The thermal treatment effects on bovine blood neutrophil granulocytes apoptosis and necrosis *in vitro*

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Abstract. The aim of the study was to evaluate the effect of selected temperatures on viability (apoptosis and necrosis) of bovine blood neutrophil granulocytes (neutrophils) *in vitro*. The following temperatures were tested: –80, –20, 4, 23, 37°C. Heparinised bovine blood was incubated for 1, 4 and 24 h under respective temperature. Apoptosis and necrosis of neutrophils were detected by light microscopy, transmission electron microscopy (TEM) and flow cytometry (FCM). From selected temperatures, 4°C impaired the neutrophil viability least. The proportion of apoptotic and necrotic neutrophils amounted to (mean ± SD) 5.25 ± 3.53% and 0.83 ± 0.38%; 7.09 ± 2.07% and 1.64 ± 0.50%; 35.39 ± 12.53% and 5.46 ± 1.46%; after 1, 4 and 24 h incubation, respectively. The temperature (4°C) is the best alternative for short-term storage.

Key words: Neutrophil — Apoptosis — Necrosis — Temperature — Blood

Introduction

Neutrophils represent an important component of the non-specific defence system of the body against invading microorganisms. Their migration from the blood to the tissues, where they put their phagocytic function into effect, occurs mainly in cases of acute inflammation. Afterwards they undergo apoptosis (physiological cell death) and are removed by the tissue macrophages (Paape et al. 2003) or cytolysis (Ryšánek and Sládek 2006; Ryšánek et al. 2006).

In relation to their function in the inflammatory response, neutrophils are often present in aggressive environments. The protective programme of the cells in response to undesirable conditions in the environment is called "heat shock" or "stress response". The cells in all organisms stop the normal model of gene expression with an increasing temperature. Heat shock increases the level of B1 and B2 RNA transcription. B2 RNA blocks the transcription of mRNA in a reaction with RNA polymerase II, which decreases its activity (Allen et al. 2004).

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The signal of cell damage is the release of heat shock proteins from the cells, many of them known as molecular chaperones, essential components for the synthesis of proteins and maturation of cells (Georgopoulos and Welch 1993). They participate in the process of polypeptide chain composition, the transfer as well as the labelling of proteins, the physiological function of which is completed (Masopust et al. 2003).

The adoption of thermotolerance is achieved by exposure to sublethal hyperthermia and subsequent return to an environment of 37°C in most cells. Such cells are able to survive subsequent metabolic stress, which could be lethal under normal circumstances (Minowada and Welch 1995). Callahan et al. (1999) have reported that neutrophils have impaired functions and viability after the effect of sublethal heat shock. Maridonneau-Parini et al. (1988) reported that production of superoxides was decreased during the exposure of neutrophils to heat stress.

By Payne et al. (1994), the effective temperature for the induction of physiological cell death (apoptosis) in human neutrophils is 37°C. These authors further report that the conditions of cultivation, such as 37°C and RPMI-1640 medium without protein supplementation, have many features, which simulate *in vivo* conditions and therefore they are a suitable model for the study of programmed cell death in neutrophils.

In relation to the storage and freezing of cells, there is a question concerning the effect of temperatures below the freezing point on the cells, especially their fundamental vital functions and viability itself.

Intracellular ice formation (IIF) represents the production of ice crystals, which occurs in the cells during fast freezing. IIF is considered lethal in cryobiology (Mazur 1984) therefore it is necessary to avoid IIF during cell and tissue cryopreservation used for transfusions and transplantations.

Successful cryopreservation may be performed in isolated cells in a suspension. These cells are usually severely impaired during freezing *in situ*. For example, in isolated kidney cells (Chagnon and Pavilanis 1966), heart cells (Alink et al. 1977), liver cells (Fuller et al. 1980) and corneal cells (Armitage and Juss 1996), it is possible to restore viability partially after freezing. Nevertheless, trials in freezing whole tissues or organs were unsuccessful (Jacobsen and Pegg 1984; Wilson and Bourne 1989).

Nishimura et al. (2001) have reported that almost 100% of frozen (–196°C) and thawed granulocytes were dead within approximately 2 h in a medium (RPMI-1640 + 20% heat inactivated human serum). They did not specify further, however, whether it was apoptosis or necrosis.

