Cold-induced increases in erythrocyte count, plasma cholesterol and plasma fibrinogen of elderly people without a comparable rise in Protein C or Factor X

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1. Six elderly (66–71 years) and six young (20–23 years) subjects (half of each group women) were cooled for 2 h in moving air at 18°C to investigate possible causes of increased mortality from arterial thrombosis among elderly people in cold weather. Compared with thermoneutral control experiments, skin temperature (trunk) fell from 35.5 to 29.5°C, with little change in core temperature.

2. Erythrocyte count rose in the cold from 4.29 to 4.69 x 10^12/l, without a change in mean corpuscular volume, indicating a 14% or 438ml decline in plasma volume; increased excretion of water, Na⁺ and K⁺ accounted for loss of only 179ml of extracellular water.

3. Plasma cholesterol and fibrinogen concentrations rose in the elderly subjects from 4.90mmol/l and 2.97 g/l (control) to 5.45 mmol/l and 3.39 g/l in the cold, and in the young subjects from 3.33mmol/l and 1.84 g/l (control) to 3.77mmol/l and 2.07 g/l in the cold. Increases were significant for the elderly subjects, the young subjects and the group as a whole, except for cholesterol in the young subjects, and all were close to those expected from the fall in plasma volume.

4. Plasma levels of Protein C and factor X did not increase significantly in the cold in the elderly subjects, young subjects, or the group as a whole.

5. The results suggest that loss of plasma fluid in the cold concentrates major risk factors for arterial thrombosis, while small molecules, including protective Protein C, redistribute to interstitial fluid.

INTRODUCTION

Mortality from coronary and cerebral thrombosis increases greatly in winter in temperate climates, mainly among elderly people [1, 2]. Increases in erythrocyte count and plasma cholesterol concentration, which can be produced in young adults by exposure to mild cold [3], provide a possible explanation.

The present experiments were designed first to see whether cold induces such changes in elderly people, and whether the increases in erythrocyte count and plasma cholesterol concentration can be explained by loss of extracellular (EC) fluid due to cold diuresis [4] and natriuresis [5, 6] together. We exposed six elderly subjects and six young adults to 2h of moderate cooling by moving air at 18°C, which preliminary studies [7] showed to produce a clear increase in erythrocyte count, and to a thermoneutral control, study. Since plasma albumin enters interstitial fluid in only moderate amounts from the plasma [8, 9] we have looked for changes in plasma levels of Protein C, a major natural anticoagulant [10], and of Factor X, both of which have a lower Mr than albumin, as well as of fibrinogen, which has a higher Mr than albumin. No evidence seems to exist on the extent to which the smaller clotting factors of the blood enter the interstitial space, but sufficiently small molecular size might allow some of them to redistribute from the circulation through the interstitial space, and so prevent them rising substantially during cold-induced haemoconcentration.

METHODS

The subjects were six healthy volunteers aged 20–23 years and six aged 66–71 years. Half of each group were women. All gave their informed consent, and were asked to inform the experimenters if the thermal stresses at any time exceeded those they encountered in ordinary life; none reported this. The experiments were approved by the Ethics Committee of the Tower Hamlets District Health Authority. At an initial visit they were medically examined, and their height, weight and fat thickness were measured. Mean subcutaneous fat thickness...
was estimated from ultrasonic measurements made at four sites [11] by a Wells-Krautkramer pulsed Doppler device.

Before the experiments the subjects took no medication for 5 days, did not smoke or drink alcohol for 24 h, and took no food or drink for 8 h. After transport by car to the laboratory they ate at 08.00 hours a standard meal of 69.4 g of bread, toast, with 71.8 g of jam, and 200 ml of unsweetened orange juice, containing 15.8 mmol of Na\(^+\) and 47.2 mmol of K\(^+\). Each subject was studied twice, with an interval of at least 1 week. On each occasion, 30 min after reaching the laboratory, they started an initial stabilization period of 90 min recumbent in still air at 22±1°C (limits) in a T-shirt and shorts, with two blankets. They then either (a) remained in this control situation for a further 2 h or (b) were exposed on a net bed, with the blankets removed, for 2 h to turbulent air at 18±0.5°C (limits) blown by a fan 640 mm in diameter with a capacity of 24 m\(^3\)/s. The order of the experiments was crossed over in each (young and elderly) group.

