ABSTRACT

Background. In this study we have investigated the effect of cold exposure on young healthy subjects, with particular emphasis on the hematological system.

Study design. The lightly clothed subjects were seated in a thermally controlled climatic chamber for three hours.

Methods. One group (control) was exposed to three hours at 28 °C, while the other group was first exposed to one hour at 28 °C, and then two hours of decreasing temperature, ending at 6 °C. Mean body temperature decreased, blood pressure increased, and metabolism increased by 60 % in the cold exposure group compared to the control group. Blood samples were taken through an arterial catheter every hour during the exposure, one hour before and 1.5, 3 and 25 hours after the exposure.

Results. Red blood cell counts increased, due to hemo-concentration. In the cold neutrophil counts did not increase during the day, but were stable at a lower level compared to the controls.

Conclusions. It is suggested that the cold induced decrease in neutrophil count is caused by an activation and increased adhesion of the neutrophils to the endothelial wall. This finding is supported by the cold exposure-induced increases in IL-8 and Thromboxane B2 in LPS-stimulated blood, and an increase in the plasma concentration of P-selectin. (*Int J Circumpolar Health 2004;63(2):115-128*)

Keywords: Cold exposure, Blood factors, Cell counting, P-selectin

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INTRODUCTION

Cardiovascular disease (CVD) is the main cause of death in the western world. Although the per-capita mortality rate varies between countries, seasonal variations in mortality rate, with a higher rate in the winter than in the summer, are well described for many countries (1-10) and, in Norway, where mortality from CVD accounts for about 50% of all deaths, the difference between the highest (in January) and the lowest (in August) rates is 29% (5). The reason for this seasonal variation in mortality caused by cardiovascular diseases is not totally clear. Increases in respiratory diseases and influenza account for some of this difference, but there seems to be overall agreement that cold temperature is somehow involved. However, the exact mechanism of how cold leads to a cardiovascular event is still unclear. There are many reports describing seasonal variations in factors known to be risk factors for CVD, with reports of winter increases in blood pressure (11-13), hematocrit (12,14), cholesterol (15,16), fibrinogen (17-19) and PAI-1 (20).

Studies on mortality have also revealed that most deaths related to cold exposure do not occur immediately, but rather several hours, or even days later (21-23), suggesting that cold leads to hematological changes, which eventually lead to a cardiovascular event. To investigate this problem more closely, controlled cold exposure experiments have been performed in healthy subjects to study changes in hematological factors. In addition to the well known increases in blood pressure (24-28) and noradrenaline (25,28,29) during acute exposure to cold, due to an increased sympathetic activity, these studies have also reported various hematological changes during short-term cold exposure, including increased red blood cell counts (24,27,30-32), neutrophils (24,27,30,31), fibrinogen (32,33), cholesterol (28,32), TxA2 and tissue factor (31).

We have previously performed experiments with cold exposure in elderly subjects (30), without being able to obtain significant changes in these hematological factors, although we found a tendency for a mild inflammatory response. The level of cold exposure in those experiments were mild and, in the study described here, we have investigated the effect of a more severe exposure to cold on a variety of hematological factors in young healthy subjects. Ethical considerations limit our possibility to conduct such experiments in the elderly.
MATERIAL AND METHODS

Subjects
The participants were young healthy volunteers, aged between 19 and 31 years. Twelve (5 men and 7 women) subjects were exposed to cold, and 11 (6 men and 5 women) to a control temperature. Personal data is presented in Table 1. Four of the subjects participated in both experiments, which were carried out in February/March, with one year between the two series. Before the experiments, the subjects were subjected to a medical screening. During the 2 days before and 1 day after the experiment, the subjects were instructed to avoid exposure to cold, excessive exercise, intake of alcohol and high-fat meals. For the women, experiments were performed during the period ranging from one day after and one day before menstruation. Permission to carry out the experiments was granted by the medical ethical committee for northern Norway (Health Region V).