The conditions for the preservation of leukocyte viability, which cannot be processed immediately for subsequent experiments, were investigated by Hodge et al. (1999). The optimal condition is considered to be the usage of heparin as an anticoagulant, storage conditions of 4°C and the addition of a nutritive medium.

The necessity to store and keep blood over the long term, or blood elements of livestock, comes e.g. with the current question of animal welfare, which cannot be exposed to frequent blood sampling. Optimal conditions for the storage and cryopreservation of bovine neutrophils have not been described so far. The question remains, whether the same effect of non-physiological temperatures can be expected in the viability of bovine blood neutrophils, such as in human and laboratory animal neutrophils. For this purpose bovine blood neutrophils in this study were exposed to similar temperatures used by Payne et al. (1994), and to temperatures under the freezing point. The goal was to determine, which of the used temperatures has the lowest negative effect on neutrophil viability and is therefore useful for storage of these cells under the conditions *in vitro*.

Materials and Methods

Animals and experimental design

The experiments were carried out in six clinically healthy Holstein × Bohemian Red Pied crossbred virgin heifers aged 16 to 18 months. Heifers were used as a source of blood for subsequent study of the effect of various temperatures (–80, –20, 4, 23, 37°C). Higher temperatures than those used by Nishimura et al. (2001) were selected for freezing, considering their finding that deep freezing significantly influences the viability of granulocytes. Selected trial actions were applied to samples of whole blood with heparin, from which leukocytes were isolated after a trial exposure (see below).

Inductors of apoptosis and necrosis were used as a positive control except for the trial actions. For this purpose, the population of neutrophils was exposed for 30 min to non-physiological conditions – UV light (germicide lamp, 30 W, distance 500 mm) and temperature 60°C (water bath) then incubated for the appropriate time (below). UV light was therefore used as an inductor of apoptosis and temperature of 60°C as an inductor of cell necrosis according to Ryšánek et al. (2006).

The manifestations of apoptosis and necrosis of neutrophils were detected using flow cytometry (FCM). Furthermore, smears were prepared of fresh blood after collection and blood after trial exposure for microscopic determination of percentage portions of apoptotic and necrotic neutrophils. For the determination of cell decrease in the course of incubations, the numbers of leukocytes in Bürker's chamber were determined.

Blood sampling

The blood (100 ml) was drawn from the jugular vein into a sterile flask with an anticoagulant, namely Heparin (Léčiva a.s., Dolní Měcholupy, Czech Republic) 1000 IU in 10 ml of phosphate buffered saline (PBS) (Sigma, Saint Louis, MO, USA).

Blood incubation

Before incubation, the total leukocyte count and viability (the Trypan Blue test) were determined in Bürker's chamber. Blood was incubated *in vitro* 1, 4 and 24 h at 4, 23 and 37°C in plates (Corning Ultra Low Attachment Products, Life Sciences, Acton, MA, USA). Blood stored at –80 and –20°C was placed in freezing boxes in Eppendorf plastic tubes. After removal from the freezing boxes, it was spontaneously thawed at laboratory temperature (23°C), for approximately 10 min.

Isolation of the leukocytes

Isolation of the leukocytes from examined blood samples was carried out by FACS Lysing Solution (Beckton Dickinson Biosciences, San Jose, CA, USA) by previously described procedure (Hodge et al. 1999) immediately after experimental expositions. The steps followed after the erythrocytes lysis

and leukocytes washing (in PBS) were staining of cell in suspension and processing by FCM.

FCM

Apoptotic and necrotic neutrophils were analysed by FCM after simultaneous staining with Annexin-V labelled with FITC (fluorescein isothiocyanate) and propidium iodide (PI) as described by Vermes et al. (1995). The commercial Annexin-V-FLUOS staining kit (Boehringer Mannheim, GmbH, Germany) was used according to the manufacturer's instructions. After staining, the suspension was analysed using FCM (FACS Calibur apparatus, Beckton Dickinson, Mountain View, CA, USA) by differentiating 20,000 cells. The neutrophil region was gated in accordance with procedure published Van Oostveldt et al. (1999). Dot plots were evaluated qualitatively and quantitatively using the WinMDI 2.8 software (Trotter 2000).