During this they ate another standard meal of the same composition as before, half of it 30 min, and half 2 h, after the start of the recovery period. They returned the next morning by car, again ate the standard meal at 08.00 hours, and remained recumbent under blankets in air at 22±1°C (limits), until 10.00 hours when final readings were taken. Subjects were allowed to drink water at any time; volumes drunk were recorded. All readings and blood samples were taken just before the 2 h experimental or control period, 30 min into it, just before its end, and 2 h, 4 h and 22 h after its end. Ventilation was also measured every 30 min, and electrocardiogram was monitored for safety throughout.

Core temperature was measured by a zero-gradient aural thermometer from a thermistor probe inserted 10 mm into the external auditory meatus with servo-controlled external heating [12]. Skin temperature was measured by uncovered thermocouples on the right anterolateral surface of the middle of the trunk, middle of the dorsum of the hand, and posterior surface of the thickest part of the calf. Expired air was collected by a mouthpiece and valve assembly, and \(O_2\) was measured downstream of a mixing box by a Beckman OM11 analyser, volume by a pneumotachograph, and pulmonary ventilation and metabolic rate were calculated [13] on-line over 5 min periods by an Opus 5 computer.

In calculating respiratory water loss, from expired gas volume, expired air was taken as 90% saturated at a temperature calculated from the measured pressure, water vapour pressure and temperature of inspired air [14]. Subjects emptied their bladders at the start of each control or experimental 2 h period and again at the end of it, when the sample was measured for volume and by flame spectrophotometry for Na\(^+\) and K\(^+\).

Changes in EC and IC (intracellular) water caused by overall losses of water, Na\(^+\) and K\(^+\) were calculated on the following basis: control values for men and women respectively were taken as EC fluid 19.6% and 18.45% body weight, IC fluid 40.03% and 35.55% body weight [15], plasma volume 43.54 and 44.14 ml/kg [16]; initial concentrations of Na\(^+\) and K\(^+\) in EC fluid for both men and women, including plasma, were taken respectively as 141.2 mmol/l and 4.11 mmol/l [15], Na\(^+\) and K\(^+\) in IC fluid as 14 mmol/l and 140 mmol/l, and initial osmolarity of body fluids as 302 mosmol/l [17]; partitioning between EC and IC fluid or net water loss from the body was calculated assuming that EC/IC ratios for Na\(^+\) and K\(^+\) concentrations each remained constant, and that EC osmotic pressure remained equal to IC osmotic pressure.

Blood was taken from flowing blood in a forearm vein after only brief occlusion [18], using vacutainers and a 21-gauge needle. In the 'cold' experiments the forearm from which blood was taken was warmed by a 28 W heater pad to ensure free flow of blood; this always occurred. Of the 16 ml of blood taken on each of six occasions in each experiment, two samples of 3 ml each were taken into 0.072 ml of 0.17 mol/l EDTA. After 20 min one was assayed in a Technicon Counter for erythrocyte count and mean corpuscular volume (MCV), and packed cell volume (PCV) was calculated from these. Changes in plasma volume due to haemoconcentration or dilution were calculated for each subject, assuming a constant number of circulating erythrocytes [19] as:

\[ (1 - PCV_f) \times MCV_f / (1 - PCV_i) \times MCV_i \]

