillated between $1,3 \times 10^5/l$ and $10,8 \times 10^5/l$. Although we did not evaluate this parameter in our small sample of patients, we have never observed such wide variation in neutrophils levels. To get comparable results it is necessary to maintain a time schedule for saliva sampling since the level of salivary neutrophils during the day tends to fluctuate as a result of the consumption of liquids and food. The connection between the level of neutrophils in saliva and blood is also confirmed by increasing levels of neutrophils after administration of growth factors. Lieschke et al.¹⁹ found a several times greater increase in levels of neutrophils in saliva as well as in blood in G-CSF treated patients where levels of neutrophils in saliva increased a few days earlier than in peripheral blood, compared to untreated patients.

Croatian authors advert to different phagocytic function of salivary neutrophils in comparison to neutrophils in peripheral blood²⁰. They compare phagocytic function of salivary neutrophils of healthy volunteers with their activity in patients suffered from oral lichen planus and acute recurrent aphthous ulceration. Our experience is similar (the nurses study): oral mucositis turned to better just after appearance of neutrophils in oral fluid²¹.

The radicality of the stomatological preparation of the

Graph 6. Neutrophil levels in saliva from patient with parodontitis. High entering neutrophil levels in saliva and resulting steep decrease as a proof of good response to Listerine treatment.

patients before stem cell transplantation and before surgery must be also carefully considered. It seems that while this preparation is being planned, the type of transplantation and the aggressiveness of the chemotherapy should be taken into account. Strictly radical methods are recommended in patients before allogeneic transplantation. Our experience confirms Starosta's and Faber's view^{22, 23} that before ASCT less radical preparation is necessary – it is unnecessary to extract teeth, even if they are not quite perfectly endodontically treated unless they display X ray confirmed peri-apical finding. In any event, it is important to eliminate the risks of mechanical irritation of soft tissues through sharp tooth edges, dental fillings, dentures or plaque to obviate mucosal lesions.

Our results confirmed that parodontal diseases can influence the salivary neutrophil level.

CONCLUSION

In spite of a small group of analyzed patients we can conclude that monitoring the level of the neutrophils in saliva after ASCT appears to be practical and useful. A non-invasive easy sampling of biological material can be used for earlier signalling of successful engraftment.

Good cooperation between the stomatologist and the hemato-oncologist results in both the reduced frequency and gravity of oral mucositis as well as in eliminating the risks of potential odontogenic treatment complications. Oral health in this connection serves as an indicator of general health and signalizes a risk of complications after high-dose chemotherapy. The practical use of determining the level of neutrophils in saliva as an indicator of treatment success in hemato-oncology can also lead to modifications in the treatment protocol and to essential financial savings. To confirm these conclusions, a study of a larger set of patients will be necessary.

