Rīgas Stradiņa universitāte

Arnis Vilks

RELATIONSHIP OF GENERAL AND REGIONAL ANAESTHESIA WITH ACTIVATION BETA-HERPESVIRUSES AND IMMUNOLOGICAL CHANGES IN PROLONGED MICROVASCULAR FREE FLAP SURGERY

Summary of the Doctoral Thesis
for obtaining the degree of a Doctor of Medicine

Speciality – Anaesthesiology and Intensive Care

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The Doctoral Thesis was carried out at the Centre of Plastic and Microsurgery of Rīga Eastern Clinical University Hospital clinic "Gaiļezers", the Department of Anaesthesiology and Reanimation of Rīga Stradiņš University and August Kirchenstein Institute of Microbiology and Virology of Rīga Stradiņš University.

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The Doctoral Thesis is available in the RSU library of and at RSU webpage: www.rsu.lv.

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ABBREVIATIONS USED IN THE THESIS

AKTH	adrenocorticotropic hormone
CMV	cytomegalovirus
$CD4^+$	T helper cells / regulatory cells
$CD8^+$	T cytotoxic cells / suppressor cells
$CD16^+$	natural killer cells
$CD38^+$	activated lymphocytes
CVP	central venous pressure
DNS	deoxyribonucleic acid (DNA)
ELISA	enzyme-linked immunosorbent assay
$EtCO_2$	end tidal CO_2
FiO_2	fraction of inspired oxygen
GA	general anaesthesia
GVHD	graft versus host disease
HHV-6	human herpesvirus 6
HHV-7	human herpesvirus 7
n	sample size
NF1	neurofibromatosis type 1
p	p-value
PCR	polymerase chain reaction
RA	regional anaesthesia
SaO_2	oxygen saturation
Th	T helper cells

INTRODUCTION

Relevance of the problem

Microvascular free flap surgery is a complex method of wound closure for large wounds not amenable to linear (primary) closure. Microvascular free flap surgery involves the transfer of free tissue (muscle, bone or a combination) to a site of tissue loss where its circulation is restored via microvascular anastomoses (Shaw, 1983; Thorne, 2006).

Microvascular free flap surgery is usually long-term, large-scale and traumatic.

Surgical operations cause a variety of immunological disturbances. The overall picture is one of a generalised state of immunosuppression in the post-operative period, the degree this occurs ir proportional to the magnitude of tissue damage (Decker, 1999; Welch, 1981).

The main causes of immunocompromised responses in surgical patients are well known to be related to the neuroendocrine stress exerted through activation of the autonomic nervous system and the hypothalamic pituitary-adrenal axis. Activation of the hypothalamic pituitary-adrenal axis is the key response to stress and plays a central role in mediating the effect of surgery on the immune system (Chrousos, 1995; Kennedy, 1999). Surgery induced immunosuppression is caused by the effect on the cellular components of the immune system and is related to the magnitude of the surgery.

Not only surgery, but also a method used in anesthesia may have potential impact on immune function. For example, there is evidence that the general anaesthetic propofol inhibits neutrophil and macrophage chemotactic and phagocytosis (Chen, 2003; Wu, 2005). There is evidence that spinal

anesthesia is less immunosuppressive and has less impact on T lymphocyte proliferation (Le Cras, 1998). For example, NK cell function after extensive ovarian cancer surgery returned to normal within 21 days (Brøchner, 2016).

Human herpesvirus-6 (HHV-6) and human herpesvirus-7 (HHV-7) are ubiquitous beta-herpesviruses widely distributed in the general population. Their primary infection usually occurs in the early years of life and remains latent in the host for the lifelong period.

At present more attention is being paid to trying to find and evaluate the role of beta-herpesviruses infection in the development of various chronic diseases, but there is still no final answer to this question.

HHV-6 and HHV-7 can be activated by immunosuppressant factors and these viruses possess the ability to affect the body's immune system. HHV-6 and HHV-7 belong to *Betaherpesvirinae* subfamily *Roseolovirus* genus.

HHV-6 was isolated in 1986 from the interleukin-2 – stimulated peripheral blood mononuclear cells obtained from the AIDS patients and patients with lymphoproliferative diseases. HHV-6 was specified in two varieties and later as two species – HHV-6A and HHV-6B.

Since its discovery HHV-6 has been associated with a wide range of chronical diseases – multiple sclerosis, chronic fatigue syndrome, acquired immunodeficiency syndrome and cancer.

HHV-6B causes *Roseola infantum*, febrile illness and encephalitis in infants and it reactivates in transplant patients, causing complications such as encephalitis, pneumonia and liver damage. In renal transplant patients, HHV-6 is associated with the development of chronic allograft nephropathy and graft versus host disease (Caiola, 2012; Chapenko, 2009).

HHV-7 clinical role is poorly documented, but it is possibly due to the development of *Pityriasis rosea* (Black, 1999; Rebora, 2010).

Up to now no studies have been conducted in order to investigate the possible effects of surgery and anaesthesia on reactivation of HHV-6 and HHV-7 infection and its impact on the outcome of the surgery.

Scientific actuality

An average of 30 microvascular free flap surgeries per year are performed in the Microsurgery centre of Rīga Eastern Clinical University Hospital "Gaiļezers" Although injuries are usually obtained by healthy people, for unknown reasons, many of these patients during postoperative period suffer from a variety of complications, including septic complications.

This research studied the relationship of prolonged microvascular free flap surgery under two different methods of anaesthesia – general and regional, with activation of HHV-6 and HHV-7 infection, changes in numbers of immune cells and their influence on the postoperative period course.

Scientific novelty of treatment results

1. For the first time in the world the incidence of HHV-6 and HHV-7 infection was analysed in surgical patients who have undergone prolonged microvascular free flap surgery.
2. It was investigated and analysed how the prolonged microvascular free flap surgery carried out under general anaesthesia affects the activation of HHV-6 and HHV-7 infection in comparison to prolonged microvascular free flap operations carried out in regional anaesthesia, and how the activation of HHV-6 and HHV-7 infection affects the postoperative period course and surgical outcome.

The aim of the study

To explore the use of different methods of anaesthesia – general and regional – in prolonged microvascular free flap surgeries, their relationship with the activation of HHV-6 and HHV-7 infection and changes in cellular immune response in order to find the optimal application of anaesthesia in microvascular free flap operations.

