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ORIGINAL RESEARCH PAPER

Leukocyte apoptosis in winter swimmers

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A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article, E – Critical revision of the article, F – Final approval of the article

Abstract

Background: With regular cold baths, winter swimmers (individuals swimming in low-temperature water) develop an adaptation to the cold. Apoptosis is a fundamental process of the immune system; its main role consists in maintaining cell homeostasis to prevent the development of pathological conditions. The aim of this study is to investigate the effect of winter swimming on the apoptosis of peripheral blood leukocytes. It focuses on a unique and interesting aspect of winter swimming, providing vital insights into the immune system's physiological adaptations to harsh environments. The study investigates a relatively under-researched area, namely, the impact of winter swimming on leukocyte apoptosis, and thus makes a novel and relevant contribution to the fields of sports physiology and immunology, as understanding how regular exposure to cold water influences immune cell apoptosis can provide broader insights into the human body's adaptive mechanisms in the case of extreme environments.
Material and methods: The study group consisted of 9 male winter swimmers. After a bath, blood samples were collected from the ulnar vein. Blood smears were stained with the Hemacolor method. By light microscopy (1000×) under immersion, apoptotic forms were counted in the whole preparation relative to 100 leukocyte forms.
Results: Apoptotic leukocyte forms were very rare in the participants' blood. Out of the 9 subjects, only 3 individuals exhibited 2%–3% of leukocyte apoptotic forms.
Conclusions: The findings demonstrate that low water temperature does not cause significant leukocyte apoptosis in winter swimmers, which is an important finding. This suggests that regular exposure to cold water may improve immunological resilience, which is a favourable adaptation for those who participate in this sport.
Keywords: winter swimming, blood, apoptosis

Introduction

Winter swimmers regularly bathe in ice holes, lakes, rivers, and seas during winter. They have implemented this type of alternative medicine, treating it as a hobby. This extreme sport positively affects the lives of winter swimmers by toughening their bodies. Cold water immersion is associated with physiological changes in the respiratory, circulatory, and endocrine systems, and also influences the morphological and rheological properties

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of the blood [1]. The human body is characterized by homeothermy, which denotes keeping a constant body temperature under various conditions and promotes maintaining homeostasis in the body. Extreme body cooling under aquatic conditions can lead to hypothermia; the human body defends itself against this by activating such mechanisms as constriction of cutaneous blood vessels and increased metabolic heat production by exertion or muscle shivering [1,2]. With regular cold baths, winter swimmers develop adaptation to the cold, which translates into resistance to respiratory infections, reduced cardiac output and heart rate, as well as increased vasoconstriction within the skin [3].

The aim of this study was to investigate the effect of winter swimming on the apoptosis of peripheral blood leukocytes in winter swimmers.

Material and methods

The study group consisted of 9 male winter swimmers swimming in low-temperature water (2–7.2°C) during the winter season, lasting from November to March. The participants were affiliated to the Krakow Society of Winter Swimmers "Kaloryfer" in Krakow (Poland). After a bath, a qualified nurse collected blood samples from the ulnar vein of each winter swimmer into Vacuette tubes, after which they were transported to the Blood Physiology Laboratory of the Central Research and Development Laboratory, University of Physical Education in Krakow. A limitation of the study is that only a small number of winter swimmers took part.

The study was approved by the Ethics Committee of the Regional Medical Chamber in Krakow and carried out in accordance with the principles of the Declaration of Helsinki. All subjects provided their informed consent to participate in the study.

Cytological examination

2 ml of blood was collected from each participant in accordance with the current standards. In the Blood Physiology Laboratory of the of the Central Research and Development Laboratory, University of Physical Education in Krakow, smears were performed on a basic slide. The smears were allowed to dry for 12 hours to prefix the cells on a basic slide. After 12 hours, the blood smears were stained using the Hemacolor method (a modification of the May-Grünwald-Giemsa method), Merck catalog No. 107961. The preparation was washed with a buffer solution of pH 6–8. Then, by light microscopy (1000×) under immersion, apoptotic forms were counted in the whole preparation relative to 100 leukocyte forms encountered in the blood smear. The results were reported in percentages.