where PCV\(_f\) is final PCV and PCV\(_i\) is control PCV, and MCV\(_f\) is final MCV and MCV\(_i\) is control MCV. The other EDTA-treated sample was centrifuged and the plasma was assayed for cholesterol by an enzymic, colorimetric method (Cholesterol C-system, high performance) from Boehringer Mannheim Ltd. Further samples of 2.5 ml each were taken into a vacutainer containing 0.5 ml of 0.105 mol/l sodium citrate and 1500 kallikrein inhibitory units of aprotinin/ml, and two vacutainers each containing 0.072 ml of 0.17 mol/l EDTA. The tubes were centrifuged as before and the plasma was decanted into 1.5 ml Sarstedt tubes and stored at −80°C until assayed. Within 1 h before assay plasma samples were thawed at 37°C, and then maintained at room temperature (21–23°C). Plasma in which fibrinolytic activity was inhibited by aprotinin was used for assays of fibrinogen, Protein C, Factor X and anti-Factor Xa. Fibrinogen was determined using the method of Claus [20]. Protein C was determined by the synthetic chromogenic substrate method [21]. Factor X was determined using a one-stage prothrombin time assay (Immuno AG). Anti-Factor Xa was assayed using a clotting time method [22].

All assays on a given subject were made using the same batch of reagents to minimize errors in the
Haemoconcentration in the cold

Table 1. Physical characteristics of the subjects. Values are means ± SEM. Abbreviation: NS, not significant.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Age (years)</th>
<th>Height (m)</th>
<th>Weight (kg)</th>
<th>Surface area (m²)</th>
<th>Mean subcutaneous fat thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All subjects</td>
<td>12</td>
<td>45.2 ± 7.1</td>
<td>1.68 ± 0.02</td>
<td>71.0 ± 3.2</td>
<td>1.81 ± 0.04</td>
<td>10.26 ± 2.9</td>
</tr>
<tr>
<td>Elderly subjects</td>
<td>6</td>
<td>68.7 ± 0.7</td>
<td>1.64 ± 0.03</td>
<td>77.6 ± 4.9</td>
<td>1.86 ± 0.06</td>
<td>13.73 ± 6.50</td>
</tr>
<tr>
<td>Young subjects</td>
<td>6</td>
<td>21.7 ± 0.5</td>
<td>1.70 ± 0.04</td>
<td>64.5 ± 2.2</td>
<td>1.76 ± 0.05</td>
<td>6.79 ± 1.90</td>
</tr>
</tbody>
</table>

*Difference (elderly versus young) NS P=0.04 NS P=0.03*

Intra-subject comparisons. Statistical comparisons were made by student's t-test, with pairing when appropriate. In order to minimize errors due to multiple comparisons, only data obtained just before the start and the end of experiments, and 22 h after they finished, were compared statistically. Values are means ± SEM unless otherwise stated.

RESULTS

Subjects

Table 1 shows that the elderly subjects were on average 47 years older, were heavier and had thicker subcutaneous fat than the young adults. The weights (see the Methods section) represent a plasma volume of 3.114 ± 0.143 litres, an EC fluid volume of 13.51 ± 0.60 litres and an IC fluid volume of 26.82 ± 1.21 litres for the group as a whole.

Changes in temperature and metabolic rate

Two hours of exposure to cold caused (Fig. 1) little change in body core temperature, but a large fall in trunk skin temperature, in both elderly and young subjects. Calf skin temperature (not in Fig. 1) fell from 33.2 ± 0.4 (control) to 22.3 ± 0.26°C in the cold, and hand skin temperature fell from 32.8 ± 0.5 to 22.5 ± 0.2°C (n=12, P<0.001 in both cases). Metabolic rate increased from 43.6 ± 1.3 W/m² (control) to 49.3 ± 5.9 W/m² at the end of the 2 h of cold exposure (P=0.001). There were no significant differences between elderly and young subjects in any of these values or in their changes in the cold, but on average falls in trunk skin temperature in the cold were larger, and recovered more slowly during subsequent passive rewarming, in the elderly subjects (Fig. 1).