Tasks of the research

1. To determine the presence and state of the HHV-6 and HHV-7 infection before and after prolonged microvascular free flap surgery in patients to whom general or regional anaesthesia was applied.
2. To determine the immunocompetent cells (lymphocytes and their subpopulations – CD4+, CD8+, CD16+ CD38+) before and after prolonged microvascular free flap surgery in patients to whom general or regional anesthesia was applied.
3. To determine presence and state of the HHV-6 and HHV-7 infection before and after short-term reconstructive surgery in patients to whom general or regional anaesthesia was applied.
4. To determine the immunocompetent cells (lymphocytes and their subpopulations – CD4+, CD8+, CD16+ CD38+) before and after short-term reconstructive surgery in patients to whom general or regional anaesthesia was applied.
5. To prepare recommendation othe anaesthesia method in prolonged microvascular free flap surgery.

The hypotheses of the study

1. Prolonged general anaesthesia in microvascular free flap surgeries compared to the prolonged regional anaesthesia significantly affects cellular immune response that may be associated with the activation of HHV-6 and HHV-7 infection.
2. HHV-6 and HHV-7 activation after prolonged microvascular free flap surgery adversely affects the postoperative period course and outcome of the surgery.

1 MATHERIAL AND METHODS

The study was performed at the Centre of Plastic and Microsurgery of Rīga Eastern Clinical University Hospital clinic "Gaiļezers", the Department of Anaesthesiology and Reanimation of Rīga Stradiņš University and August Kirchenstein Institute of Microbiology and Virology of Rīga Stradiņš University.

1.1 Inclusion criteria and study group

In this prospective study 89 patients were enrolled. The cohort was divided in a study group of 58 patients and and a control group, which included 31 patients.

All patients of the study group underwent prolonged microvascular free flap surgery in order to close large tissue defects. In the study group, 35 patients received general anaesthesia according to the same plan.

In the study group, 23 patients received regional anaesthesia. Regional anesthesia was done with the simultaneous use of two methods – spinal anesthesia and *Plexus brachialis* block.

All patients of the control group underwent short up to one hour long superficial plastic surgery. In the control group, 16 patients received general anaesthesia according to the same plan.

In the control group, 15 patients received regional anesthesia – *Plexus brachialis* block or spinal anesthesia.

Inclusion criteria:

1) at least 18 years of age (except for one 5-year-old boy who was on the enrollment date);

2) tissue defect was closed with microvascular flaps (study group);

3) short (up to one hour) plastic surgery (control group);

4) the patient is not pregnant;

5) the patient is a non-lactating woman;

6) the patient is not immunocompromised;

7) the patient without an autoimmune disease;

8) the patient without immunosuppressive therapy;

9) the patient without cardiopulmonary decompensation;

10) the patient without a mental disease;

11) the patient without oncology or more than 5 years after radical treatment, cured, without relapses of tumor or metastasis receive, not receiving adjuvant treatment;

12) the patient without HIV;

13) the patient without HCV;

14) the patient without addiction to drugs;

15) the patient without chronic alcoholism;

16) the patients without renal failure;

17) the patient without impaired hepatic function.

This thesis contains a unique clinical case that represents different results of two similar surgeries due to congenital pseudoarthrosis as a result of *Neurofibromatosis* type 1 (NF-1) and its different outcomes associated with active or latent HHV-6 and HHV-7 infection in 6-year-old boy. Anaesthesia, diagnostics of HHV-6 and HHV-7 infection and estimation of immune cell count was identical to the other patients enrolled in the study.

The general characteristics of patients of the study group are summarised in the Table 1.1.

Table 1.1

The characteristics of patients of the study group

Parameters	General anaesthesia (n = 35)	Regional anaesthesia (n = 23)
Age (years)	42.31 (± 13.45)	42.04 (± 15.26)
Sex, M/F (n)	22/13	15/8
ASA 1	11 (34.43 %)	13 (56.52 %)
ASA 2	21 (57.00 %)	9 (39.13 %)
ASA 3	3 (8.57 %)	1 (4.35 %)
Number of previous surgeries	2.80 (± 1.93)	1.78 (± 1.24)
Duration of microvascular free flap surgery (h)	320.57 (± 86.94)	341.74 (± 119.23)

Reasons for the surgery in the study group are summarised in the Table 1.2.

Table 1.2

Reasons for the surgery in the study group

Reasons for the surgery	General anaesthesia (n = 35)	Regional anaesthesia (n = 23)
Upper extremity trauma	4 (11.43 %)	8 (34.78 %)
Lower extremity trauma	23 (65.71 %)	12 (52.17 %)
Extensive surgery (onco.)	7 (20.00 %)	3 (13.04 %)
Status after scalp injury and skull fracture	1 (2.86 %)	0 (0.00 %)

The characteristics of patients of the control group are summarised in the Table 1.3.

Table 1.3

The characteristics of patients of the control group

Parameters	**General anaesthesia (n = 35)**	**Regional anaesthesia (n = 23)**
Age (years)	51.75 (± 13.89)	38.00 (± 14.21)
Sex, M/F (n)	10/6	9/6
ASA 1	13 (81.25 %)	14 (93.33 %)
ASA 2	3 (18.75 %)	11 (6.67 %)
ASA 3	0	0
Duration surgery (min)	45 (± 10.08)	41.33 (± 10.43)

Reasons for the surgery in the control group are summarised in the Table 1.4.

Table 1.4

Reasons for the surgery in the control group

Reasons for the surgery	**General anaesthesia (n = 16)**	**Regional anaesthesia (n = 15)**
Trauma	8 (50.00 %)	13 (86.96 %)
Benign tumour	3 (18.75 %)	2 (13.04 %)
Compressed nerve	2 (12.50 %)	0 (0.00 %)
Circulation disorders	3 (18.75 %)	0 (0.00 %)

1.2 Methods

From the patients of both study and control group blood samples with an anticoagulant (EDTA) for the detection of latent / persistent or active viral infection were collected before the surgery and 14 days after the surgery.

Nested polymerase chain reaction (nPCR) was used for the detection of HHV-6 and HHV-7 sequences in peripheral blood and plasma DNAs. The presence of viral sequences in peripheral blood DNAs was a marker of latent /

persistent viral infection and in plasma DNAs – of active viral infection (plasma viremia).

HHV-6 and HHV-7 load determined in accordance with the manufacturer's instructions with real-time PCR method as a template using the DNA isolated from full blood, and using HHV-6 Real-TM Quant (SACACE biotechnologies, Italy) and HHV-7 PrimerDesign Ltd. (Genesig, United Kingdom) commercially available working sets.

From all the patients of both study and control groups in the morning before the surgery peripheral blood samples were collected in order to evaluate changes of CD4+, CD8+, CD38+, CD16+ cell count. Repeated blood sample collection was carried out on 14 post-operative day. Blood was taken from a vein with a vacuum tube with K3 EDTA. The minimum amount of blood test was one ml. Principle of the method – peripheral blood lymphocyte subpopulation content was determined by FACS Calibur cytofluometer laser (Becton Dickinson) and by using corresponding monoclonal antibodies.