Microscopy

Light microscopy
Slides stained by the Pappenheim method (May-Grünwald, Giemsa-Romanowski stain) were examined by light microscopy with oil immersion (Olympus BH2; Olympus Optical Co., Ltd., Tokyo, Japan). Apoptosis and necrosis of neutrophils were assessed by the enumeration of at least 200 neutrophils according to morphological features previously described (Sládek and Ryšánek 2000).
Transmission electron microscopy (TEM)
The samples for TEM were prepared by previously described procedure (Sládek and Ryšánek 1999).

Statistical methods

The proportions of apoptotic and necrotic neutrophils from

Table 1. The effects of thermal and UV light treatments, various duration of incubation on portions of apoptotic and of necrotic blood neutrophils (statistical significance of differences compared to fresh blood)

Temperature (°C)	Incubation (h)	Apoptotic neutrophils An.-V^+/PI^- (%)		Necrotic neutrophils An.-V^+/PI^+ (%)		Statistical significance	
		arithmetic mean	SD	arithmetic mean	SD	An.-V^+/PI^-	An.-V^+/PI^+
23 (a)	Fresh	2.86	1.34	0.14	0.11	–	–
–80 (b)	1	16.35	7.71	72.44	11.60	a : b**	a : b**
	4	15.33	4.44	76.81	8.05	a : b**	a : b**
	24	14.98	5.37	78.71	4.50	–	–
–20 (c)	1	24.15	11.31	60.17	13.69	a : c**	a : c**
	4	18.49	7.43	73.93	8.90	a : c**	a : c**
	24	17.10	6.36	72.27	9.23	–	–
4 (d)	1	5.25	3.53	0.83	0.38	n.s.	a : d**
	4	7.09	2.07	1.64	0.50	a : d**	a : d**
	24	35.39	12.53	5.46	1.46	–	–
23 (e)	1	7.12	3.87	0.49	0.29	n.s.	a : e*
	4	10.66	4.68	0.56	0.27	a : e**	a : e*
	24	32.14	10.51	2.43	1.33	–	–
37 (f)	1	11.09	8.99	0.50	0.31	n.s.	a : f*
	4	16.10	7.26	0.85	0.29	a : f**	a : f**
	24	42.55	14.94	10.67	10.84	–	–
60	1	23.64	14.88	39.36	15.63	–	–
	4	35.53	17.70	50.61	22.18	–	–
	24	37.11	22.58	50.75	17.56	–	–
UV	1	24.12	7.23	0.87	0.34	–	–
	4	30.30	14.49	0.63	0.37	–	–
	24	53.32	10.40	12.80	5.19	–	–

An.-V^+, Annexin-V positivity; PI^-, propidium iodide negativity; PI^+, propidium iodide positivity; * $p < 0.05$; ** $p < 0.01$; n.s., non-significant.

