https://doi.org/10.15360/1813-9779-2024-1-2350

The Effect of Transfusion and Hypoxia on Cells in an *in vitro* Model of the Neurovascular Unit

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For citation: *Artem A. Ivkin, Evgeny V. Grigoriev, Elena D. Khilazheva, Andrey V. Morgun.* The Effect of Transfusion and Hypoxia on Cells in an *in vitro. Obshchaya Reanimatologiya = General Reanimatology.* 2024; 20 (1): 37–42. https://doi.org/10.15360/1813-9779-2024-1-2350 [In Russ. and Engl.]

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Summary

Up to 57% of patients develop postoperative delirium after surgery for congenital heart defects (CHD). To reduce cerebral damage in pediatric patients during CHD surgery it is important to find out what inflicts the worse damage: would it be a systemic inflammatory response (SIR) triggered by transfusion, or hypoxia developed in non-transfused patients? *In vitro* evaluation of hypoxia and SIR effects on the neurovascular unit (NVU) cells might contribute to finding the answer.

The aim of the study was to compare the effect of varying severity hypoxia and SIR on the functional activity of NUV cells *in vitro*.

Materials and methods. An *in vitro* NVU model was designed including neurons, astrocytes and endotheliocytes. The effect of hypoxia on NVU was evaluated in the control (C) and 4 study groups (H 1–4), formed based on O_2 content in the medium. The C group NVU were cultivated in standard conditions: N_2 — 75%, O_2 — 20%, CO_2 — 5%; H1: N_2 — 99%, O_2 — 1%; H2: N_2 — 98%, O_2 — 2%; H3: N_2 — 97%, O_2 — 3%; H4: N_2 — 96%, O_2 — 4%. The significance of the differences was 0.0125. The effect of interleukin-6 (IL-6) content on NVU was measured by adding to culture medium pediatric patients' serum with known minimal or maximal SIRS-response. The assessment was made in the Control — an intact NVU model, and 2 study groups — «Minimum» and «Maximum», i. e. samples with minimum or maximum IL-6 content in culture, respectively. The significance of the differences was 0.017. The cells were incubated at a normothermia regimen for 30 minutes. Then, the functional activity of NVU cells was evaluated by measuring transendothelial resistance (TER) for 24 hours and Lucifer Yellow (LY) permeability test at 60 and 90 minutes after the start of the experiment.

Results. After incubation under hypoxic conditions, TER changes occurred in all studied groups. However, they were statistically significant only in the group with 1% oxygen content in the medium. TER decrease in this group was observed after 2, 4 and 24 hours. LY permeability also changed at 60 and 90 minutes, similarly — in NVU cultivated with 1% oxygen in the medium. Minimal TER values were documented at 4 hours after patients' serum was added to NVU cells culture medium, and TER increased at 24 hours in both study groups. Cellular permeability to LY changed significantly after 1 hour exposure in both groups — with minimum and maximum IL-6 content in the medium. Although at 90 minutes, there was no difference between the 3 groups in LY permeability tests.

Conclusion. Intensive SIR demonstrated short-term but more deleterious than hypoxia, effect on cells in the NVU model. Hypoxia disrupted functional activity of NUV cells only at 1% O_2 concentration in the medium.

Keywords: transfusion; hypoxia; neurovascular unit; systemic inflammatory response; interleukin-6; cerebral damage; cardiopulmonary bypass; children; cardiac surgery

Conflict of interest. The authors declare no conflict of interest.

Funding. The research was supported by No. 22-15-00258; https://rscf.ru/project/22-15-00258/ grant from the Russian Science Foundation.