To detect the level of IL-1β (interleukin-1β), IL-2 (interleukin-2), IL-6 (interleukin-6) and TNF-α (tumor necrosis factor α), IL-1β working set of ELISA (*enzyme-linked immunosorbent assay*) (IBL International GMBH, Germany) was used in accordance with the manufacturer's recommendations.

2. RESULTS

2.1. Frequency of HHV-6 infection before and after surgery in the study and control groups

In GA (n = 35) patients of the study group before the prolonged microvascular free flap surgery latent / persistent HHV-6 infection was found in 14 (40.00 %), active HHV-6 infection in two (5.71%) patients. Detecting the viral load by real-time PCR, before the surgery, it was found that in all 16 patients with latent / persistent or active HHV-6 infection, viral load was < 10 HHV-6 copies/1 × 10^6 cells and < 10 HHV-6 copies/1 × μg DNA. In 19 patients (54.29 %) HHV-6 infection was not detected.

In contrast, in GA patients (n = 16) of the control group before the surgery latent / persistent HHV-6 infection was found in two (12.50 %) patients and viral load was < 10 HHV-6 copies/1 × 10^6 cells and < 10 HHV-6 copies / 1 × μg DNA. Active infection in GA patients of control group was not detected. In 14 patients (87.50%) HHV-6 infection was not detected.

In RA (n = 23) patients of the study group before the prolonged microvascular free flap surgery latent / persistent HHV-6 infection was found in six (26.09 %), active HHV-6 infection in two (8.70 %) patients. Detecting the viral load by real-time PCR before surgery was found that six patients had viral load < 10 HHV-6 copies/1 × 10^6 cells and < 10 HHV-6 copies/1 × μg DNA. In two patients high viral load was detected. In the first patient, viral load was 7620.23 HHV-6 copies/1 × 10^6 cells and 1154.58 HHV-6 copies/1 × μg DNA and this patient had active HHV-6 infection. In the second patient, viral load was 6676.3 HHV-6 copies/1 × 10^6 cells and 1011.56 HHV-6 copies/1 × μg DNA and latent / persistent HHV-6 infection.

In 15 patients (65.29 %) HHV-6 infection was not detected.

In RA patients (n = 15) of the control group before the surgery latent / persistent HHV-6 infection was found in two (13.33 %) patients and viral load was < 10 HHV-6 copies/1 × 10^6 cells and <10 HHV-6 copies/1 × μg DNA. Active infection in RA patients of control group was not detected. In 13 patients (86.67 %) (87.50 %) HHV-6 infection was not detected.

After prolonged microvascular free flap surgery in one GA patient of the study group the activation of HHV-6 infection was detected.

Detecting the viral load in patient with activation of HHV-6 infection, viral load did not change and remained < 10 HHV-6 copies/1 × 10^6 cells and < 10 HHV-6 copies/1 × μg DNA.

In remaining patients in whom after surgery remained latent / persistent or active HHV-6 infection, viral load was < 10 copies of HHV-6/1 × 10^6 cells and < 10 copies of HHV-6/1 × μg DNA.

Comparing frequencies of active HHV-6 infection in GA patients of the study group before and after the surgery, it was found that frequency of active HHV-6 infection increases after the surgery without statistical significance ($p = 0.31$).

After short surgery in control group in GA patients the activation of HHV-6 infection was not detected. All patients with latent / persistent infection viral load was < 10 copies of HHV-6/1 × 10^6 cells and < 10 copies of HHV-6/1 × μg DNA.

After prolonged microvascular free flap surgery in RA patients of the study group the activation of HHV-6 infection was not detected.

Also in RA patients of the control group after short surgery activation of HHV-6 infection was not detected.

2.2. Frequency of HHV-7 infection before and after surgery in the study and control groups

In GA (n = 35) patients of the study group before the prolonged microvascular free flap surgery latent / persistent HHV-7 infection was found in 26 (74.29 %), active HHV-7 infection in three (8.57 %) patients. Detecting the viral load by real-time PCR, before the surgery, it was found that in 27 patients with latent / persistent or active HHV-7 infection, viral load was < 10 HHV-7 copies/1 × 10^6 cells and < 10 HHV-6 copies/1 × μg DNA. In two patients high viral load was of HHV-7 detected. One patient with active HHV-7 infection viral load was 5419.5 HHV-7 copies/1 × 10^6 cells and 821.1310 HHV-7 copies/1 × μg DNA, other patient with latent / persistent HHV-7 infection had the viral load of 6937.33 HHV-7 copies/1 × 10^6 cells and 1509.71 HHV-7 copies/1 × μg DNA.

In six patients (17.14%) HHV-7 infection was not detected.

In contrast, in GA patients (n = 16) of the control group before the surgery latent / persistent HHV-7 infection was found in three (18.57 %) patients and viral load was < 10 HHV-7 copies/1 × 10^6 cells and < 10 HHV-7 copies/1 × μg DNA. Active HHV-7 infection in GA patients of control group was not detected. In 13 patients (81.25 %) HHV-7 infection was not detected.

In RA (n = 23) patients of the study group before the prolonged microvascular free flap surgery latent / persistent HHV-7 infection was found in 16 (69.75 %), active HHV-7 infection in four (17.39 %) patients. Detecting the viral load by real-time PCR before surgery was found that 20 with active and latent/persistent HHV-7 infection had viral load < 10 HHV-7 copies / 1 × 10^6 cells and < 10 HHV-7 copies/1 × μg DNA.

In three patients (13.04 %) HHV-7 infection was not detected.

In RA patients (n = 15) of the control group before the surgery latent / persistent HHV-7 infection was found in two (13.33 %) patients and viral load was < 10 HHV-7copies/1 × 10^6 cells and <10 HHV-7 copies/1 × μg DNA. Active infection in RA patients of control group was not detected. In 13 patients (86.67 %) (87.50%) HHV-7 infection was not detected.

After prolonged microvascular free flap surgery in seven GA patients of the study group the activation of HHV-6 infection was detected.

Comparing frequencies of active HHV-7 infection in GA patients of the study group before and after the surgery, it was found that frequency of active HHV-7 infection after the surgery increases statistically significant ($p = 0.01$). In one patient with latent / persistent HHV-7 infection before and after surgery viral load increased from < 10 copies of HHV-7/1 × 10^6 cells and < 10 copies of HHV-7/1 × μg DNA before the surgery to 10981.46 copies of HHV-7/1 × 10^6 cells and 1663.85 copies of HHV-7/1 × μg DNA

After short surgery in control group in GA patients the activation of HHV-6 infection was not detected and viral load of HHV-7 remained constant.