| Table 1. Personal data of the subjects |
|--------------------------------------|
| Subject | Gender (M/F) | Age (years) | BP (mm Hg) | BW (kg) | Height (m) | BMI (kg/m²) |
| Control | |
| a | F | 21 | 130/80 | 88 | 1.65 | 32.3 |
| b* | F | 27 | 120/80 | 80 | 1.67 | 28.7 |
| c | F | 24 | 120/75 | 64 | 1.69 | 22.4 |
| d* | M | 20 | 120/80 | 74 | 1.78 | 23.4 |
| e* | M | 25 | 120/70 | 66 | 1.81 | 20.2 |
| f | M | 25 | 135/80 | 74 | 1.75 | 24.2 |
| g | M | 24 | 110/70 | 65 | 1.79 | 20.3 |
| h | F | 24 | 100/60 | 51 | 1.59 | 20.2 |
| i | F | 20 | 120/80 | 71 | 1.69 | 24.9 |
| j | M | 31 | 130/85 | 88 | 1.92 | 23.9 |
| k* | M | 23 | 130/90 | 75 | 1.74 | 24.8 |
| Cold | |
| a | M | 21 | 130/70 | 75 | 1.83 | 22.4 |
| b* | M | 19 | 120/70 | 71 | 1.78 | 22.4 |
| c* | M | 22 | 140/90 | 77 | 1.74 | 25.4 |
| d* | M | 24 | 130/75 | 64 | 1.81 | 19.5 |
| e | M | 24 | 118/53 | 79 | 1.88 | 22.4 |
| f | F | 20 | 110/60 | 60 | 1.59 | 23.7 |
| g* | F | 26 | 139/88 | 74 | 1.67 | 26.5 |
| h | F | 24 | 130/80 | 68 | 1.73 | 22.7 |
| i | F | 22 | 100/65 | 56 | 1.60 | 21.9 |
| j | F | 23 | 110/70 | 72 | 1.76 | 23.3 |
| k | F | 20 | 110/75 | 65 | 1.70 | 22.5 |
| l | F | 20 | 119/72 | 66 | 1.67 | 23.7 |

BP = Blood pressure; BW = Body weight; SFT = skin fold thickness; BMI = Body mass index; * = subjects involved in both experiments.
The Climatic Chamber

During the experiments, the subjects sat in an upright position in a specially constructed chair placed in a 10-m³ temperature controlled room (Figure 1). Air movement was minimized by using a wind-break constructed from a light-weight transparent plastic material (walls 110 cm in height). The top of the wind-break was covered with a light-weight cloth material. Air temperature and air movement inside the wind-break could be regulated by a thermally controlled air circulation system. For blood sampling, the subjects stuck an arm through a small opening in the side-wall of the wind-break. A separate ventilated cage for the head was constructed out of aluminium with a transparent plastic front. Measurements of the exposure temperature were made with thermocouples placed at 8 different positions inside the wind break (Twb) and 4 inside the head cage (Thc). The average exposure temperature (Ta) was then calculated by use of equation 1, where Twb and Thc represent average temperatures at these sites.

\[ Ta = 0.93T_{wb} + 0.07T_{hc} \]  

(Eq.1)

A loudspeaker and a microphone were located inside the head cage, allowing communication with the subjects during the experiment. During the exposure, the subjects were able to watch a video film. The subjective feeling of comfort was recorded using an electronic visual analog scale (VAS), ranging from 0 to 100 %, were 100 % represents maximum discomfort. The subjects were asked to set the scale every 15 minutes, and
the scale was returned to 0 between each measurement. Two video cameras were installed inside the climatic chamber; one was directed towards the subject’s face, and the other at the arterial catheter on the subject’s wrist (see below).

**Experimental protocol**

In the control experiments the subjects sat in the climatic chamber for 3 hours, and the temperature was kept at 28 °C throughout this period. In the cold exposure experiments, the chamber was kept at 28 °C during the first hour, and the temperature was then gradually decreased to 6 °C during the next two hours (Figure 2, upper left panel). During the experiments the male subjects wore shorts and, the women, shorts and a bikini top.