Results

Out of the 9 subjects, 3 individuals exhibited 2–3% of leukocyte apoptotic forms. These are illustrated in Figure 1.

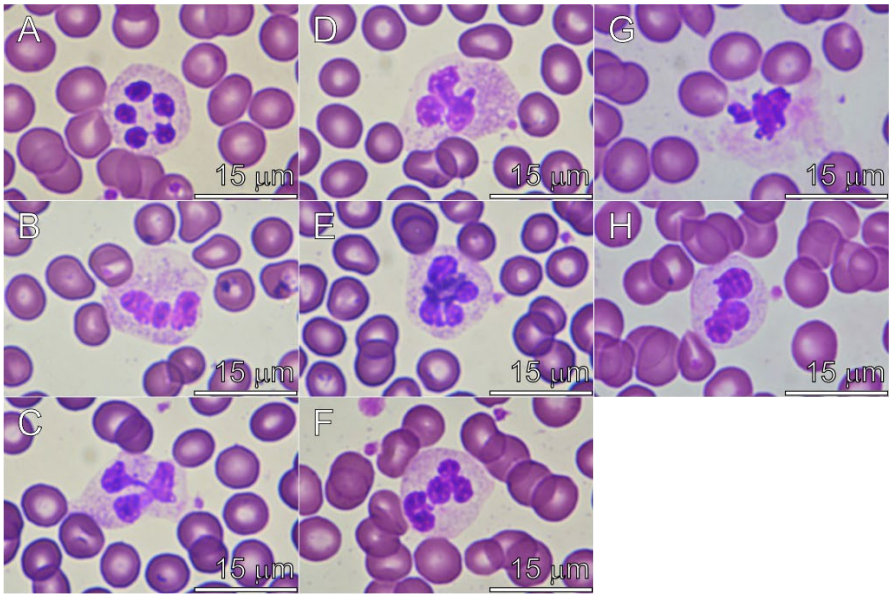

Figure 1 (A) A neutrophilic granulocyte. Clustered lobes of the cell nucleus, partially degenerating. Developing apoptosis. (B) A neutrophilic granulocyte. The lobes of the nucleus close together, invisible "bridges" between the nucleus lobes. Early stage of apoptosis. (C) An eosinophil with a degenerating nucleus. Pre-apoptotic form. (D) A neutrophilic granulocyte with a degenerating nucleus. Pre-apoptotic form. (E) A neutrophilic granulocyte. Aging form, nonapoptotic. (F) A neutrophilic granulocyte. Early apoptosis. (G) A neutrophilic granulocyte. Loss of granulation, complete apoptosis: cell disintegration. (H) A neutrophilic granulocyte. Early apoptosis

Discussion

The aim of this study was to investigate the effect of winter swimming on peripheral blood leukocyte apoptosis in individuals swimming in cold waters. Previously, no study had been conducted with this purpose. Apoptosis plays an important role in the human body; it is a fundamental process of the immune system [4]. Between 5,000 and 8,000 leukocytes, or white blood cells, are formed in the red bone marrow. Taking into account the proportion and type of granules in the cytoplasm, leukocytes can be divided into granulocytes (granular) and agranulocytes (nongranular). Granulocytes are characterized by a segmented nucleus and apparent granules. They can