After prolonged microvascular free flap surgery in RA patients of the study group the activation of HHV-7 infection detected in one patient.

Comparing frequencies of active HHV-7 infection in GA patients of the study group before and after the surgery, was found that frequency of active HHV-7 infection increases after the surgery without statistical significance ($p = 0.28$).

In RA patients of the control group after short surgery activation of HHV-7 infection was not detected.

2.3. Impact of surgery and anaesthesia on changes of immune cell number

Comparing the average number of lymphocytes and lymphocyte subsets (CD4+, CD8+, CD16+, CD38+) and imunoregulatory indexes (CD4+/CD8+) between GA and RA subgroups of the study group before prolonged free flap surgery it was found that none of these parameters are statistically significantly different ($p > 0.05$) (Table 2.1).

Table 2.1

Number of immune cells in the study group before surgery

Sub-group	Lympho-cytes	CD4+	CD8+	CD16+	CD38+	CD4/ CD8
VA	1786.97 ± 558.45	800.58 ± 265.57	507.39 ± 219.07	240.94 ± 16.22	591.33 ± 218.72	1.74 ± 0.71
AR	1670.00 ± 643.62	821,76 ± 454,23	430.47 ± 214.61	231.90 ±130.23	502.19 ± 185.79	2.08 ± 0.94

Also comparing the average number of lymphocytes and lymphocyte subsets (CD4+, CD8+, CD16+, CD38+) and imunoregulatory indexes (CD4+/CD8+) between GA and RA subgroups of the 19ontrol group before prolonged free flap surgery it was found that none of these parameters are statistically significantly different ($p > 0.05$) (Table 2.2).

Table 2.2

Number of immune cells in the study group before surgery

Subgroup	Lymphocytes	$CD4^+$	$CD8^+$	CD16+	CD38+	CD4/CD8
VA	2563.10 ±844.93	1187.3 ±437.25	680.25 ±228.22	408.75 ±238.22	732.56 ±272.19	1.86 ±0.70
AR	2650.20 ±835.02	1186.40 ±420.06	761.60 ±381.19	294.20 ±122.51	750.40 ±314.07	1.73 ±0.55

Comparing the average number of lymphocytes and lymphocyte subsets (CD4+, CD8+, CD16+, CD38+) and imunoregulatory indexes (CD4+/CD8+) between GA subgroups of the study group and control group before surgery it was found that none of these parameters are statistically significantly different ($p > 0.05$) (Table 2.3).

Table 2.3

Number of immune cells in GA patients of the study and control groups before surgery

Group	Lympho-cytes	$CD4^+$	$CD8^+$	$CD16^+$	$CD38^+$	CD4/ CD8
Study	1786.97 ± 558.45	800.58 ±265.57	507.39 ±219.07	240.94 ±156.22	591.33 ±218.72	1.74 ±0.71
Control	2563.10 ± 844.93	1187.3 ±437.25	680.25 ±228.22	408.75 ±238.22	732.56 ±272.19	1.86 ±0.70

Comparing the average number of lymphocytes and lymphocyte subsets (CD4+, CD8+, CD16+, CD38+) and imunoregulatory indexes (CD4+/CD8+) between RA subgroups of the study group and control group before surgery it was found that none of these parameters are statistically significantly different ($p > 0.05$) (Table 2.4).

Table 2.4

Number of immune cells in AR patients of the study and control groups before surgery

Group	Lympho-cytes	$CD4^+$	$CD8^+$	$CD16^+$	$CD38^+$	CD4/ CD8
Study	1670.00 ±643.62	821.76 ±454.23	430.47 ±214.61	231.9 ±130.23	502.19 ±185.79	2.08 ±0.94
Controle	2650.20 ±835.02	1186.40 ±420.06	761.60 ±381.19	294.20 ±122.51	750.40 ±314.07	1.73 ±0.55

Comparing the average number lymphocytes and lymphocyte subsets (CD4+, CD8+, CD16+, CD38+) and imunoregulatory indexes (CD4+/CD8+) before and after prolonged microvascular free flap surgery in GA subgroup of

study group, statistically significant decrease in the number of lymphocytes, CD8+ cells and increase of imunoregulatory index (CD4+/CD8+) after the surgery was observed (p = 0.049; p = 0.03; p = 0.005) (Table 2.5).

Table 2.5

Number of immune cells in GA patients of study group

Time	Lympho-cytes	$CD4^+$	$CD8^+$	$CD16^+$	$CD38^+$	CD4/ CD8
Before operation	1786.97 ±558.45	800.58 ±265.57	507.39 ±219.07	240.94 ±156.22	591.33 ±218.72	1.75 ±0.72
After operation	1676.67 ±569.87	777.79 ±294.47	460.18 ±235.84	219.30 ±119.94	602.12 ±241.13	2.01 ±0.95

Comparing the average number of lymphocytes and lymphocyte subsets (CD4+, CD8+, CD16+, CD38+) and imunoregulatory indexes (CD4+/CD8+) before and after prolonged microvascular free flap surgery in RA subgroup of study group, statistically significant increase in the number of CD38+ cells and increase of imunoregulatory index (CD4+/CD8+) after the surgery was observed (p = 0.017) (Table 2.6).

Table 2.6

Number of immune cells in AR patients of study group

Time	Lymho-cytes	CD4+	CD8+	CD16+	CD38+	CD4/ CD8
Before operation	1670.00 ±643.62	821.76 ±454.42	430.48 ±214.61	231.90 ±130.23	502.19 ±185.79	1.86 ±0.10
After operation	1687.14 ±475.82	801.95 ±236.92	414.86 ±156.26	264.10 ±181.94	624.81 ±187.60	2.01 ±0.95

Comparing the average number of lymphocytes and lymphocyte subsets (CD4+, CD8+, CD16+, CD38+) and imunoregulatory indexes (CD4+/CD8+) before and after short surgery in control group irrespective of anaesthesia method applied, statistically significant changes were not observed (p > 0.05) (Table 2.7, Table 2.8).

