Method to test influence of bio-implants on blood cells by consecutive measurements

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Abstract

The study was directed to the development of a method for evaluation of the changes in 15 blood parameters of venous blood specimens under the contact with biomaterials. Plain titanium plates and also titanium deposited with a hydroxyapatite ceramic layer were chosen for testing. Titanium free blood specimens were used as a reference. Five consecutive tests of experimental and reference blood samples were performed simultaneously. One way analysis of variance was used to determine significant difference of the blood parameters from baseline values. The results demonstrated the method as sensitive to reveal changes in erythrocytes, subpopulations of white blood cells and platelets measured by an ABX Micros OT automated hematology analyzer.

Key words: bio-implant, blood cells, blood contact *in vitro* method.
Abbreviations: EP, experimental; GRA, granulocytes; HCT, hematocrit; HGB, hemoglobin; LY, lymphosytes; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; MCV, mean cell volume; MON, monocytes; MPV, mean platelet volume; PCT, plateletcrit; PDW, platelet distribution width; PLT, platelets; RBC, red blood cells; RDW, red cell distribution width; RP, reference; Ti, titanium plate; Ti:HAP, titanium deposited with hydroxyapatite ceramic layer; WBC, white blood cells.

Introduction

Bio-implants are widely used in modern medicine (Slucki, Vetra 2001; Hepch, Jones 2007). The choice of biomaterial is determined by several different factors. The most important of them is biocompatibility. This precondition is true if the biomaterial is compatible with body tissues and does not cause any change in the key functions of the organism.

Biomaterials, placed temporarily or permanently in the animal or human, are in contact with body tissues including blood and, therefore, may affect blood cells. It is important to consider material response and host response, which can be determined by *in vitro* as well as *in vivo* tests (Exbrayat et al. 1987; Cannas et al. 1988; Kononen et al. 1992; Maloney et al. 1993; Groth, Atlankov 1996; Black, Hasting 1998; Hepch, Jones 2007). *In vitro* tests (among them blood contact tests) as screening tests have to be first provided. The main host responses are considered to be blood coagulation and lyses of erythrocytes and this blood contact tests are usually used.

Methods investigating biocompatibility *in vitro* are frequently based on immunochemical methods as well as investigations that are carried out in cell culture, requiring relatively expensive equipment and which are time-consuming. There are presently no investigation techniques based on blood cell hematological parameters that would enable to assess the bio-implant impact in dynamics.

The aim of the study was to develop a novel method of evaluating compatibility of bio-implants *in vitro* with a wider spectrum of obtained blood parameters (15) over a shorter period of time which is less expensive.

Materials and methods

Two types of bio-implants, titanium plate and titanium deposited with hydroxyapatite ceramic layer (Ti:HAP) were chosen for testing. The Ti 2 mm thick plates (1 × 2 cm^2) were cut off titanium (grade A) tape. After that the Ti plates (Ti) were washed carefully in acetone and ethanol and finally dried in air.

Also, a second type of bio-implants was manufactured for testing. Titanium specimens were deposited with HAP by means of the Pulse Laser Deposition technology (Ti:HAP plate). The thickness of the HAP layer was a couple of micrometers.

The experimental design included three series of experiments: the first was carried out with titanium plates and blood of healthy persons, the second with Ti:HAP plate and blood of healthy persons and the third with Ti:HAP and blood of the patients with a low haemoglobin level.

Blood specimens were tested by an ABX Micros OT automated hematology analyzer, delivering a standard package of blood characteristics (Invitros 2000) including

the count of red (RBC) and white (WBC) blood cells, platelets (PLT) as well as the percentages of three WBC subpopulations – lymphocytes (LY), monocytes (MON) and granulocytes (GRA), values of mean platelet volume (MPV), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), content of hemoglobin (HGB), red cell distribution width (RDW), plateletcrit (PCT), platelet distribution width (PDW) and hematocrit (HCT) (Lejniece 2005). Subpopulations of white blood cells were recognized according to their size: LY between 30 – 100 fL, MON between 100 – 150 fL and GRA between 150 – 450 fL (Invitros 2000). MPV, PDW, PCT were parameters related to platelets whereas HGB, HCT, MCV, MCH, MCHC, RDW to erythrocytes.

Typically, blood samples stored with K_2-EDTA at room temperature for 48 h are aged (Vogelaar et al. 2002; LaFayette et al. 2007; Vienken 2007). According to a hematology analyzer manual, samples of blood can not be stored longer than 2 h at room temperature (Invitros 2000). On the other hand, a sufficient period of time is required for exposure of bio-implant to result in changes in blood cells to occur. Therefore, the duration of the experiments was chosen to be an hour. The experiment was performed at room temperature.

