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THE EFFECT OF TEMPERATURE ON APOPTOSIS OF BOVINE BLOOD EOSINOPHIL GRANULOCYTES *IN VITRO*

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Abstract

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The aim of the study was to evaluate the effect of temperature on apoptosis of bovine blood eosinophil granulocytes *in vitro*. Heparinised bovine blood was incubated for 1, 4 and 24 h under following temperatures: 4, 23 and 37 °C. UV irradiation was used as positive control of apoptosis. Eosinophil granulocytes apoptosis was detected by flow cytometry after simultaneous staining with Annexin-V and propidium iodide. From selected temperatures, 4 °C induced the eosinophil granulocytes apoptosis least. The proportion of apoptotic eosinophil granulocytes amounted to (mean ± SD) 1.65 ± 0.46%; 1.76 ± 0.36%; 4.78 ± 1.70% after 1, 4 and 24 h incubation, respectively.

eosinophil, apoptosis, temperature, blood

Eosinophil granulocytes (eosinophils) are important cells in many allergic and parasitic inflammatory responses (Rothenberg, 1998). Aged eosinophils undergo apoptosis (physiological cell death) and then have to be removed to maintain homeostasis of cells. Apoptotic eosinophils are recognized and then phagocytosed by tissue macrophages without release of their histotoxic contents (Stern et al., 1992; Stern et al., 1996).

Eosinophils are often present in aggressive tissue environment. The protective programme of the cells in response to unsuitable conditions in the environment is called heat shock or stress response. The cells 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). The signal of cell damage is the release of heat shock proteins from the cells (Georgopoulos and Welch, 1993).

The adoption of cell thermotolerance is achieved by exposure to sublethal hyperthermia and subsequent return to temperature 37 °C. Such cells are able to survive metabolic stress, which could be lethal under normal conditions (Minowada and Welch, 1995).

The conditions for the preservation of human leukocytes viability were investigated by Hodge et al. (1999). The optimal condition is considered to be usage of heparin as an anticoagulant and storage temperature 4 °C. This result correspond with our previous experimets with bovine neutrophils (Sláma et al., 2007). Light and transmission electron microscopic studies showed that lower body hyperthermia (43 °C for 30 min) induced apoptosis of rat eosinophils (Allan and Harmon, 1986).

Optimal conditions for the storage of eosinophils have not been described so far. The question remains, whether the same effect of temperatures can be expected in the apoptosis of bovine blood eosinophils, such as in bovine blood neutrophils. For this purpose bovine blood eosinophils were exposed to similar temperatures (4, 23, 37 °C) used in our previous study of the thermal treatment effects on bovine blood neutrophils apoptosis (Sláma et al., 2007). The goal was to determine, which of the used temperatures has the lowest negative effect on eosinophil apoptosis. Detection of eosinophils and their viability by flow cytometry (FCM) is also important in diagnostics of many allergic and parasitic inflammatory responses.

MATERIAL 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 donor of blood for subsequent study of the effect of temperatures (4, 23, 37 °C) on apoptosis of eosinophils. Selected trial actions were applied to semple of whole blood with heparin, from which leukocytes were isolated after incubation *in vitro* 1, 4 and 24 h at 4, 23 and 37 °C in plates (Corning Ultra Low Attachment Products, Life Science, Acton, MA, USA).

UV irradiation was used as a positive control of eosinophils apoptosis. For this purpose, the blood was exposed for 30 min to UV irradiation (germicide lamp, 30 W, distance 500 mm) and then incubated for the appropriate time (1, 4 and 24 h). UV irradiation was therefore used as an inductor of cell apoptosis according to Ryšánek et al. (2006).

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).

Isolation of the leukocytes

Isolation of the leukocytes from examined blood samples was carried out by FACS Lysing Solution (Becton Dickinson Biosciences, San Jose, CA, USA) by previously described procedure (Hodge et al., 1999) immediately after experimental exposition. The steps followed after the erythrocytes lysis and leukocytes washing (in PBS) were staining of cell in suspension and processing by FCM.

FCM and cell sorting

The manifestations of eosinophils apoptosis were detected using FCM. Apoptotic and necrotic eosinophils were analysed by FCM after simultaneous staining with Annexin-V labelled with FITC (fluorescein isothiocyanate) and propidium iodide (PI) as described 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 cell suspension was analysed using FCM (FACS Calibur apparatus, Becton Dickinson, Mountain View, CA, USA). Dot plots were evaluated qualitatively and quantitatively using the WinMDI 2.8 software (Trotter, 2000). The region of eosinophils was gated in according to cell sorting using FCM (FACSAria, Becton Dickinson Biosciences, San Jose, CA, USA). Leukocyte regions were evaluated by light microscopy. Slides stained by the Pappenheim Metod (May-Grünwald, Giemsa-Romanowski stain) were examined by light microscopy with oil immersion (Olympus BH2, Olympus Optical Co., Ltd., Tokyo, Japan).