Table 2.7

Number of immune cells in GA patients of control group

Time	Lympho-cytes	CD4+	CD8+	CD16+	CD38+	CD4/ CD8
Before operation	2563.10 ±844.93	1187.37 ±437.25	680.25 ±228.22	408.75 ±238.22	732.56 ±272.19	1.86 ±0.70
After operation	2688.84 ±947.31	1218.88 ±540.88	723.31 ±267.33	395.25 ±170.20	728.63 ±271.33	1.76 ±0.58

Table 2.8

Number of immune cells in control group patients who received regional anaesthesia

Time	Lympho-cytes	CD4+	CD8+	CD16+	CD38+	CD4/ CD8
Before operation	2650.20 ±835.02	1186.40 ±420.06	761.60 ±381.19	294.20 ±122.51	750.40 ±314.07	1.73 ±0.55
After operation	2721.70 ±889.16	1189.00 ±424.47	836.80 ±471.00	330.93 ±138.02	691.66 ±221.17	1.69 ±0.68

2.4. Changes of immune cells in relationship to HHV-6 and HHV-7 infection

The relationship of the number lymphocytes and lymphocyte subsets (CD4+, CD8+, CD16+, CD38+) and imunoregulatory indexes (CD4+/CD8+) HHV-6 and HHV-7 infection with were studied by not dividing patients of study group into GA and RA subgroups. Nonparametric Mann–Whitney test was used for statistical calculation.

Comparing the average number of lymphocytes and lymphocyte subsets (CD4+, CD8+, CD16+, CD38+) and imunoregulatory indexes (CD4+/CD8+) in patients of the study group with active HHV-6 and / or HHV-7 and latent / persistent HHV-6 and / or HHV-7 infection before surgery, statistically significant changes were not observed ($p > 0.05$) (Table 2.9).

Table 2.9

Number of immune cells in patients with active and latent / persistent HHV-6 and / or HHV-7 infection before surgery

Patients	Lymfpho-cytes	$CD4^+$	$CD8^+$	$CD16^+$	$CD38^+$	CD4/ CD8
Active infection after surgery (n = 11)	1490 (1280–2060)	838 (678–948)	412 (320–515)	185 (133–216)	492 (453–741)	1.84 (1.33–2,58)
Latent / persistent infection before surgery (n = 32)	1860 (1605–2053)	873.5 (648.5–1002)	488 (393–609)	228 (160–340)	581 (397–657)	1.82 (1,41–2.39)

Comparing the average number of lymphocytes and lymphocyte subsets (CD4+, CD8+, CD16+, CD38+) and imunoregulatory indexes (CD4+/CD8+) in patients of study group with active HHV-6 and / or HHV-7 infection after the surgery, statistically significant changes were not observed ($p > 0.05$) (Table 2.10).

Table 2.10

Number of immune cells in patients with active HHV-6 and / or HHV-7 infection after the surgery

Patients	Lymphocytes	$CD4^+$	$CD8^+$	$CD16^+$	$CD38^+$	CD4/CD8
Active infection before surgery (n = 9)	1500 (970–2060)	742 (396–948)	355 (285–602)	165 (93–408)	554 (310–763)	1,53 (1.35–3.7)
Latent / persistent infection after surgery (n = 23)	1660 (1315–2120)	747 (617–966.5)	377 (320–770)	232 (150.5–374.5)	595 (478.5–742.0)	1.7 (1.54–2.49)

Comparing the average number of lymphocytes and lymphocyte subsets (CD4+, CD8+, CD16+, CD38+) and imunoregulatory indexes (CD4+/CD8+) before and after the surgery in patients of study group which activation of HHV-6 and / or HHV-7 infection after the surgery was detected, statistically significant changes were not observed ($p > 0.05$) (Table 2.11).

Table 2.11

Number of immune cells in patients with activation of HHV-6 and / or HHV-7 infection after the surgery

Patients	Lympho-cytes	CD4+	CD8+	CD16+	CD38+	CD4/CD8
Latent / persistent infection before surgery (n = 9)	1470 (1270–1950)	630 (588–975)	459 (338–505)	147 (135–215)	405 (368–662)	1.38 (1.32–1.96)
Active infection before surgery (n = 9)	1544 (970–2060)	742 (396–948)	335 (285–602)	165 (93–408)	554 (310–763)	1.53 (1.35–3.76)

2.5. Effects of surgery and anaesthesia on the concentration of IL-1β, IL-2, IL-6 and TNF-α

Effects of surgery and anaesthesia on concentration of IL-1β, IL-2, IL-6 and TNF-α were studied by not dividing patients of the study group into GA and RA subgroups. Nonparametric Mann–Whitney test was used for statistical calculation.

Comparing the average number of concentration of IL-1β, IL-2, IL-6 and TNF-α before the surgery in patients with latent / persistent HHV-6/HHV-7 infection statistically significant differences were not observed ($p > 0.05$) (Table 2.12).

Table 2.12

Concentration of IL-1β, IL-2, IL-6 and TNF-α in patients with latent / persistent HHV-6/HHV-7 infection before and after surgery

Patients	IL-1β (pg/ml)	IL-2 (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)
Latent / persistent infection before surgery (n = 18)	< 0.3	193.6 ± 20.2	12.1 ± 11.3	< 5
Latent / persistent infection after surgery (n = 18)	< 0.3	184.4 ± 17.5	10.9 ± 8	< 5

Comparing the average number of concentration of IL-1β, IL-2, IL-6 and TNF-α before the surgery in patients with active HHV-6/HHV-7 infection statistically significant differences were not observed ($p > 0.05$) (Table 2.13).

Table 2.13

Concentration of IL-1β, IL-2, IL-6 and TNF-α in patients with active HHV-6/HHV-7 infection before and after surgery

Patients	IL-1β (pg/ml)	IL-2 (pg/ml)	IL-6 (pg/ml)	TNF- α (pg/ml)
Active infection before surgery (n = 9)	< 0,3	187 ± 11,9	5,3 ± 4,5	< 5
Active infection after surgery (n = 9)	< 0,3	195 ± 16,1	6,8 ± 5,6	< 5

Comparing the average number concentration of IL-1β, IL-2, IL-6 and TNF-α before and after the surgery in patients with reactivation of latent / persistent HHV-6/HHV-7 infection after the surgery statistically significant decrease of concentration of IL-2 ($p = 0,003$). Changes of concentration of IL-1β, IL-6 and TNF-α after the surgery were not observed ($p > 0.05$) (Table 2.14).

Table 2.14

Concentration of IL-1β, IL-2, IL-6 and TNF-α in patients with activation of HHV-6/HHV-7 infection after surgery

Patients	IL-1β (pg/ml)	IL-2 (pg/ml)	IL-6 (pg/ml)	TNF- α (pg/ml)
Latent / persistent infection before surgery (n = 6)	< 0.3	213 ± 12.6	9.4 ± 5.1	< 5
Active infection after surgery (n = 6)	< 0.3	191 ± 14	10 ± 7.4	< 5

2.6. Possible relationship of HHV-6 and HHV-7 infection with the outcome of the surgery

Comparing surgical outcomes (flap ischemia, infectious complications, primary healing) in GA and RA patients of the study group, it was found that in GA patients flap ischemia was in 3 (8.75%) patients, infectious complications in 10 (28.57%) patients, but in 22 (62.86%) patients wound was healed without complications.