Introduction

Contemporary cardiac surgery and anesthesiology are rapidly evolving, with an increasing number of procedures being performed using minimally invasive or endovascular techniques. However, correction of congenital heart disease (CHD) in children often requires an extensive surgical intervention and the use of cardiopulmonary bypass (CPB). These surgical procedures carry the risk of potential brain damage in children due to various pathological factors, mainly associated with CPB. The effects of CPB can be both direct (embolism, hypoxia, hemodynamic changes) and indirect, through the initiation of a systemic inflammatory response (SIR) as a result of blood contact with the extracorporeal circuit, hemolysis, and disturbances in thermoregulation [1–4]. Furthermore, the infant brain, especially in the first year of life, exhibits various specific patterns, such as active neuronal differentiation and synaptogenesis, rapid glial cell growth and myelination, high hydrophilicity and metabolic rate [5, 6]. All these patterns make the brain vulnerable to

any pathological factors, which explains high incidence of postoperative cognitive disorders. For instance, postoperative delirium occurs in up to 57% of patients following congenital heart disease correction [7]. Importantly, the impact of any brain damage and dysfunction in childhood on a child's further development has received little attention. Meanwhile, studies in children who have undergone cardiac surgery show reduced cognitive performance one year later [8].

Therefore, it is important to explore ways to prevent brain injury during the correction of congenital heart disease in children. One promising approach is to minimize the perioperative use of transfusions. This strategy is based on the understanding that components of donor blood can induce a systemic inflammatory response that manifests in the brain as neuroinflammation, ultimately leading to damage of the entire neurovascular unit (NVU) which includes neurons, astrocytes, and endothelial cells [2, 9].

However, it is important to understand that by refusing transfusions of RBC-rich donor blood components, we increase chances of hemic hypoxia as a result of the hemodilution that occurs during CPB. Thus, a critical question is: what poses a greater risk to the NVU — the increased SIR following transfusion administration, or the hypoxia resulting from refusal of transfusion? Due to patient risk, this question cannot be answered in a clinical trial. However, there is a way to study the effect of different levels of hypoxia and systemic inflammatory response on the functional activity of NVU cells. The aim of the study was to compare the effect of different degrees of hypoxia and SIR on the functional activity of NVU cells using an in vitro cell model [10].

Materials and Methods

Serum samples were available from 78 pediatric patients, aged 1 month to 6.5 years (13 [9–23] months) and weighing between 3.3 and 21.5 kg (8.7 [6.9–11.0] kg), who underwent surgery for correction of congenital septal heart defects under CPB at the Research Institute for Complex Problems of Cardiovascular Diseases. The level of interleukin-6 (IL-6), an important SIR marker, was measured in each sample [11]. Three blood samples from this pool with the highest and lowest IL-6 levels were selected for the in vitro phase of the study. The frozen serum was delivered to the V. F. Voino-Yasenetsky Krasnoyarsk State Medical University (KSMU) in accordance with the temperature control protocol.

The KSMU laboratory has developed a method to assess brain damage during cardiac surgery based on a cellular model of the neurovascular unit that can be cultured under various conditions that mimic the intraoperative period.

Developing primary cultures of brain cells *in vitro*. Primary cultures of brain endothelial cells, astrocytes, and neurons obtained from Wistar rats were used. The animals were housed in cages with free access to food and water. A constant temperature of 21±1°C was maintained. The light cycle was 12 h day/12 h night. Animal studies were conducted in accordance with the principles of humane care as set forth in the European Community Directive (2010/63/EC). The total number of animals used was 10.

Several steps were required to obtain an in vitro cell model of the NVU:

1. Isolation of brain endothelial cells.
2. Isolation and culture of neurospheres.
3. Obtaining astrocytes and neurons from the obtained neurospheres by targeted differentiation into astrocytes and neurons.
4. Establishing an in vitro model of NVU. For this purpose, a mixture of endothelial cells and neurons was placed at the bottom of the wells of the culture plate, then culture inserts (Corning-Costar, USA) were installed on which astrocytes were placed. The cell mixture was cultured in a medium consisting of DMEM with PBS, glutamine, antibiotic mixture at 37°C with 5% CO_2 [12].

After establishing a cell model of NVU, the main experiment was performed.

Experimental design. To determine the effects of IL-6 on the NVU cell model in vitro, blood serum samples with minimal and maximal concentrations of IL-6 were added to the culture medium.

Groups were formed according to the level of IL-6 in the sample:

1. «Control» —intact model of NVU.
2. «Minimum» — samples with minimal level of IL-6 in the culture.
3. «Maximum» — samples with maximum level of IL-6 in culture.

The number of replicates in serum incubation groups was 10.