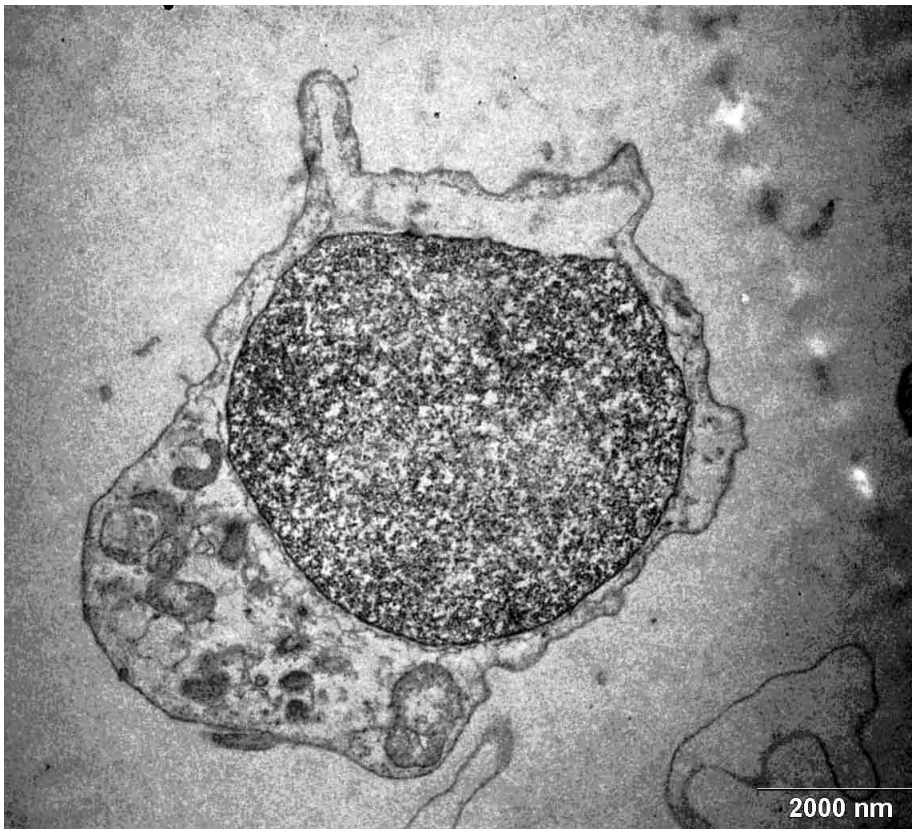

Figure 1. TEM: apoptotic neutrophil in the stage of secondary necrosis. Nucleus is compact, without segmentation. Primary magnification: ×10,000.

blood of the six heifers are shown as arithmetic means and standard deviations. The normality of a data distribution was proved by Kolmogorov test. The statistical significance of differences in the proportion of apoptotic and necrotic neutrophils were determined by the paired Student's *t*-test. The data were processed by the STAT Plus software (Matoušková et al. 1992).

Results

FCM

Freshly collected blood revealed 2.86 ± 1.34% apoptotic and 0.14 ± 0.11% necrotic neutrophils. The portions of apoptotic and necrotic neutrophils and the statistical significance in the difference at various temperatures and times of incubation as well as during exposure to UV light and temperature of 60°C are provided in Table 1. It is obvious from the table that the portion of apoptotic neutrophils after 4 h of incubation at 4°C (7.09 ± 2.07%) corresponds to the portion of apoptotic neutrophils after incubation of 1 h at 23°C (7.12 ± 3.87%). The portion of apoptotic neutrophils after 4 h of incubation at 23°C (10.66 ± 4.68%) then corresponds to the portion of apoptotic neutrophils after 1 h of incubation at 37°C (11.09 ± 8.99%).

Statistically highly significant ($p < 0.01$) elevation of apoptotic neutrophils proportion at temperatures of 4, 23 and 37°C was observed compared with the fresh blood only at 4 h of incubation. After 1 h of incubation, however, no statistically significant difference was observed.

Compared to fresh blood, there was a statistically highly significant ($p < 0.01$) elevation of the portion of apoptotic and necrotic neutrophils at all time points of incubation at temperatures of –80 and –20°C.

When comparing fresh blood and blood exposed to the effects of UV light, there was a statistically highly significant ($p < 0.01$) elevation of the portion of apoptotic neutrophils observed at all time points.

The effect of the temperature of 60°C and subsequent blood incubation resulted in an elevation of the portion of necrotic neutrophils. After 1 h of incubation, a statistically significant difference ($p < 0.05$) was observed and after 4 h of incubation a highly significant statistical difference ($p <$

0.01) in the portion of necrotic neutrophils was observed in comparison with fresh blood.

Blood samples exposed to the effects of UV light, temperatures of 60°C and 37°C (incubated for 4 h) underwent analysis in light and electron microscopes.

Light microscopy

In freshly collected anticoagulant blood there were no apoptotic or necrotic neutrophils recorded using light microscopy. In comparison, neutrophils from blood samples exposed to UV irradiation revealed 9.75 ± 2.17% apoptotic cells and 2.25 ± 0.87% necrotic cells after 4 h of incubation. Under light microscopy, apoptotic neutrophils were observed mainly in the stage of karyopycnosis. Blood exposed to the effects of 60°C contained mainly necrotic cells in the population of neutrophils and all leukocytes, respectively.

TEM

Similarly as in the case of light microscopy, fresh blood contained mostly neutrophils with an intact ultrastructure in the TEM image. Exposure to UV light in the *in vitro* condition resulted in the induction of apoptosis, which was characterised by typical ultrastructural features: the first feature of neutrophil apoptosis was the loss of pseudopodia. Then the fusion of nuclear segments in one spherical unit followed. Neutrophils further converted from the stage of karyopycnosis to secondary necrosis (Fig. 1).

The exposure of cells to a high temperature (60°C) significantly induced (primary) necrosis of the neutrophils. Necrotic neutrophils were present at various stages of karyolysis and cytolysis.