In RA patients flap ischemia was found in 5 (21.74%) patients, infectious complications in 3 (13.04%) patients, and in 15 (62.22%) patients, the wound healed without complications.

Comparing the frequencies of complications (flap ischemia, infectious complications) between GA and RA patients of the study group, independent sample t-test indicated that the frequencies of complications between GA and RA subgroups are not statistically significantly different (p = 0.20) (Figure 2.1).

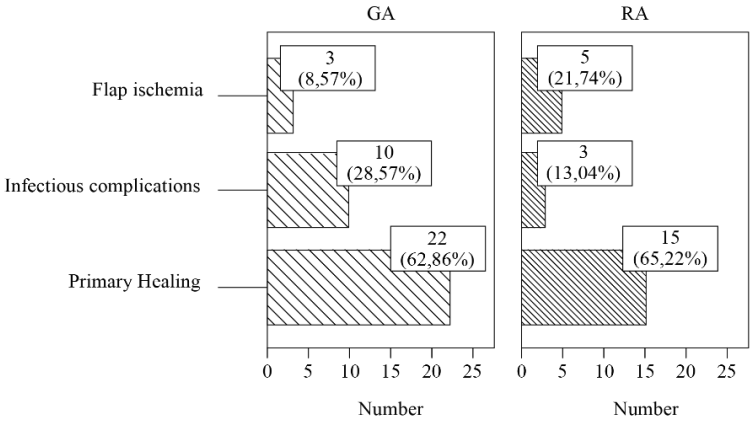

Figure 2.1. **Surgical outcome in GA and AR patients of the study group**

Duration of the post-postoperative period in the GA subgroup of the study group was 30.14 (± 27.72) days, in the RA subgroup – 14.52 (± 10.55) days (Figure 2.2). The comparison between the post-operative period durations between VA and RA subgroups based on independent sample t-test proved that post-operative duration of the GA subgroup is statistically significantly longer than the RA subgroup ($p < 0.01$).

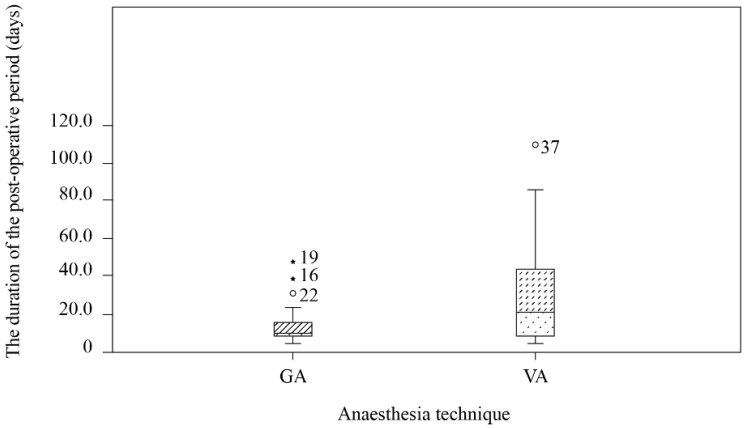

Figure 2.2. **Duration of post-operative period**

In the GA subgroup of the study group 21 (60.00%) patient in the postoperative period was treated in the intensive care unit, in turn, in RA subgroup only 3 (13,04%) patients needed treatment in the intensive care unit. The differences are statistically significant ($p = 0.001$).

The average duration of treatment in the intensive care unit during postoperative period for GA subgroup patients was 6.76 (± 10.83) days, while for the patients of RA subgroup 2 (± 1.00) days. This difference was not statistically significant ($p = 0.46$).

All patients of the study group, regardless of the anaesthetic technique applied, were evaluated for both latent / persistent and active HHV-6 and HHV-7 infection in relationship with incidence of the post-operative surgical complications – flap ischemia, thrombosis, partial or localized necrosis and wound infection.

While using the Pearson χ^2 test, it was found that neither the latent / persistent nor active HHV-6/HHV-7 infection have any statistically significant ($p = 0.36$) influence on the development of surgical complications in post-operative period, the following tendency was observed, in patients with activation of HHV-6/HHV-7 infection after surgery complications were 1.77 times more often than in patients if HHV-6/HHV-7 infection had remained latent / persistent after the surgery (OR = 1.77; 95% CI 0.50–6.2) (Figure 2.3).

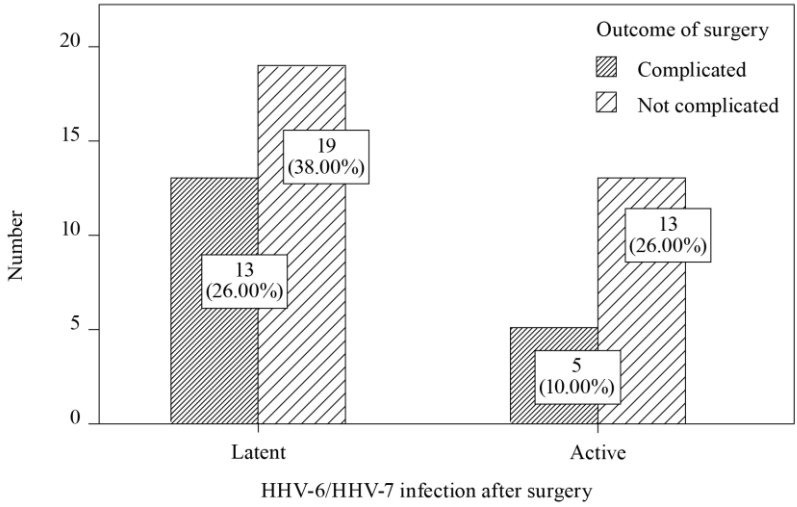

Figure 2.3. **Relationship of HHV-6/HHV-7 infection with postoperative complications**

All patients of the study group, regardless of the anaesthetic technique applied, were evaluated for both latent / persistent and active HHV-6 and HHV-7 infection in relationship with incidence of the post-operative wound infection.

Using the Pearson χ^2 test, it was found that neither the latent / persistent nor active HHV-6/HHV-7 infection have any statistically significant ($p = 0.37$) influence on development of wound infection in post-operative period, the following tendency was observed, in patients with activation of HHV-6/HHV-7 infection after surgery development of post-operative wound infection was 1,78 times more often than in patients if HHV-6/HHV-7 infection had remained latent / persistent after the surgery (OR = 1.78; 95% CI 0.49–6.48) (Figure 2.4).