According to the conditions of hypoxic incubation of NVU, the following groups were formed:

1. Control (C), standard culture conditions: N_2 — 75%, O_2 — 20%, CO_2 — 5%.
2. Hypoxia 1 (H1): N_2 — 99%, O_2 — 1%, CO_2 — 0%.
3. Hypoxia 2 (H2): N_2 — 98%, O_2 — 2%, CO_2 — 0%.
4. Hypoxia 3 (H3): N_2 — 97%, O_2 — 3%, CO_2 — 0%.
5. Hypoxia 4 (H4): N_2 — 96%, O_2 — 4%, CO_2 — 0%.

The number of replicates in the hypoxic incubation groups was 5.

The incubation time was 30 minutes. The temperature conditions were normothermic (37.0°C).

Evaluation of NVU performance. To evaluate the effect of hypoxia and blood serum with different levels of IL-6 on cell culture, transendothelial resistance and endothelial layer permeability were measured in the model of NVU as indicators of blood-brain barrier function.

Transendothelial resistance (TER) in the in vitro cell model was measured after 1, 2, 4, and 24 hours of culture. Direct measurement of transendothelial electrical resistance (TEER) was performed with an EVOM2 epithelial voltmeter using a STX2 electrode (World Precision Instruments, USA).

Table 1. TER parameters during incubation of cells in hypoxia.

Stage	Values of TER in groups				
	C	H1	H2	H3	H4
0 h	199.5 [197.25–201.5]	197.0 [196.75–199.25] *P*=0.1533	197.5 [196.25–199.25] *P*=0.3316	192.5 [190.75–195.0] *P*=0.0407	195.0 [194.75–196.5] *P*=0.1169
1 h	204.25 [202.75–206.0]	191.0 [189.75–193.0] *P*=0.03	191.0 [189.5–192.5] *P*=0.031	191.0 [189.75–193.25] *P*=0.033	193.0 [191.5–195] *P*=0.034
2 h	200.0 [196.75–203.5]	185.0 [182.25–187.25] *P*=0.0105	191.5 [188.75–194.75] *P*=0.0407	192.0 [187.5–195.0] *P*=0.066	193.0 [190.75–194.5] *P*=0.0408
4 h	194.5 [193–195.75]	175.5 [174.5–176.75] *P*=0.0105	177.0 [175.75–178.25] *P*=0.0151	183.5 [182.0–185.5] *P*=0.0329	189.0 [187.5–191.25] *P*=0.1441
24 h	203.0 [200.75–205.5]	141.0 [138.75–143.0] *P*=0.0105	162.0 [160.0–164.0] *P*=0.021	179.5 [175.0–183.25] *P*=0.0152	192.0 [191.0–192.75] *P*=0.0531

Note. For Tables 1, 3, Fig.1, *a*: C — control group; H — Hypoxia group 1–4.

The fluorescent dye Lucifer Yellow (LY) was added to the culture medium at a final concentration of 50 μM to measure permeability. The medium was removed from the lower compartment of the wells after 60 and 90 minutes, and the optical density of the mixture was measured using a spectrofluorometer SM 2203 (SOLAR, Belarus). Relative permeability was calculated based on the variation of LY concentration between experimental and control groups.

Statistical analysis of data was performed using BioStat Pro 5.9.8 software. Data in the text, tables, and figures were reported as median *(Me)*, upper *(Q1)* and lower quartile *(Q3)* because most variables had non-normal distribution (Shapiro–Wilk test, $P<0.05$). The Mann–Whitney test was used for one-way comparisons of quantitative variables. Differences were considered significant when $P<0.05$. Bonferroni correction was used to compare more than one group.

The effect of hypoxia on NVU was evaluated in 4 study groups and one control group, and the differences were significant at $P=0.0125$.

The effect of IL-6 on NVU was evaluated in two study groups and one control group, and the difference was significant at $P=0.017$.

Results and Discussion

When assessing the effect of hypoxia on NVU (Table 1, Fig. 1, *a*), transendothelial resistance (TER) decreased insignificantly in all groups after 1 hour of hypoxia exposure, by 7% on average.