**Physiological measurements**

Skin and rectal temperatures were measured continuously by use of thermocouples. Skin temperature was measured at 13 different places (forehead, neck, chest, back, abdomen, upper arm, fore-arm, hand, thigh backside, thigh frontside, leg backside, leg frontside, foot) and the average body temperature (\(T_b\)) was calculated by use of equation 2, where \(T_{\text{skin}}\) represents the average of the 13 measurement sites.

\[
T_b = 0.1T_{\text{skin}} + 0.9T_{\text{rec}} \quad \text{(Eq.2) (34)}
\]

Metabolic rate (M) was determined by indirect calorimetry, using an open-circuit ventilation system. M was calculated from the air flow rate through the ventilated head cage and oxygen extraction measured by means of an oxygen analyser (Ametek Oxygen Analyzer, Model S-3A/1, Applied Electrochemistry, U.S.A.), assuming a caloric equivalent of 20.35 kJ l\(^{-1}\) O\(_2\) (S.T.P.D). Heart rate and blood pressure were measured automatically every 10 minutes with a standard clinical arm cuff system (Omega 1400, Invivo Research Laboratories Inc.).

**Blood Sampling and analysis**

On the morning of the experiment, a catheter was placed in a radial artery. Blood samples (18 ml) were taken through the catheter 1 hour before the experiment, every hour during, and 1.5 and 3 hours after the experiment. A venous blood sample (Median Cubital vein)
was taken the day after. The blood was analysed for a number of different parameters. Blood collected in EDTA tubes were, after 30 minutes, analysed for red and white blood cell differentiation using a coulter counter (Sysmex K-1000, Digitana AG, Switzerland). For the analysis of noradrenaline, 3 ml of blood was collected in EDTA tubes containing 40 µl reduced glutathion, centrifuged, and the plasma was analysed by HPLC. Plasminogen activator inhibitor antigen (PAI-1) and P-selectin were analysed by use of commercially available ELISA kits (PAI-1: Biopool AB, Sweden., P-selectin: R&D systems Inc. Minneapolis, USA). P-selectin was assayed later, when the results of the effects of cold exposure on neutrophils were available.

In vitro stimulation of blood
To test the white blood cells’ and blood platelets’ ability to be activated, 1 ml of heparinized blood was stimulated with 5 ng lipopolysaccharide (LPS) (*Escherichia coli* 026:B6, Difco Laboratories. Detroit, MI, USA) (35). The aliquots were incubated for 2 h at 37 °C. The reaction was stopped by adding 100 µl 2% EDTA, pH 7.35. Plasma was separated by centrifugation (1500g, 5ºC) and analysed for Thromboxane B2 and interleukin 8 (IL-8) using ELISA kits (Biotrak, Amersham International plc, Buckinghamshire, England). Thromboxane A2 is unstable, with a short half-life, so Thromboxane B2, which is an inactive metabolite, is used to indicate the activity of this substance.

Statistics and Data processing
Cold exposure leads to a certain degree of hemo-concentration. Results for blood parameters that do not freely filter out of the capillaries were, accordingly, corrected by a factor based on the measured hematocrit. The value measured at T0 (after 1 h at 28 °C, and before the cooling) was used as the reference point. In figures 3-5, in which the blood parameters are presented, the values are given as absolute changes from time 0. All values are presented as means ± SEM. Values from the two experimental groups were compared using a two-sample t-test, and p < 0.05 was considered significant.
Physiological measurements