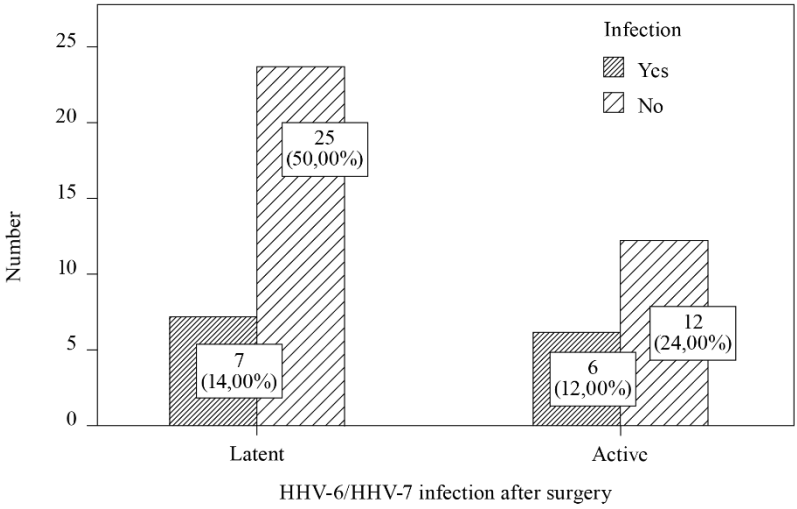

Figure 2.4. **Relationship of HHV-6 / HHV-7 infection with post-operative wound infection**

Though statistically significant relationship between status of HHV-6 and HHV-7 infection, development of post-operative wound infections and duration of postoperative period failed to identify, detailed analysis of those 13 patients, who already before the operation had active HHV-6 or HHV-7

infection, the following tendency was observed – 7 patients had chronic osteomyelitis prior to the surgery. It is more common than the overall number of patients.

All patients of the study group, regardless of the anaesthetic technique applied, were evaluated for both latent / persistent and active HHV-6 and HHV-7 infection in relationship with the duration of post-operative period.

While using the independent sample t-test, it was found that neither the latent/persistent nor active HHV-6/HHV-7 infection have any statistically significant ($p = 0.36$) influence on duration of post-operative period. The following tendency was observed in patients with activation of HHV-6/HHV-7 infection after surgery duration of post-operative period was longer ($M = 25.57 \pm 22.96$) compared to patients with HHV-6/HHV-7 if infection had remained latent / persistent after the surgery (21.12 ± 12.40) (Figure 2.5).

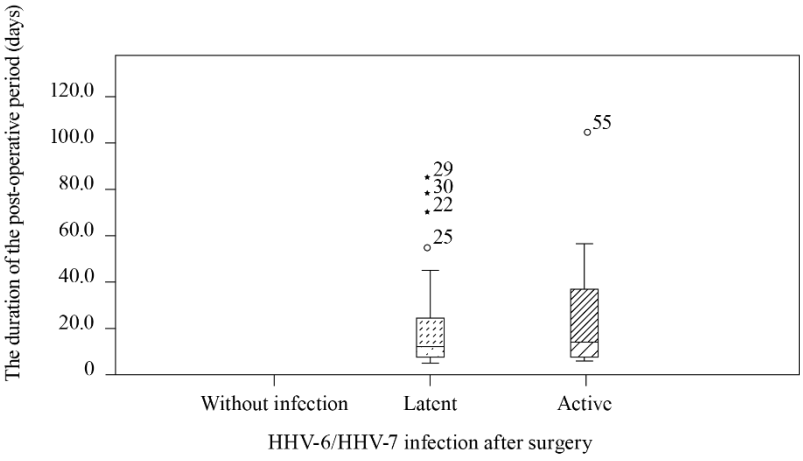

Figure 2.5. **Relationship of HHV-6 / HHV-7 infection with duratinon of post-operative period**

All patients of the study group, regardless of the anaesthetic technique applied, were evaluated for relationship of status of HHV-6 and HHV-7 infection (active or latent / persistent) before surgery with the physical status of patients before surgery (ASA grade).

In accordance with the independent sample t-test it was found that the physical status (ASA grade) before the surgery was not statistically significantly related to the status (active or latent / persistent) of HHV-6/ HHV-7 infection before surgery ($p = 0.93$).

All patients of the study group, regardless of the anaesthetic technique applied, were evaluated for relationship of status of HHV-6 and HHV-7 infection (active or latent / persistent) after surgery with the physical status of patients before surgery (ASA grade).

Also the relationship of the status (active or latent / persistent) of HHV-6/HHV-7 infection after surgery with a physical status of the patients before surgery was not statistically significant ($p = 0.87$).

All patients of the control group, irrespective of the method of anaesthesia applied, left the hospital on the day of the surgery.

For all patients of the control group the post-operative period proceeded without complications.

2.7. Case report

This thesis contains a unique clinical case of a boy with NF-1 who at the age of 5 and 6 received two microvascular free flap surgeries due to congenital tibial pseudoarthrosis.

At the age of 6 months the patient was diagnosed NF-1. It was observed that he had more than six *café au lait* spots and a right lower limb deformity – congenital tibial pseudoarthrosis.

At the age of 5, in the Latvian Centre of Plastic and Reconstructive Surgery, tibial microvascular reconstruction with contralateral fibula free flap was performed to the boy. Total duration of surgery was 6 hours. The child received balanced general anaesthesia according to accepted standards of free flap surgery. Isoflurane was the inhalation agent of choice because of its beneficial effects on systemic vascular resistance. A regional block to supplement general anaesthesia was not used.

A day before and on the tenth day after surgery EDTA anti-coagulated peripheral blood samples from the patient were collected to detect HHV-6 and/or HHV-7 infection and the number of peripheral blood CD4+, CD8+, CD38+, and CD16+ positive cells to detect the status of immune system. Samples taken before and after the surgery, revealed active HHV-6 and HHV-7 infection and decreased number of CD4+ and NK cells.

After 6 months, the child was hospitalised due to non-healing wounds and a few fistulas on the right shin and X-rays showed absorbing of fibula transplant. The patient underwent surgery-debridement and osteonecrectomia.

One year after the first microvascular free flap surgery the child was re-hospitalised in the Latvian Centre of Plastic and Reconstructive Surgery for elective plastic of congenital tibial pseudoarthrosis with *femur-vastus medialis* osteomuscular free flap microvascular surgery. Total duration of surgery was 6.5 hours.

Balanced general anaesthesia according to accepted standards of free flap surgery was applied. Isoflurane was the inhalation agent of choice. A regional block to supplement general anaesthesia was not used.