Decline in the number of leukocytes

After isolation from fresh blood, there were 4.86 ± 0.72 × 10^9/l leukocytes in the suspension of cells. Concerning the number of leukocytes in the cell suspension after isolation from the blood, there is a decline of cells at various incubation periods (Table 2). The greatest decline of leukocytes was observed under a temperature of –20 °C after 24 h of incubation ($p < 0.01$). The least decline of leukocytes count was detected under a temperature of 4°C after 1 h incubation.

Discussion

The goal of this study was to determine which of the non-physiological temperatures has the lowest negative effect on the viability of neutrophils in bovine blood under the conditions of *in vitro* cultivation. This is the first study, which has determined the effects of temperature in relation to the induction of apoptosis and necrosis of cultured blood cattle neutrophils.

As reported by Payne et al. (1994), there are no apoptotic neutrophils in fresh blood. In this study, we have nevertheless observed 2.86% of apoptotic cells (Annexin-V positive) in the population of neutrophils, which is comparable to the ratio of 1.8% of Annexin-V positive neutrophils in full blood of dairy cows, observed by Van Oostveldt et al. (1999). The presence of apoptotic neutrophils in fresh blood may be due to the isolation technique used, which is in accordance with our previous results of the effect of isolation techniques on the viability of blood neutrophils (Sláma et al. 2006).

From the incubation temperatures above the freezing point (4, 23, 37°C), the apoptosis of neutrophils was induced mostly by the temperature of 37°C. The same results were observed in human neutrophils by Payne et al. (1994), who determined, similarly to Hodge et al. (1999), the temperature of 4°C as an optimum to maintain the viability of granulocytes in human blood.

The results of this study also indicate that temperature of 4°C seems to be the temperature with the least significant negative impact on the viability of cultivated bovine blood neutrophils *in vitro*. It is necessary, however, to comment closely on the presence of apoptotic neutrophils through-

Table 2. Significance of the differences in leukocyte count. Fresh blood (mean ± SD: 4.86 ± 0.72 × 10^9/l) compared with blood incubated for 1 to 24 h at various temperatures and after UV irradiation

Temperature (°C)	Incubation (h)			Statistical significance
	1	4	24	
–80	2.29 ± 0.45	1.99 ± 0.65	1.77 ± 0.83	**
–20	2.32 ± 0.46	1.57 ± 0.35	1.31 ± 0.31	**
4	4.37 ± 0.32	4.04 ± 0.36	3.70 ± 0.68	*
23	4.08 ± 0.59	3.96 ± 0.28	2.92 ± 0.99	*
37	4.02 ± 0.50	4.00 ± 0.66	2.80 ± 0.39	*
60	2.46 ± 0.36	1.89 ± 0.52	1.64 ± 0.74	**
UV	4.30 ± 0.68	3.55 ± 0.38	2.07 ± 0.62	**

* $p < 0.05$; ** $p < 0.01$.

out the entire period of experimental cultivation, since a statistically significant difference in the portion of apoptotic neutrophils between the temperature of 4°C and the temperatures of 23 and 37°C was observed only during incubation for 4 h. After 1 h of incubation, the lowest portion of apoptotic neutrophils was observed just at the temperature of 4°C; it was not, however, statistically significantly different. This was observed during a longer period of cultivation (4 h); these differences are not statistically significant in the case of 24 h of incubation, however. This apparent disproportion has its reasons, since cultivation of neutrophils *in vitro* results in the occurrence of secondary necrosis (Payne et al. 1994). The secondary necrosis of apoptotic neutrophils could therefore contribute significantly to the decrease in the portion of these cells. Furthermore, during long-term incubation (24 h), the portion could also be influenced by a decrease in the total number of cultivated cells, which was observed in the trial. The fact that apoptotic neutrophils could transfer to the stage of secondary necrosis in a few hours (Payne et al. 1994; Haslett 1999; Guejes et al. 2003) indicates that this is a relatively fast action. Afterward, these necrotic cells (primary apoptotic) disintegrate and are removed from the cell suspension during the isolation procedure. Therefore the portion of apoptotic and necrotic neutrophils was recorded only in cells, which remained intact and could be detected in the FCM and Bürker chamber. The same fact, i.e. a paradoxically higher portion of intact leukocytes during cultivation due to secondary necrosis and lysis of cells, was observed by Ryšánek et al. (2006).