Statistical methods

The proportion of apoptotic and necrotic eosinophils from blood of six heifers are shown as arithmetic means and standard deviations (SD). The statistical significance of differences in the proportion of apoptotic eosinophils were determined by the paired Student's *t*-test. The data were processed by the STATISTICA 7.1 software (StatSoft CR s.r.o., Prague, Czech Republic).

RESULTS

Purity of eosinophil region established by cell sorting and light microscopy was 97.87 ± 0.42%. Dot plot with region of eosinophils is in Fig. 1 (red colour). Eosinophils from this region are in Fig. 2 (light microscopy).

In freshly collected blood there were 4.13 ± 0.26% eosinophils in differential leukocyte count (measured by FCM).

Freshly collected blood revealed 1.32 ± 0.37% apoptotic and 0.27 ± 0.14% necrotic eosinophils. The portions of apoptotic and necrotic eosinophils and statistical significance in the difference at selected temperatures and times of incubation as well as exposure to UV irradiation are provided in Tab. I.

Statistically highly significant ($p < 0.01$) elevation of apoptotic eosinophils proportion at temperatures 4 and 23 °C was observed compared with the fresh blood only at 24 h of incubation. Compared to fresh blood, there was a statistically highly significant ($p < 0.01$) elevation of the portion of apoptotic eosinophils at all time points of incubation at temperature 37 °C.

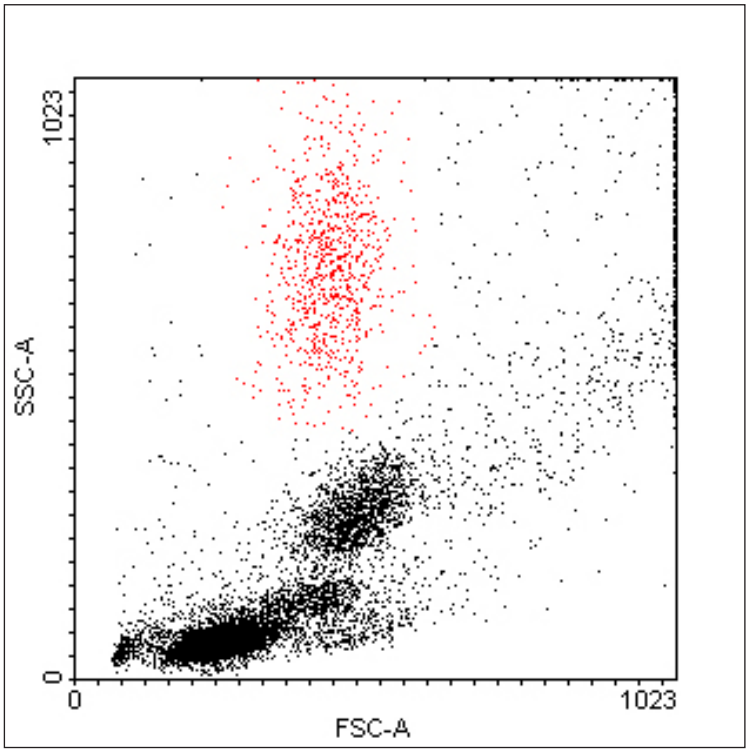
When comparing eosinophils from fresh blood and eosinophils from blood exposed to the effects of UV irradiation, there was a statistically highly significant ($p < 0.01$) elevation of the portion of apoptotic eosinophils observed at all time points.

DISCUSSION

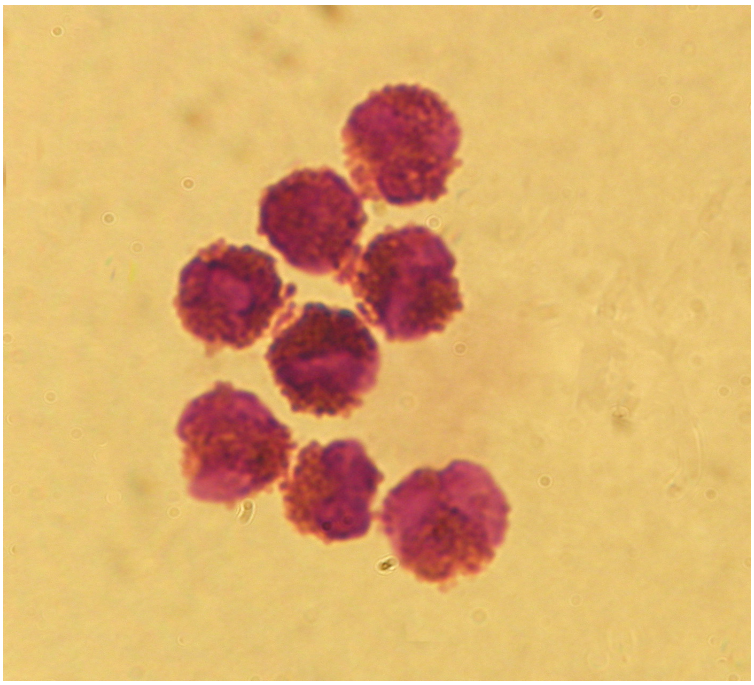
The aim of this study was to determine which of the selected temperatures has the lowest negative effect on the apoptosis of eosinophils in bovine blood under the conditions of *in vitro* cultivation. This is the first study, which has determined the effects of temperature in relation to the induction of apoptosis of cultured bovine blood eosinophils.