Results from the physiological measurements are presented in figure 2. In the cold exposure experiment, the mean body temperature ($T_b$) dropped by 1.0 °C from $T_0$ to $T_{180}$. As there were no statistically significant changes in $T_{rec}$, the changes in $T_b$ were predominantly due to a fall in skin temperature. In both groups, the metabolic rate (M) decreased slightly, reaching its minimum value at the end of the first hour at 28 °C. After 30-60 minutes, M started to increase in the
cold exposure group and, at the end of the cold exposure period, the metabolism was 60% higher than in the control experiment. This represents an increase of 82% relative to the value at the start of the cooling. During the last 30 minutes, all subjects in the cold exposure experiment shivered. Towards the end of the control experiment, there was a slight increase in M. The mean arterial blood pressure also increased during the cooling, and was significantly higher at the end of the experiment compared to the controls. The visual analog scale (VAS) showed that the subjects in the cold exposure experiments were much more uncomfortable during the cooling period, although none of the subjects asked to leave the chamber before the end of the experiment. At the end of the exposure, the average VAS was at 70% of maximum discomfort. The subjects in the control group also reported being slightly uncomfortable towards the end of the experimental period.

**Blood parameters**

The results from the blood analyses are presented in figures 3 - 5. All the data are presented as changes relative to the values measured at time 0. The absolute values at time 0 are given in table 2. Plasma noradrenaline increased during the cold experiment and did not return to the control level within 3 hours after the cooling. Red blood cell counts increased during the cooling. This is not assumed to correspond to an increase in total number of cells, but solely due to hemoconcentration, and the values for neutrophils were corrected for this effect. The hemo-concentration-corrected neutrophil count increased in the control group, and this increase follows the normal diurnal rhythm for leukocytes (36,37). In the cold exposure experiments, the neutrophil count stabilised at a lower level, and did not increase in the afternoon, as a normal rhythm would predict. PAI-1 also has a diurnal rhythm, with a peak in the early morning (38). In both cold and control experiments, the values decreased during the day. In the cold experiment, however, the values increase, although not significantly, during the cooling period, returning to their normal rhythm thereafter.

There was a marked difference between the groups in the LPS-induced IL-8 and Thromboxane B₂ (TxB₂) after cold exposure, indicating an activation, or sensitization, of leukocytes and blood platelets.

Plasma P-selectin was significantly increased at 2 and 3 hrs of the cold exposure.
Figures 3 a–c. Time-course of changes in noradrenaline, neutrophil count and red blood cell count (RBC) during control (open circles, n=11) and cold exposure (filled circles, n=12) experiments. Data is presented as relative changes from time 0, which represents the onset of cooling one hour after the subjects entered the climatic chamber, and is given as mean ± SEM. The vertical dashed lines indicate the start of the control period inside the climatic chamber (time -1 hour) and the onset of cold exposure (time 0 hour) and end of the experiment (time 2 hour). The results for the neutrophil counts have been corrected for hemo-concentration. * represents significant difference between the two groups, p < 0.05.

Figures 4 a–c. Time-course of changes in PAI-1, IL-8 and Thromboxane B2 during control (open circles, n=11) and cold exposure (filled circles, n=12) experiments.
DISCUSSION

Some caution must be applied in interpreting the results of this study for several reasons. First, there are relatively large inter-individual variations in many of the blood parameters investigated. Secondly, most of the hematological parameters measured have natural diurnal variations and, thirdly, only a third of the subjects were involved in both groups. Since, for logistic reasons, the low number of subjects used in this study, including the use of different subjects in the two groups, was unavoidable, the hematological data is presented as relative rather than absolute values. However, the reliability of treating the data in this way is dependent on the fact that there were no statistically significant differences between the two groups in the absolute reference values, in this case at time zero (Table II).

The cold exposure in these experiments was rather severe. This can be seen in the results of the VAS measurements, were the subjects reported 70% of maximum discomfort. There was also a slight increase in the reported discomfort in the control experiments. This is probably due to the increasing discomfort of sitting still for a long period of time. The metabolic rate (M) decreased to its lowest value in both groups during the first hour at 28 °C and we believe this value to be close to resting metabolism levels. The slight increase of M in the control group towards the end of the experiment was probably due to the subjects being more restless and uncomfortable. The large in-
Increases in noradrenaline show that there was a marked increase in sympathetic activity. These physiological changes occurring during cooling, together with the observed hemo-concentration, as indicated by increased red blood cell counts, were as expected (31,39-44).