be neutrophilic (neutrophils), acidophilic (eosinophils), or basophilic (basophils). Agranulocytes do not exhibit granularity, and their nucleus is rounded or kidney-shaped. Among agranulocytes, one distinguishes monocytes and lymphocytes [5,6]. Neutrophils – neutrophilic granulocytes – are about 12 μm in size, with a segmented nucleus depending on the cell maturity; the "younger" they are, the fewer lobes they have. They possess granules of two types: primary (nonspecific) and secondary (specific). They contain lysozyme, defensins, cathepsins, and myeloperoxidase. They are responsible for the first line of defence against microbial infection; their presence indicates acute inflammation. The defensive activities of neutrophils include microorganism phagocytosis, microorganism killing, and the ability to move (chemotaxis). Their lifespan ranges from several hours in the blood to about 2–3 days after migrating to tissues. Eosinophils – acidophilic granulocytes – account for 40% of all leukocytes. They have a diameter of 14 μm and are distinguished by an "ocular" nucleus. Eosinophil granules involve major basic proteins, eosinophil cationic protein, and eosinophil-derived neurotoxins. The function of eosinophils is to kill multicellular organisms and neutralize inflammatory mediators [5–7]. Basophils – basophilic granulocytes – make up only 1% of leukocytes. They are 12 μm in size, are associated with the development of allergic reactions, and possess receptors for IgE antibodies. Their primary function is to prevent blood clotting [5,6]. Lymphocytes – nongranular leukocytes – constitute the smallest blood component (8 μm). In turn, monocytes, with a diameter of 20 μm, are considered to be the largest morphological elements of blood. Their nucleus is kidney-shaped. They remain in blood for 1–2 days, have phagocytosis capacity, and are involved in the production of immunity-related factors. They also transform into large phagocytic cells capable of absorbing and digesting bacteria or the remains of damaged cells [5–7].

Programmed cell death (i.e., apoptosis, a term introduced in 1972 by Kerr et al.) is a physiological, active mechanism that determines the proper functioning of the organism. The process of apoptosis creates a pattern of eliminating superfluous cells or tissues in the body, which allows the normal number and quality of cells to be maintained [8,9]. Cells that degenerate in the course of apoptosis present characteristic morphological and biochemical changes. The loss of intracellular water and electrolytes in a single cell results in the cell shrinking, as well as in changes in shape, size, and cytoplasm density. The cell surface becomes corrugated; chromatin condensation occurs and nuclear DNA is fragmented [10,11]. Apoptotic bodies are formed, which are surrounded by cytoplasmic membrane and contain a DNA fragment and "healthy" organelles [11]. They are subsequently phagocytosed by macrophages and the surrounding cells. No inflammatory

process develops, as the cell membrane does not lose its continuity or function, which is of crucial importance [8].

There are two main apoptosis pathways: extrinsic (receptor-mediated) and intrinsic (mitochondrial). Both activate the caspase cascade, which is responsible for triggering the process. Caspases are cysteine protease enzymes that destroy enzymatic and structural proteins, which leads to complete cell disintegration [4,11].

Intrinsic apoptosis is initiated by heat shock, oxidative stress, or DNA damage. The mitochondrial membrane loses its continuity, forming a mitochondrial permeability transition pore, through which cytochrome c enters the cytoplasm. Cytochrome c then combines with Apaf-1 proteins and procaspase-9 to form a complex called the apoptosome. The apoptosome stimulates caspase-9, which subsequently activates the executive caspase – caspase-3. Flavoprotein, which constitutes an apoptosis-inducing factor, and endonuclease G enter the cell nucleus, contributing to DNA fragmentation and nuclear chromatin condensation [12–15].

Extrinsic apoptosis occurs by the activation of the death receptor in the cell membrane. Death receptors include proteins involving tumour necrosis factor (TNF), e.g. TNFR1, TNFR2, Fas/CD95/Apo-1, or TRAIL/Apo2. The interaction between membrane receptors and ligands leads to changes in the structure of intracytoplasmic domains, so-called death domains. The death signal then travels to the adaptor protein of the Fas-associated death domain protein [8,10,15,16].

In the erythropoietic system, Cowling and Dexter [17] demonstrated erythropoietin, the stem cell factor, and insulin-like growth factor 1, reducing the apoptotic death of erythroid progenitor cells. They indicated that a deficiency of these growth factors stopped heme synthesis, which contributed to progenitor cell death.