Changes in erythrocyte parameters

Fig. 1 also shows that erythrocyte count increased in the cold in both the elderly and young adults. Neither it nor its changes differed significantly between elderly and young subjects. The erythrocyte count increased in the cold on the whole group from 4.29 ± 0.10 to 4.69 ± 0.10 × 10⁹/l (P<0.001). MCV did not significantly change in the elderly subjects, young subjects or the group as a whole, or differ between elderly and young subjects. For the elderly subjects it was 88.1 ± 3.1 fl at the end of control exposure; for the young subjects it was 91.3 ± 0.9 fl (control) and 91.5 ± 2.2 fl (cold). PCV (from erythrocyte count and MCV) rose in the elderly subjects from 38.0 ± 1.4% (control) to 43.2 ± 1.6% (cold), (P=0.007); and in the young subjects from 39.1 ± 1.7 to 42.0 ± 1.3% (P=0.04). Plasma volume, calculated from PCV and MCV, fell on average by 14% or 438 ± 89 ml (P<0.001) (583 ± 113 ml, P=0.004, in the elderly subjects; 293 ± 117 ml, P=0.05, in the young subjects). The falls would increase the concentration of a solute confined to plasma by a factor of 1.176 ± 0.042 (1.229 ± 0.062 in the elderly subjects; 1.125 ± 0.054 in the young subjects).

Fig. 1. Aural (zero-gradient) and trunk skin temperatures, and erythrocyte count, during control experiment and cold exposure. ●, Control, clothed in still air at 22°C throughout; ▲, exposure to turbulent air at 18°C for 2 h. Values are means and SEM (n=6) for 0, 2 and 24h only. Statistical significance: *P<0.05, **P<0.01, ***P<0.001) compared with control (given for 0, 2 and 24 h only).
Table 2. Fluid and electrolyte exchanges during cold exposure. Values are means ± SEM. One elderly subject drank 20 ml of water in the control experiment and 3 ml in the cold. Net water loss includes this and urinary and respiratory losses.

<table>
<thead>
<tr>
<th></th>
<th>Elderly subjects</th>
<th>Young subjects</th>
<th>Difference cold versus control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Cold</td>
<td>Control</td>
</tr>
<tr>
<td>Urine volume (ml)</td>
<td>162 ± 17</td>
<td>496 ± 117</td>
<td>176 ± 52</td>
</tr>
<tr>
<td>Respiratory loss of water (ml)</td>
<td>25.5 ± 1.8</td>
<td>29.2 ± 3.5</td>
<td>20.6 ± 1.2</td>
</tr>
<tr>
<td>Net water loss (ml)</td>
<td>168 ± 14</td>
<td>52 ± 117</td>
<td>190 ± 52</td>
</tr>
<tr>
<td>Urinary Na⁺ (mmol)</td>
<td>17.9 ± 2.0</td>
<td>45.2 ± 14.3</td>
<td>23.4 ± 3.0</td>
</tr>
<tr>
<td>Urinary K⁺ (mmol)</td>
<td>9.1 ± 0.9</td>
<td>14.5 ± 1.4</td>
<td>9.5 ± 1.9</td>
</tr>
<tr>
<td>Urinary K⁺ (mmol)</td>
<td>234 ± 1.4</td>
<td>125 ± 6.0</td>
<td>125 ± 6.0</td>
</tr>
<tr>
<td>EC water loss* relative to</td>
<td>± 87</td>
<td>± 60</td>
<td></td>
</tr>
<tr>
<td>control (ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Calculated for each subject (see the Methods section) from water Na⁺ and K⁺ losses.