After 2, 4, and 24 hours, there was a decrease in TER in all groups, but it was significant only in the group with 1% oxygen in the medium. In this group, the TER decreased by 7.5% after 2 hours, by 9.8% after 4 hours, and by 30.5% after 24 hours. In general, this confirms the fact that even short-term hypoxia has an effect on TES values, but it also shows that the effect of hypoxia on NVU varies depending on the percentage of oxygen in

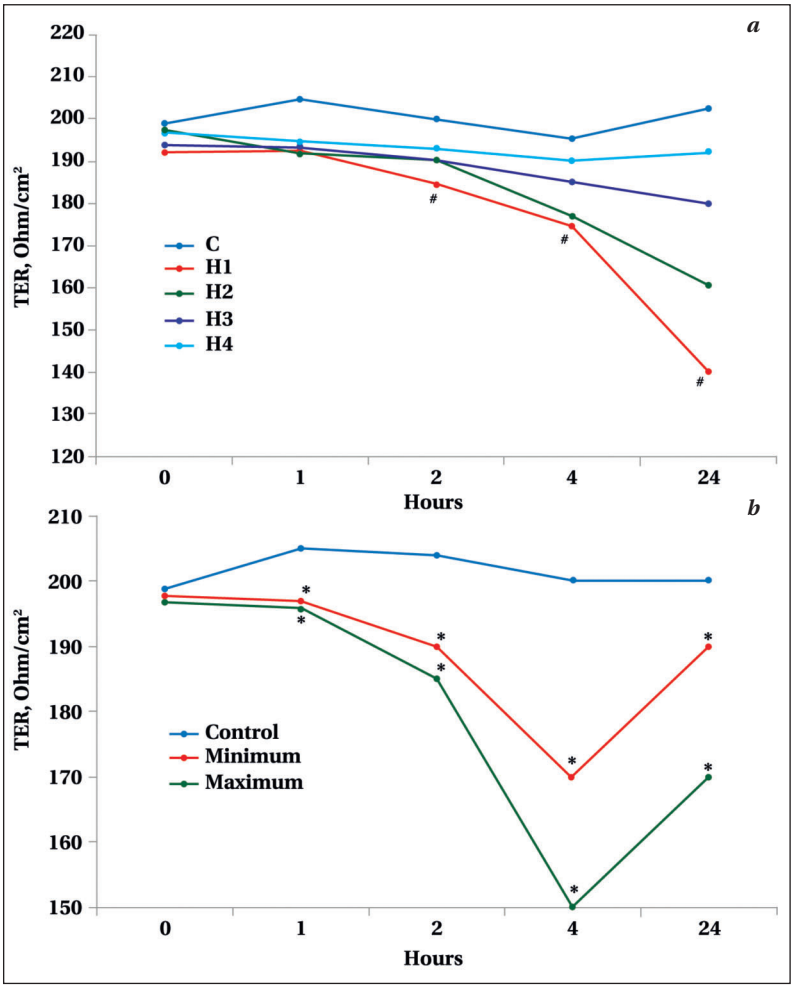

Fig. Effect of hypoxia *(a)* and serum from patients with different levels of IL-6 *(b)* on the transendothelial resistance index.
Note. # — $P<0.0125$, * — $P<0.017$, Mann–Whitney test with Bonferroni correction.

the medium. Furthermore, the absence of significant changes in TER in the 2, 3, and 4% oxygen groups suggests that moderate short-term hypoxia did not affect the functional activity of the NVU.

When evaluating the effect of IL-6 on TER (Fig. 1, *b*, Table 2), we found that at 2 hours, TER was significantly decreased by 5% in the minimum group and by 7.5% in the maximum group compared to the control (Fig. 1, *b*). At 4 hours after IL-6 exposure, the TER decreased by 15% in the minimum group and by 25% in the maximum group compared with the control. After 24 hours, the TER values increased but still remained significantly lower than in the control, maximum and minimum groups. Therefore, the presence of IL-6 in blood serum resulted in a decrease in TER, which was reversible and had a stronger and faster impact than hypoxia, albeit for a shorter period of time. Meanwhile, a significant difference in TER values between the minimum and maximum groups was observed at the following time points: 2 hours (P=0.00640), 4 hours (P=0.00017), and 24 hours (P=0.00016), indicating varying levels of NVU damage based on the concentration of IL-6 in the medium and, thus, the severity of systemic inflammation.