Factors that inhibit the process of apoptosis also include the hematopoietic growth factor in granulocyte-macrophage progenitor cells. More precisely, the granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), and interleukin 3 (IL-3) have an impact on both the development and death in the granulocyte-monocyte hematopoietic lineage [18,19].

Vermes and Haanen [20] confirmed that GM-CSF, G-CSF and IL-3 regulated the development and differentiation of neutrophils, increasing their survival, and simultaneously suppressed apoptotic death. Biffl et al. [21] also demonstrated that IL-3 delayed neutrophil apoptosis. However, eosinophil death was decreased by IL-5.

The results of studies conducted in normal and leukemic myeloid cell

populations demonstrate the occurrence of apoptotic death in the absence of growth factors or modifications at the level of apoptosis resulting from interactions among the cytokines on which these cells depend in terms of survival [6,18,22,23].

Studies have been performed in the immune system concerning the effects of cytokines on myeloid-derived B cells, thymus-derived T cells, and natural killer (NK) cells. Koury [18] observed mature T cells undergoing apoptosis in the absence of either IL-3 or IL-6. Haanen and Vermes [24] detected IL-2-dependent T lymphocytes that had undergone apoptotic death after being deprived of access to this cytokine. In turn, Lømo et al. [25] demonstrated that transforming growth factor β induced apoptosis in resting peripheral blood lymphocytes *in vitro*, and IL-4 partially inhibited apoptotic death caused by transforming growth factor β.

The impact of cytokines on the above-mentioned cells has been observed in *in vitro* studies, which allow the examination of several arbitrary cytokines. Both inhibition and increase of programmed cell death are of significance in the immune system [19,26].

Apoptosis is induced by biological factors (TNF, glucocorticoids, growth factor deficiency), physical factors (ionizing radiation, temperature shock), or chemical factors (cytostatics, oxidative stress caused by oxygen free radicals) [8,9]. Seki et al. [27] investigated the effect of γ-radiation on the level of apoptotic death in lymphocyte subpopulations. NK cells proved to be the most radioresistant to apoptosis, T lymphocytes $CD8^+$ and B lymphocytes exhibited poor sensitivity, while T lymphocytes $CD4^+$ turned out to be relatively resistant to apoptotic death. Delic et al. [28] also observed apoptotic death in human lymphocytes after fractional γ-ray irradiation *in vivo*. Grelli et al. [29] identified apoptotic death of lymphocytes induced by prostaglandin E2 $9PGE_2$. Another physical factor increasing apoptotic death is hyperthermia, which affects the bone marrow, thymus, spleen, and lymph nodes [30]. Natural cell suicide is inherent in the proper function of the immune system. During leukocyte maturation in the thymus, cell selection occurs to prevent intolerance of own antigens [4]. Increased apoptosis contributes to the development of degenerative diseases, such as Alzheimer's disease or Parkinson's disease, or infectious diseases, e.g. AIDS or hepatitis [9,11]. In turn, a decrease in this process leads to the onset of malignancies, autoimmune diseases, or viral infections [8]. Cytokine proteins exert a large impact on immune system cells. Their role in this system consists in regulating cell growth and maturation, as well as in controlling cell death. The influence of cytokine on immune system cells, the populations of myeloid-derived B cells, thymus-derived T cells, and NK cells is complex [19,31].

This study, however, revealed that apoptotic leukocyte forms were very rare in the participants' blood: they were only detected in 3 winter swimmers. Methods for detecting apoptosis are constantly being modified, as understanding the relationships between molecules involved in its regulation contributes to improvements in diagnosis.

Conclusions

The findings demonstrate that low water temperature does not cause significant leukocyte apoptosis in winter swimmers, which is an important finding. This suggests that regular exposure to cold water may improve immunological resilience, which is a favourable adaptation for those who participate in this sport.

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