REFERENCES

1. Faber E, Pytlík R, Slabý J, Zapletalová J, Kozák T, Raida L, Papajik T, Žikešová E, Marešová I, Hamouzová M, Indrák K, Trněný M. Individually determined dosing of filgrastim after autologous peripheral stem cell transplantation in patients with malignant lymphoma – results of a prospective multicenter controlled trial *Europ J of Haematol* 2006; 77(6): 493–500.
2. Bender JS, Thang H, Glogauer M. Novel rinse assay for the quantification of oral neutrophils and the monitoring of chronic periodontal disease. *J Periodontol, Res* 2006; 41: 214–220.
3. Cheretakis C, Dror Y, Glogauer M. A noninvasive oral rinse assay to monitor engraftment, neutrophil tissue delivery and susceptibility to infection following HSCI in pediatric patients. *Bone Marrow Transplant* 2005; 36: 227–232.
4. Wright DG, Meierovics AI, Foxley JM. Assessing the delivery of neutrophils to tissues in neutropenia. *Blood* 1986; 67: 1023–1030.
5. Vokurka S, Bystrická E, Koza V, et al. The comparative effects of providone-iodine and normal saline mouthwashes on oral mucositis in patients after high-dose chemotherapy and APBSCT – results of a randomized multicentre study. *Support Care Cancer* 2002; 13: 554–558.
6. Fine DH. Mouthrinses as adjunct for plaque and gingivitis management. A status report for the Am J Dent 1988; 1: 259–263.
7. Miler TF, Silva A, Ferreira SM, Jabra-rizk MA, Kelley JJ, Depaola LG. Efficacy of Listerine antiseptic in reducing viral contamination of saliva. *J Clin Periodontol* 2005; 32: 341–346.
8. Minah GE, Depaola LG, Overholser CD, Miller TF, Niehaus C, Lamm RA, Ross NM, Dills S.S. Effects of 6 months use of an antiseptic mouthrinse on supragingival dental plaque microflora. *J Clin Periodontol* 1989; 15: 347–352.
9. Walker C, Clark W, Tyler K, Ross N, Dill S. Evaluation of microbial shifts following long-term antiseptic mouthrinse use. *J Periodontol* 1997; 48: 646–649.
10. Klinkhamer JM. Quantitative evaluation of gingival and periodontal disease. The orogranulocyte migratory rate. *J Periodontol* 1968; 6: 207.
11. Ljungman P, Urbano-Ispizua A, Cavazzana-Calvo M, et al. Allogeneic and autologous transplantation for haematological diseases, solid tumours and immune disorders: definitions and current practice in Europe. *Bone Marrow Transplant* 2006; 37: 439–449.
12. Loe H. Silness Journal Periodontal disease in pregnancy. Prevalence and severity. *Acta odont scan* 1963; 25: 533.
13. Figueredo CM, Fischer RG, Gustafsson A. Aberrant neutrophil reactions in periodontitis. *J Periodontol* 2005; 76: 951–955.
14. Gorgung A, Knight DR, Wright GD. Use of oral mucosal neutrophil counts to detect the onset and resolution of profound neutropenia following high-dose myelosuppressive chemotherapy. *Am J Hematol* 2003; 72: 13–19.
15. Burlage FR, Pijpe J, Coppes RP, et al. Variability of flow rate when collecting stimulated human parotid saliva. *European Journal of Oral Sciences* 2005; 113(5): 386–90.
16. Vissink A, Spijkervet FK, Van Nieuw, Amerongen A. Aging and saliva: a review of the literature. *Special Care in Dentistry* 1996; 16(3): 95–103.
17. Wu AJ, Baum BJ, Ship JA. Extended stimulated parotid and submandibular secretion in healthy young and old population. *Journals of Gerontology* 1995; 50A(1): 45–8.
18. Schenkels LCPM, Veerman ECI, Nieuw Amerongen AV. Biochemical composition of human saliva in relation to other mucosal fluids. *Critical Reviews in Oral Biology & Medicine* 1995; 6: 161–75.
19. Veerman ECI, Van Den Keijbus Pam, Nieuw Amerongen AV. Human glandular salivas: their separate collection and analysis. *European Journal of Oral Sciences* 1996; 104: 346–52.
20. Lieschke GJ, Ramenghi U, O'Connor MP, Sheridan W, Szer J, Morstyn G. Studies of oral neutrophils levels in patients receiving G-CSF after autologous marrow transplantation, *British Journal of Haematology* 1992; 82: 589–595.
21. Lukač J, Mravak – Stipetič et al. Phagocytic functions of salivary neutrophils in oral mucous membrane diseases. *J Oral Pathol Med* 2003; 32: 271–4.
22. Rubenstein E, Peterson D, Schubert M, et al. Clinical practice guidelines for prevention and treatment of cancer therapy – induced oral and gastrointestinal mucositis. *Cancer* 2004; 100: 2026–2046.
23. Starosta M, Faber E. Stomatitis u nemocných po autologní transplantaci hemopoetických kmenových buněk, *Praktické zubní lékařství* 2000; 5:131–136.
24. Bartáková V. Problémy stomatologického ošetřování u vybraných skupin rizikových pacientů, *Česká Stomatologie* 1986; 4: 255–260.