Changes in fluid and electrolytes

Table 2 shows that urine volume more than doubled and the Na⁺ and K⁺ output approximately doubled during the 2 h of cold exposure compared with control values. An increase in respiratory water loss in most subjects, associated with an increase in ventilation from 9.2 ± 0.6 to 10.7 ± 1.3 l/min (not significant) in the elderly subjects and from 7.4 ± 0.4 to 10.3 ± 0.9 l/min (P = 0.02) in the young subjects, further increased water loss in the cold. There were no significant differences between the elderly subjects and young adult in these values or in their changes in the cold. The loss of extracellular water, calculated from water, Na⁺ and K⁺ losses in the cold (see the Methods subjects) was 179 ± 53 ml for all subjects. Table 2 shows that this loss was significant for all subjects and for the elderly subjects alone. It was also significantly less (P = 0.01) then the fall in plasma volume both for all subjects and for the elderly subjects alone.

Fibrinogen and cholesterol

Fig. 2 shows that plasma fibrinogen concentration was significantly higher in the elderly subjects than in the young adults at all points at which comparisons were made, and that it increased significantly in the cold in both groups. The increase for all subjects in the cold (P = 0.003) averaged 13%, and the level reached in the cold averaged 2.73 ± 0.24 g/l, not significantly below the level of 2.88 ± 0.32 g/l expected from the shrinkage of plasma volume in the cold if no fibrinogen left the circulation. Fig. 2 shows that plasma cholesterol concentration was also significantly higher in the elderly subjects than the young subjects at all points of comparison. It also increased by the end of the 2 h of exposure to cold, significantly in the elderly subjects; the increase for all subjects in the cold (P = 0.004) averaged 12%, and the level reached in the cold, 4.61 ± 0.40 mmol/l, was not significantly below the level, 4.91 ± 0.51 mmol/l, expected from the shrinkage of plasma volume if no cholesterol left the circulation.
Plasma Protein C concn. and Plasma Factor X concn. for the group as a whole, or for the elderly subjects separately. It also shows that at the end of cold exposure plasma levels of both Protein C and Factor X for the group as a whole were significantly below the levels for young subjects separately, during exposure to cold. It could not, and must represent movement of plasma fluid to the interstitial space. Both of the components of plasma fluid loss are likely to result from engorgement of central blood vessels. Pulmonary blood vessels become markedly distended with blood in the cold [23, 24], as peripheral vasoconstriction moves blood out of peripheral vessels [25], and this can be expected to increase the filtration of plasma fluid into the interstitial space of the lungs. Raised central venous pressures due to the central shift of blood are likely to cause the falls in plasma antidiuretic hormone [26] and aldosterone [27] levels in the cold that mediate increased water and Na⁺ excretion. The increase in K⁺ excretion that we observed in the cold cannot be explained by falls in plasma levels of these hormones; its cause is uncertain, but increased K⁺ excretion has been observed during postural manoeuvres designed to increase central blood volume [5].

The increase in plasma fibrinogen concentration that occurred in both elderly and young subjects in the cold, and the absence of a comparable increase in plasma Protein C level, are of particular interest. Fibrinogen aggregates platelets once agents such as adenosine or granulocyte elastase have exposed binding sites on their surface, as well as being a substrate for fibrin production; like raised erythrocyte count and plasma cholesterol concentration, it is a well-established risk factor for arterial thrombosis [28–30]. Protein C, which inhibits several steps in the clotting process, protects against intravascular clotting [10], and the failure of its plasma level to rise in line with erythrocyte count and plasma fibrinogen and cholesterol concentrations is likely to be important in cold-related thrombosis. The fact that the magnitude of the increase in plasma fibrinogen concentration was almost in line with the fall in plasma volume in the cold, suggests that the increase was produced passively by fluid, but not fibrinogen, leaving the circulation. Even albumin (M₉, 69 000) enters interstitial fluid about three times as readily as fibrinogen (M₉, 340 000) [31] reaching 0.23–0.38 of its plasma concentration in the interstitial fluid of the dog paw [32]. The failure of plasma levels of Protein C (M₉, 62 000) [33] and Factor X (M₉, 55 000) [34] to increase significantly in the cold suggests that their molecules were small enough to redistribute rapidly out of the plasma to the interstitial fluid; plasma forms only 23% of the total EC space [15, 16]. The behaviour of plasma cholesterol concentration, in increasing in the cold to a similar degree as plasma fibrinogen concentration, is consistent with its incorporation into large lipoprotein particles [35]. The higher plasma levels of cholesterol and fibrinogen reached in the cold by the elderly subjects than the young subjects might produce a greater risk of thrombosis, apart from that due to the higher background level of atheroma in the