In total, 106 venous blood samples taken from cubital vein of healthy persons and patients with a low hemoglobin level (86 and 20, respectively; both gender, aged 25 to 60 years) for ordinary clinical examination were collected in Vacutainer tubes (Becton-Dickinson) with K_2-EDTA. The remaining 3 mL of each sample remained anonymous and were used in the study. Particular requirements of prior consent are not needed if persons remaine anonymous according to ISO 15189 Medical laboratory, section C.9.

Initially, the baseline measurements (1st test) of the collected specimens from each person were performed. Then each was divided into two parts: "reference" (RP) and "experimental" (EP). Thus, blood samples of the control group were taken from the same persons as the experimental samples. The bio-implants were positioned within the EP, whereas, the RP were bio-implants free. The ratio of bio-implant/blood volume was 1:10.

Both EP and RP were tested simultaneously four times within one hour: at 10, 20, 40 and 60 minutes after start of the experiment. The first series of experiments was carried out with Ti, the second with Ti:HAP. Blood samples from patients with low haemoglobin level were selected for the third series of testing.

Differences in blood parameters over time of exposure of bio-implants compared to baseline values of blood parameters [DIF] were determined. In this case the initial test values were taken to be zero. This approach allowed to eliminate the effect of individual differences in the baseline values of blood parameters. DIF of the blood parameters calculated from the values at the corresponding time of testing in EP and RP samples were compared.

For statistical comparisons one way analysis of variance (ANOVA) was used. Significance for F statistic was at the $P \leq 0.05$ level. Groups "RP" and "EP" were selected as factors for this analysis.

Results

Blood samples of 16 persons were used for the first series of experiments with titanium plate. Thus, 160 measurements including baseline measurement and values of consecutive four tests were involved in the calculation. Four blood parameters (DIF.HGB, DIF.HCT, DIF.MCV and DIF.MCH) changed in relation to contact with the Ti plate (Table 1). All of these parameters were related to red blood cells, and characterized by the size of red blood cells, content of hemoglobin, distribution of the histogram. In general, they referred to erythrocytes, i.e., red blood cells from different aspects.

The second series of experiments was performed using titanium plate deposited with hydroxyapatite ceramic layer (Ti:HAP). In total, 70 blood samples (700 measurements) were included in the analysis. Three blood parameters related to red blood cells (DIF.RBC, DIF.HGB and DIF.HCT), as well as two others related to platelets (DIF.PLT and DIF.PCT) differed significantly between the EP and RP treatments (Table 2). DIF.WBC in EP and DIF.WBC level in RP did not differ.

In contrast, the % proportions of the subpopulations of leukocytes in blood samples with Ti:HAP differed significantly from the blood samples in the Ti:HAP free

Table 1. Significant differences (ANOVA) in blood sample parameters between Titanium and control treatments . Analysis of variance for DIF of 15 blood parameters. *, significant

Blood parameter	F statistic	Significance (p-value)
DIF.RBC [10^{12} L^{-1}]	2.375	0.125
DIF.HGB [g L^{-1}]	5.406	0.021*
DIF.HCT [%]	3.799	0.053*
DIF.MCV [μm^3]	9.200	0.003*
DIF.MCH [pg]	4.829	0.029*
DIF.MCHC [g dL^{-1}]	0.739	0.391
DIF.RDW [%]	0.136	0.713
DIF.PLT [10^9 L^{-1}]	0.018	0.895
DIF.PCT [%]	0.001	0.971
DIF.MPV [μm^3]	0.366	0.546
DIF.PDW [%]	0.135	0.713
DIF.WBC [10^9 L^{-1}]	0.069	0.792
DIF.LY [%]	3.556	0.061
DIF.MON [%]	1.756	0.187
DIF.GRA [%]	1.533	0.218

Table 2. Significant differences (ANOVA) in blood sample parameters between Ti:HAP and control treatments. Analysis of variance for DIF of 15 blood parameters. *, significant