The results from the neutrophil count contrast with previous studies, where increases in neutrophils during cold exposure have been reported (24,27,30,31). There is a marked diurnal rhythm in human leukocyte counts (36,37). Our control group follows this rhythm, while in the cold exposure group it seems as if the normal increase is suppressed. One reason for this might be that the cold exposure activates the neutrophils, causing them to leave the circulation, probably by adhesion to the endothelium. The increase in IL-8 and Thromboxane B2 supports this. IL-8 is a chemokine produced by leukocytes and the endothelium. It acts as a signalling substance, increasing during inflammation, to recruit cells to the site of injury. Receptors for IL-8 are found on leukocytes, the most important being the neutrophils (45-48). Thromboxane B2 is the inactive metabolite of Thromboxane A2. Thromboxane A2 is a potent vasoconstrictor and is involved in platelet aggregation. The indirect measurement of these substances, showing an increase after the cooling compared to the control, indicates that the leukocytes are activated and produce these substances more easily when they are stimulated, in this case by LPS. This has also been shown in earlier studies (31). The measurements of PAI-1 did not show any significant differences between

| Table II. Absolute values for blood parameters at T0. (mean ± SEM) |
|-------------|-----------------|-----------------|
| Noradrenaline (pg • ml⁻¹) | Control experiment | Cold exposure experiment |
| Neutrophils (10⁹ • µl⁻¹) | 340.1 ± 33.8 | n.s 334.9 ± 28.9 |
| Erythrocytes (10⁹ • µl⁻¹) | 4.29 ± 0.12 | n.s 4.42 ± 0.12 |
| PAI-1 (ng • ml⁻¹) | 8.84 ± 0.76 | n.s 8.10 ± 0.93 |
| IL-8 (ng • ml⁻¹) | 3.32 ± 0.54 | n.s 4.29 ± 1.47 |
| TxB2 (ng • ml⁻¹) | 15.8 ± 1.9 | n.s 16.3 ± 1.8 |
| P-selectin (ng • ml⁻¹) | 45.7 ± 4.2 | n.s 41.7 ± 5.3 |

n.s = no significant difference between the two groups
the two groups. PAI-1 is a well-known risk factor for CVD, and it has a marked diurnal rhythm, with a peak in the morning (38). Although our results do not show a significant difference between the control and cold exposure groups, there was a tendency for PAI-1 to increase during cold exposure, returning to the normal rhythm afterwards. Similar results have been reported previously (31).

To more closely investigate the possible activation of leukocytes, we analysed plasma for P-selectin, a substance known to be involved in cell adhesion. P-selectin is expressed on the surface of activated endothelium and blood platelets. It binds to a receptor on the surface of leukocytes, causing them to adhere to the endothelium. This interaction causes the leukocytes to roll along the endothelium and, possibly, to migrate into the tissue (46,48-50). Studies in vitro have shown that cold induces changes in the filtrability and adhesion of leukocytes (51). We observed that P-selectin increased during the cold exposure, supporting the theory of cold-induced cell adhesion. Moreover, this finding of a cold exposure-induced increase in plasma levels of P-selectin has recently been confirmed in a study in mice (Bøkenes - unpublished results).

In conclusion, the findings of a cold exposure-induced increase in plasma levels of the adhesion molecule P-selectin, coupled with cold-induced sensitization of leukocytes, as indicated by the LPS-induced increases in IL-8 and Thromboxane B₂, support the hypothesis that exposure to cold may promote thrombogenesis. While the magnitude of the observed changes in the healthy subjects used in this study cannot be regarded as being pathological, a situation is imagined in which an elderly person with an already activated immune system due, for example, to a sub-clinical infection, or atherosclerotic heart disease, may end up in a higher risk situation for developing a cardiovascular event when exposed to cold. However, while the results of this study further emphasize the effect of cold exposure on the hematological system, the relationship between cold exposure and leukocyte activation requires further investigation.

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