The occurrence of a lower number of apoptotic neutrophils at a temperature below 37°C (if we do not consider temperatures below the freezing point) is also in accordance with the results of experiments performed by Mizuno et al. (2000) in neutrophiles from laboratory mice. They observed a lesser occurrence yet of apoptotic neutrophils under mild hypothermia (35°C). Pryde et al. (2000) report that the inhibition of the apoptotic process of human neutrophils at 15°C could depend on the failure of the pro-apoptotic Bax protein, which is associated with decreased morphologic signs typical for apoptosis.

Interesting facts also occurred in the course of cell freezing. A greater portion of apoptotic neutrophils was observed at all time points at the temperature of –20°C compared to the temperature of –80°C. This could indicate that in the case of blood freezing under a lower temperature, freezing occurs faster and the blood elements are then exposed to lower temperatures for a shorter period prior to the interruption of metabolic activity. Another matter of interest is the decreased portion of apoptotic neutrophils in the blood at a temperature below the freezing point with an increasing period of cell incubation. This result, which is paradoxical at first sight, could have its reasons: The portion of necrotic neutrophils increased simultaneously with the decreasing portion of apoptotic neutrophils. The neutrophils could therefore probably move during the procedure of isolation using FACS Lysing Solution from the stage of apoptosis to the stage of secondary necrosis, as mentioned above.

Temperatures of –20 and –80°C influence the viability of bovine neutrophils during spontaneous freezing under the listed temperatures and during gradual freezing under the laboratory temperature (20 to 23°C) significantly more than temperatures of 4, 23 and 37°C. The difference is especially obvious in the number of necrotic neutrophils (60 to 74% at –20°C and 72 to 79% at –80°C). Nishimura et al. (2001) reported in their experiments that almost 100% of granulocytes frozen (–196°C) in liquid nitrogen and thawed were dead in approximately 2 h, regardless the cryoprotective agent (DMSO) was applied for protection of the cells integrity. In this study, the frozen blood samples were spontaneously thawing at laboratory temperature (23°C) and than undergone erythrocyte lysis and FCM analysis. The results obtained in this conditions show a trend, which is in an accordance with the data of Nishimura et al. (2001). Concurrently, the time from thawing to sample analysis in FCM was around 1 h.

As reported by Sweeney et al. (1997) and Ryšánek et al. (2006), short effects of UV light may be used for the induction of cell apoptosis. UV light was therefore used in this study as a confident inductor of apoptosis. As shown by the results obtained with light microscopy, neutrophils exposed to the effects of UV light reveal morphological features of all three stages of apoptosis, but with a maximum predominance of karyopycnosis. TEM confirms this fact and further estimates the presence of secondary neutrophil necrosis. Payne et al. (1994) and Haslett (1999) report that due to the absence of macrophages capable of phagocytate apoptotic neutrophils under the conditions *in vitro,* these cells undergo secondary necrosis within several hours. Apoptotic neutrophils undergoing secondary necrosis are morphologically typical due to the destruction of nuclear and cytoplasmic components with all signs of necrosis (Payne et al. 1994).

It is known in general that high temperatures may be used for the induction of cell necrosis. Zheng et al. (2004) report that the exposure of human neutrophils for 30 min to a temperature of 43°C results in approximately 3% positive neutrophils for PI, and thereby necrotic neutrophils. A temperature of 60°C, which was used in our study, was also used by Ryšánek et al. (2006) for the induction of neutrophil necrosis.

The high temperature (60°C) used in experiments unambiguously induced the (primary) necrosis of neutrophils. Karyolysis and cell cytolysis in TEM were clearly visible. It was clear from the dot plots obtained after analysis in FCM that most neutrophils were positive on PI, as was also proved in the study by Ryšánek et al. (2006).

Finally, it should be stated that temperatures under the freezing point influenced the viability of neutrophils more

significantly than temperatures above 0°C. Considering this finding, it is necessary to find cryoprotective substances for the needs of neutrophil freezing, which have a minimal influence on the viability and associated function of these cells.

The least effect of temperatures above the freezing point was shown by the temperature of 4°C, which therefore seems to be the most suitable temperature for the short-term storage of blood and blood neutrophils, respectively.

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