We observed 1.32% apoptotic eosinophils in fresh blood of heifers. This result is comparable to 2.86% of apoptotic neutrophils in blood of heifers (Sláma et al., 2007) and to the ratio of 1.8% of apoptotic neutrophils in blood of dairy cows (Van Oostveldt et al., 1999). The presence of apoptotic cells in fresh blood may be due to the isolation technique used, which is in accordance with our previous study (Sláma et al., 2006).

The apoptosis of eosinophils was induced mostly by temperature 37 °C. The same results were observed in our previous experiments with bovine neutrophils (Sláma et al., 2007) and in human neu-



1: *Dot plot of bovine blood leukocytes after their isolation. Red colour represents region of eosinophils.*



2: *Light microscopy of bovine blood eosinophils after cell sorting. Magnification × 1,000.*

I: *The effects of thermal and UV irradiation treatments and various duration of incubation on portions of apoptotic and necrotic blood eosinophils. (Statistical significance of differences compared to fresh blood.)*

Temperature (°C)	Incubation (h)	Apoptotic eosinophils An.-V$^+$/PI$^-$ (%)		Necrotic eosinophils An.-V$^+$/PI$^+$ (%)		Statistical significance	
		arithmetic mean	SD	arithmetic mean	SD	An.-V$^+$/PI$^-$	An.V$^+$/PI$^+$
(a)	fresh	1.32	0.37	0.27	0.14	–	–
4 (b)	1	1.65	0.46	0.46	0.28	n.s.	n.s.
	4	1.76	0.36	0.67	0.22	n.s.	a : b*
	24	4.78	1.70	1.38	0.43	a : b**	a : b**
23 (c)	1	1.57	0.42	0.23	0.10	n.s.	n.s.
	4	2.34	0.56	0.33	0.16	a : c*	n.s.
	24	8.63	0.56	1.03	0.42	a : c**	a : c**
37 (d)	1	2.86	0.58	0.20	0.10	a : d**	n.s.
	4	4.43	1.52	0.68	0.36	a : d**	n.s.
	24	10.67	1.75	1.44	0.59	a : d**	a : d**
UV (e)	1	5.33	1.35	0.24	0.12	a : e**	n.s.
	4	6.74	1.27	0.47	0.26	a : e**	n.s.
	24	17.66	2.98	4.85	1.09	a : e**	a : e**

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

trophils by Payne et al. (1994). The occurrence of a lower number of apoptotic eosinophils at a temperature below 37 °C is also in accordance with the study of neutrophils from laboratory mice published by Mizuno et al. (2000). Morphological studies showed that lower hyperthermia (43 °C for 30 min) induced apoptosis of rat eosinophils (Allan and Harmon, 1986).

The results of this study indicate that temperature of 4 °C seems to be the temperature with the least significant negative impact on the apoptosis of cultivated eosinophils *in vitro*. We observed the same result in experimets with bovine neutrophils (Sláma et al., 2007). Hodge et al. (1999) obtain the same effect in human blood.

Ryšánek et al. (2006) reported that short effects of UV irradiation may be used for the induction of apoptosis of leukocytes from bovine mammary gland. We used UV irradiation in our previous study (Sláma et al., 2007) as an inductor of bovine blood neutrophil apoptosis. UV irradiation was also used in this study as a confident inductor of apoptosis.

The least effect of temperatures 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 eosinophils, respectively.

SOUHRN

Vliv teploty na apoptózu eozinofilních granulocytů krve skotu *in vitro*

Cílem studie bylo zhodnotit vliv teploty na apoptózu eozinofilních granulocytů krve skotu *in vitro*. Heparinizovaná bovinní krev byla inkubována 1, 4 a 24 hodin při následujících teplotách: 4, 23 a 37 °C. UV záření bylo použito jako pozitivní kontrola apoptózy. Apoptóza eozinofilních granulocytů byla detekována průtokovým cytometrem po simultánním barvení Annexinem-V a propidium jodidem. Z vybraných teplot indukují 4 °C apoptózu eozinofilních granulocytů nejméně. Procento apoptotických eozinofilních granulocytů bylo následující: 1,65 ± 0,46 %; 1,76 ± 0,36 %; 4,78 ± 1,70 % po 1, 4 a 24 hodinách inkubace.

eozinofil, apoptóza, teplota, krev

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