Samples for the detection of HHV-6 and HHV-7 infection and proportions of CD4+, CD8+, CD38+, and CD16+ subpopulations before the surgery revealed latent HHV-6 and HHV- 7 infection and decreased number of NK cells. Postoperatively, the child spent one day in ICU for maintenance of

fluid balance, analgesia and clinical monitoring of the condition of the flap. Thrombosis of flap blood vessels was observed on the eighth day and muscular part of the flap was removed on the tenth day due to necrosis; the bone was covered with local flap. No signs of osteomyelitis were observed. The child was discharged from the hospital on the twenty-fourth day. Repeated analysis revealed latent HHV-6 and HHV-7 infection and a decreased number of NK cells.

The child received balanced general anaesthesia according to accepted standards of free flap surgery. Isoflurane was the inhalation agent of choice because of its beneficial effects on systemic vascular resistance. A regional block to supplement general anaesthesia was not used.

This case demonstrates the potential impact of immunomodulatory effects caused by active HHV-6 and HHV-7 infection on outcome of the treatment.

3. DISCUSSION

In the literature review section, it is described that the general anaesthesia reduces, but does not excludes surgical stress. In addition to that, general anaesthesia affects the number and function of immune cells.

According to the literature, nowadays research of the impact of different methods of anesthesia on immune function is being conducted to patients with oncologic diseases in order to maximise the reduction of development of tumour relapses.

Prior to this study, no databases revealed studies of surgeries and the influence of different anaesthesia on activation of HHV6 and HHV-7 infection and how this infection affects the postoperative period course and surgical outcome.

In the study after prolonged microvascular free flap surgery with GA in 7 patients, activation of the latent / persistent HHV-7 infection was revealed. This was statistically significant ($p < 0.05$). This confirms the fact that the GA does not fully prevent surgical stress response, thus reinforcing the postoperative immunosuppression. We found that after prolonged microvascular free flap surgery in patients who received GA the effector phase of cellular immunity was affected – statistically significant decline CD8+ cell count and increase of imunoregulatory index (CD4+/CD8+). It was found in patients who received RA. By contrast, in patients who received RA we observed statistically significant increase of CD38+ cells, representing an active immune response.

Our findings coincide with previous research, which suggests that general anaesthesia method fully prevents surgical stress response, thus creating a post-operative immunosuppression (Stevenson, 1990).

Although statistically significant relationship between a state of HHV-6 and HHV-7 infection and development of post-operative site surgical infections and the process of progress of postoperative period course was not statistically significant, a detailed analysis of medical history of 13 patients who before the surgery have already active HHV-6 or HHV-7 infection we have found that 7 of them had chronic osteomyelitis prior to the surgery. It is considerably more often than in other patients and might be related to the active HHV-6 or HHV-7 infection.

This could be explained by the fact that a major surgery causes endocrine stress response which is characterised by elevated levels of serum cortisol, adrenaline and noradrenaline. Furthermore, the surgery-induced stress response is often accompanied by lymphopenia and granulocytosis in peripheral blood.

The data from previous studies indicate that major surgery induces a redistribution of lymphocytes from peripheral blood to lymphatic tissue. It is suggested that the endocrine stress response is of major importance (Toft, 2003).

Major surgery suppresses cellular immunity for several days. Humoral immunity remains relatively intact (Shakhar, 2003). There is a measurable decrease in the production of cytokines that favours cellular-mediated immunity such as IL-2, IL-12 and IFN-g and an increase in the production of cytokines that interferes with cell-mediated immunity, such as IL-10. There is a decrease in the number of circulating NK cells, cytotoxic T lymphocytes, dendritic cells, and T-helper cells. A peak in immunosuppression is thought to occur on the third day after the surgery (Coffey, 2003).

A lot of effort is directed towards the determination of the role of HHV-6/HHV-7 in immunocompromised patients. Viruses can affect immune function in different ways. For example, cytomegalovirus can reduce the

secretion of IL-1 and IL-2 of PBMC, as well as decrease proliferative response of the cell to the mitogen. Paramyxoviruses, respiratory syncytial virus and measles hinder the proliferative response (Flamand, 1995; Kapasi, 1988; Preston, 1992; Yanagi, 1992).

It is proved that not only stress of surgery induces, but also immunosuppression stress, anaesthetic and analgesic agents, widely used in anaesthesia and intensive care may directly affect immune cell functions (Kurosawa, 2008).

Our results suggest that prolonged surgery and anaesthesia are immunosuppressive factors that lead to activation of latent / persistent HHV-6 and HHV-7 infection, viruses have immuno-modulatory properties that make immunosuppression more pronounced. This immunosuppression may be followed by a variety of opportunistic infections and development of other non-infectious complications. Our study confirmed that general anaesthesia demonstrates more pronounced immunosuppressive effects compared to regional anesthesia.

CONCLUSIONS

1. The application of general anaesthesia in prolonged microvascular free flaps surgeries is associated with a statistically significant activation of HHV-7 infection.
2. The application of regional anaesthesia in prolonged microvascular free flap surgeries is not statistically significant in association with the activation of HHV-6 or HHV-7 infection.
3. Prolonged microvascular free flap surgery performed under general anaesthesia suppresses the effector stage of cellular immune response.
4. The application of regional anaesthesia in prolonged microvascular free flap surgeries preserves active immune response.
5. Short reconstructive surgery regardless of the method of anaesthesia applied – general or regional – is not associated with the activation of HHV-6 or HHV-7 infection and changes in the number of immune cells.
6. After prolonged microvascular free flap surgery neither active nor latent / persistent HHV-6 and HHV-7 infection impact on post-operative period course and outcome of surgery is statistically significant; however, patients with post-operative activation of HHV-6 and HHV-7 infection are more likely to get complications, as well as the duration of their post-operative period course is extended.
7. In individual cases the role of active HHV-6 and HHV-7 infection in the postoperative period course and outcome in surgery is significant, as described in our clinical case of a boy with neurofibromatosis.

PRACTICAL RECOMMENDATIONS

1. For the patients who need to receive a long-term microvascular free flap surgery it is important to assess the potential risk of immunosuppression caused by this surgery and complications related to anaesthesia and immunosuppression.
2. It is important to assess the immune function in the perioperative period of the patients who suffered from septic complications after an injury and require a long-term microvascular free flap surgery.
3. To avoid the risk of general anaesthesia related immuno-suppression in situations when the primary localisation of lesion of tissue and the patient's co-morbidities and degree of their decompensation allow the usage of regional anaesthesia, it is recommended together with surgeons to consider the choice of a donor site location suitable for the application of regional anaesthesia.

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