### Table 3. Effect of body cooling on plasma Protein C and Factor X correlations. Values are mean ±SEM, measured at end of a 2h control experiment or 2h of cold exposure, or expected from plasma loss at end of 2h of cold exposure. Statistical significance: *P = 0.05, †P = 0.04 compared with measured.

<table>
<thead>
<tr>
<th>Protein C</th>
<th>Young subjects</th>
<th>Difference all subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Cold</td>
<td>Control</td>
</tr>
<tr>
<td>Measured</td>
<td>80 ± 3</td>
<td>120 ± 11</td>
</tr>
<tr>
<td>Expected</td>
<td>99 ± 6</td>
<td>135* ± 15</td>
</tr>
<tr>
<td>Plasma Factor X</td>
<td>Young subjects</td>
<td>Difference all subjects</td>
</tr>
<tr>
<td>Control</td>
<td>Cold</td>
<td>Control</td>
</tr>
<tr>
<td>Measured</td>
<td>94 ± 5</td>
<td>96 ± 6</td>
</tr>
<tr>
<td>Expected</td>
<td>110 ± 12</td>
<td>measured (cold)</td>
</tr>
</tbody>
</table>

Protein C and Factor X

Table 3 shows that there was no significant change in plasma levels of Protein C or Factor X for the group as a whole, or for the elderly subjects or young subjects separately, during exposure to cold. It also shows that at the end of cold exposure plasma levels of both Protein C and Factor X in the group as a whole were significantly below the levels expected as a result of the loss of plasma volume in the cold, implying that they had left the plasma in significant amounts during the haemoconcentration. Plasma anti-Factor Xa concentration never differed significantly from zero for the group as a whole or for the elderly subjects or young subjects separately.

Lack of long-term changes

Twenty-two hours after the end of cold exposure, none of the variables measured differed significantly from control values.

Men and women

Erythrocyte count was relatively low in the young women; values for young and elderly subjects together were: men, 4.50 ± 0.11 × 10¹²/L; women, 4.09 ± 0.14 × 10¹²/L; difference, P = 0.04, at the start of the control study. There were otherwise no significant differences initially or in the cold between men and women, for any of the temperatures, fluid and electrolyte exchanges, or haematological parameters measured.

DISCUSSION

The results show first that exposure to cold increases erythrocyte count and plasma cholesterol concentration in elderly subjects, and does so by at least as much as in young adults. Although part of the haemoconcentration in the cold could be explained by excretion of water, Na⁺ and K⁺, most of it could not, and must represent movement of plasma fluid to the interstitial space. Both of the components of plasma fluid loss are likely to result from engorgement of central blood vessels. Pulmonary blood vessels become markedly distended with blood in the cold [23, 24], as peripheral vasoconstriction moves blood out of peripheral vessels [25], and this can be expected to increase the filtration of plasma fluid into the interstitial space of the lungs. Raised central venous pressures due to the central shift of blood are likely to cause the falls in plasma antidiuretic hormone [26] and aldosterone [27] levels in the cold that mediate increased water and Na⁺ excretion. The increase in K⁺ excretion that we observed in the cold cannot be explained by falls in plasma levels of these hormones; its cause is uncertain, but increased K⁺ excretion has been observed during postural manoeuvres designed to increase central blood volume [5].

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elderly subjects. These higher levels resulted mainly from the greater initial plasma levels of cholesterol and fibrinogen in the elderly subjects; the tendency of both these to increase with age is well established [36-38].

ACKNOWLEDGMENTS

We are indebted to the subjects for their cooperation, and to the MRC for support.

REFERENCES