Blood parameter	F statistic	Significance (p-value)
DIF.RBC [10^{12} L^{-1}]	6.66	0.010*
DIF.HGB [g L^{-1}]	6.62	0.010*
DIF.HCT [%]	4.27	0.039*
DIF.MCV [μm^3]	3.02	0.083
DIF.MCH [pg]	0.04	0.841
DIF.MCHC [g dL^{-1}]	0.25	0.618
DIF.RDW [%]	0.05	0.832
DIF.PLT [10^9 L^{-1}]	12.88	0.000*
DIF.PCT [%]	6.81	0.009*
DIF.MPV [μm^3]	0.49	0.481
DIF.PDW [%]	0.26	0.611
DIF.WBC [10^9 L^{-1}]	0.10	0.751
DIF.LY [%]	11.59	0.001*
DIF.MON [%]	18.37	0.000*
DIF.GRA [%]	3.13	0.077

Table 3. Significant differences (ANOVA) in blood sample parameters in samples with HGB < 12.5 g L^{-1} and Ti:HAP plate samples. *, significant

Blood parameter	F statistic	Significance (p-value)
DIF.RBC [10^{12} L^{-1}]	3.679	0.054*
DIF.HGB [g L^{-1}]	3.974	0.048*
DIF.HCT [%]	2.885	0.091
DIF.MCV [μm^3]	0.371	0.543
DIF.MCH [pg]	0.235	0.628
DIF.MCHC [g dL^{-1}]	0.076	0.783
DIF.RDW [%]	0.783	0.377
DIF.PLT [10^9 L^{-1}]	5.558	0.019*
DIF.PCT [%]	4.865	0.029*
DIF.MPV [μm^3]	1.974	0.162
DIF.PDW [%]	3.298	0.071
DIF.WBC [10^9 L^{-1}]	0.404	0.526
DIF.LY [%]	5.545	0.020*
DIF.MON [%]	6.996	0.009*
DIF.GRA [%]	1.156	0.284

treatment.

The final series of experiments were carried out with blood samples of 20 patients (200 measurements) with reduced levels of haemoglobin (HGB < 12.5 g L^{-1}). Titanium plates coated with hydroxyapatite ceramic layer were used in this experiment. DIF.RBC, DIF.HGB, DIF.PLT, DIF.PCT, DIF.LY and DIF.MON values differed significantly in blood samples that were in contact with bio-implant from the control (Table 3).

Discussion

It is important to monitor host response to bio-implant because of continual improvement of implants. Most of the methods described in the literature for determining the bio compatibility of implants are histomorphometrical or immunochemical. Some of them are performed *in vivo*, and others in cell cultures or by blood contact test *in vitro* (Pizzoferrato et al. 1985; Sevastyanov et al. 1987; Thomson et al. 1992; Marois et al. 1996). Blood cells react to the biomaterial immediately due to the early contact after implantation (Slucki, Vetra 2001). The adhesive properties of blood cells can be changed after contact with bio-implants (Mansurova et al.1996; LaFayette et al. 2007). Prolonged perfusion through a polypropylene tube of up to seven days in the animal model had an impact on the hematology profile (Vienken 2007). *In vitro* performed blood tests are usually directed to recognize the changes in numbers of platelets and erythrocytes as main predictors for clot development or cell lyses in blood vessels. This suggested to develop an *in vitro* method for determination of changes in blood parameters in experiments on blood samples. Another reason was the availability and relative inexpensive of the method developed for these purposes. Black and Hasting (1998) separated bio-implant testing into three types of tests: *in vitro* static, *ex vivo* dynamic and *in vivo* dynamic. According to this classification, this method can be accepted as an *in vitro* static blood contact test. However, due to several consecutive measurements during the test it should be nominated as *in vitro* dynamic type test. The duration of the experiment was chosen as half of the time allowed for a blood sample to be stored without significant changes in time (Invitros 2000). This duration was equal to an hour. The question was whether the effect of the bio-implant will be manifested in such a short time and whether there will be enough tests to allow statistical analysis. Considering that the changes in blood parameters during an hour would be minimal, the differences from baseline values of blood parameters were selected as the variables for analysis. This approach allowed not to consider individual variance in the baseline values of blood parameters. Five tests performed in an hour provided enough data for statistically significant results. One way analysis of variance allowed to reveal differences in blood parameters between the EP and RP treatments. Two types of implants were tested. Both revealed statistically significant differences in the parameters. Tests on blood samples with HGB < 12.5 g L^{-1} showed that the method is suitable for blood with different hematology profile. The developed method can be used for evaluation of erythrocyte, white blood cells and platelets response to bio-implants (Leice et al. 2008) or other materials and can be modified under

different experimental conditions.

The proposed method, due to the statistical approach, is sensitive for detection of changes in blood cells occurring within an hour of contact with bio-implant.

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