Table 2. TER during incubation of cells with serum.

Stage	Values in groups		
	Control	Minimum	Maximum
0 h	198.0 [197.0–200.0]	199.0 [197.0–199.0] P=0.25	197.0 [195.0–198.0] P=0.073
1 h	206.0 [205.0–207.0]	197.0 [196.0–198.0] P=0.00027	196.0 [196.0–197.0] P=0.00016
2 h	203.0 [202.0–204.0]	190.0 [188.0–192.0] P=0.00017	185.0 [185.0–188.0] P=0.00016
4 h	200.0 [196.0–203.0]	168.0 [168.0–170.0] P=0.00016	151.0 [148.0–152.0] P=0.00017
24 h	199.0 [198.0–202.0]	190.0 [188.0–192.0] P=0.00020	169.0 [169.0–172.0] P=0.00031

Table 3. Relative permeability of cells during incubation in hypoxia.

Stage	Values in groups				
	C	H1	H2	H3	H4
60 min	99.5 [98.0–101.5]	121.0 [117.5–124.25] *P=0.01008	119.5 [118.5–120.75] P=0.0140	100.5 [99.5–101.25] P=0.44084	101.0 [100.75–102.5] P=0.37185
90 min	99.5 [99.0–100.0]	146.0 [143.75–148.0] *P=0.00971	139.0 [137.5–141.0] P=0.01942	110.0 [107.75–112.0] P=0.03228	107.0 [106.0–109.0] P=0.05095

Note. * — P<0.0125, Mann–Whitney test with Bonferroni correction.

Table 4. Relative permeability of cells during incubation with serum.

Stage	Values in groups		
	Control	Minimum	Maximum
60 min	99.5 [98.75–100.5]	119.5 [118.75–121.0] *P=0.01046	114.5 [114.0–115.25] *P=0.01470
90 min	99.5.0 [99.0–100.5]	106.5 [105.75–107.25] P=0.05314	109.5 [108.75–110.5] P=0.03291

Note. * — P<0.017, Mann–Whitney test with Bonferroni correction.

Cell permeability to LY after exposure to short-term hypoxia increased by 21.6% after 60 minutes of incubation and by 46.7% after 90 minutes only in the Hypoxia 1 group (Table 3).

The results of the cell permeability assay correlated with the changes in TER, generally indicating the effect of only the extremely low (1%) concentration of oxygen in the medium on NVU.

The effect of patient serum on cell permeability is shown in Table 4. After 60 minutes of exposure, a 15–20% increase in relative permeability was observed in both experimental groups. Interestingly, after 90 minutes, the permeability in the control and experimental groups did not differ, confirming the assumption of a rapid but short-term and possibly reversible effect of serum with IL-6. Meanwhile, cell permeability to LY was significantly higher (P=0.01470) in the group with maximal IL-6 in the medium than in the group with minimal IL-6 in the medium, which may confirm the relationship between the severity of blood-brain barrier damage and systemic inflammation.

Conclusion

The study using the NVU cell model showed that 30 min of hypoxia had no significant effect on rat brain cells. Changes in TER and cell permeability to LY were observed only at 1% oxygen concentration in the medium. Exposure to patient serum containing IL-6 caused more severe but transient damage to NVU. The severity of the damage was determined by the concentration of IL-6 in the medium, which indicated the strength of the inflammatory response. Thus, withholding intraoperative transfusion to limit SIR and resulting hemic hypoxia may be less damaging to the patient's brain than the transfusion-induced increase in systemic inflammatory activity.

The study of TER and permeability to LY allowed us to identify trends in the effects of hypoxia and SIR in an NVU model.

Further studies using functional activity markers of the neurovascular unit (NVU), such as s100β protein, neuron-specific enolase, glial fibrillary acidic protein, occludin, claudin, and others, will offer a detailed understanding of NVU performance in response to pathological factors.

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Received 07.06.2023
Accepted 11.12.2023
Online first 14.12.2023