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Catecholamines in the Vitreous Fluid and Urine of Guinea Pigs Dying of Cold and the Effect of Postmortem Freezing and Autolysis

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ABSTRACT: Concentrations of catecholamines in vitreous fluid and urine in guinea pigs dying of cold and the effects of freezing and autolysis on these parameters were studied. The analysis was performed by high performance liquid chromatography with electrochemical detection. Noradrenaline (NA) concentration in vitreous fluid was more than 20 times higher in the cold exposed animals than in controls (44.2 ± 9.2 versus 2.0 ± 1.0 ng/mL). Autolysis alone caused an increase to 33.5 ± 7.7 ng/mL, and freezing alone to 13.4 ± 5.3 ng/mL. The highest values were in the group with exposure, freezing, and autolysis. Adrenaline (A) concentration in the vitreous fluid increased fourfold (3.9 ± 1.5 versus 0.7 ± 0.5 ng/mL) in cold exposure and twofold as a result of autolysis. Dopamine (DA) concentration in vitreous fluid was elevated only in the group with exposure, freezing, and autolysis. The increase of NA concentration in urine was fivefold during the whole exposure (from 19.4 ± 6.9 to 109 ± 57.3 ng/mL), but A was increased by twentyfold (from 10 ± 5.1 to 213.2 ± 168.7 ng/mL), whereas DA concentration did not change. The increase of average excretion of NA to urine was eightfold during the first 6 h of exposure, and that of A tenfold. According to the present results, elevated concentrations of catecholamines in the vitreous fluid and urine can be used as a diagnostic aid for hypothermia death. Concerning the values of noradrenaline in the vitreous, the increase as a result of autolysis must be taken in account when interpreting the results.

KEYWORDS: pathology and biology, hypothermia, catecholamines, vitreous humor, urine, freezing, autolysis, postmortem chemistry

In a stress situation, for example, in exposure to cold, catecholamines are released into the blood circulation and excreted from there into the urine. Dying from hypothermia as a result of dry exposure lasts several hours, according to case histories available from such accidents, during which time catecholamines are released. This allows them to be used as one marker of hypothermia death. One series of fatal accidental hypothermia deaths did indeed show high concentrations of noradrenaline (NA) and adrenaline (A) so that a combined concentration of 0.1 μg/mL could be regarded as a sign of hypothermia [*1*]. This observation was considered promising in the search for reliable markers of hypothermia death, the diagnosis of which is otherwise rather difficult because of the few morphological signs present.

The most useful postmortem chemical tests in such cases are those carried out on the urine, and vitreous fluid, since both are less affected by autolysis than blood is. Postmortem determinations of glucose and electrolytes in the vitreous fluid, for instance, give a better

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picture of the situation at death than if the measurements are made from blood [*2-4*]. Since urine is sometimes lost during the premortem agony, the vitreous fluid is left as the only usable material.

For these reasons, we decided to investigate whether determinations of catecholamines in vitreous fluid could be useful as indicators of hypothermia stress. The effect of postmortem freezing, thawing, and autolysis was also studied.

Material and Methods

Experimental Groups

Altogether 30 adult guinea pigs of both sexes (weight about 800 g) were used in the experiments. They lived in a normal animal colony at +20°C and received vegetables and water ad libitum. They were divided into five groups of six animals of both sexes for testing the effect of exposure to cold, postmortem freezing, thawing, and autolysis.

Group 1—The animals were exposed to −20°C until dead and were left to freeze. The corpses were then thawed out in plastic bags under cold tap water for 30 min and the samples drawn. This group simulated death from cold without autolysis.

Group 2—The animals were also exposed to −20°C until dead and left to freeze, after which the corpses were thawed as in Group 1 and then left at room temperature for 6 h before the samples were taken. This group tested the effect of death from cold followed by autolysis.

Group 3—The guinea pigs were killed with a blow on the neck and the corpses were immediately frozen at −20°C for 6 h and then rapidly thawed as before and the samples drawn. This group was a model for sudden death under cold conditions and the effect of postmortem freezing.

Group 4—The guinea pigs were also killed with a blow, but the corpses were kept at +20°C for 6 h and the samples taken. This was a model for sudden death under warm conditions together with the effect of autolysis.

Group 5—The guinea pigs were killed with a blow and the samples taken immediately.

Samples

Vitreous fluid was taken from both eyes with a syringe at the end of the experiment, centrifuged, and frozen until analyzed.

Urine was collected into a special bottle from all animals for 18 h before the experiment to obtain base values for the excretion and concentration of catecholamines. A second sample was taken during the first 6 h of cold exposure and a third when the exposure had been finished in Groups 1 and 2. The last urine sample was taken from the bladder after the whole procedure in all groups.

Reagents

The catecholamine standards and internal standards were obtained from Sigma Chemicals. Acetonitrile of chromatography purity was from E. Merck. The aluminum oxide (Akt I) was from Woelm Pharma GmbH & Co. All other reagents were of analytical grade. All the water used was purified with an ion exchange apparatus.

Treatment of the Samples

Vitreous fluid was taken from both eyes using a disposable syringe with an 18-gauge needle. After centrifugation in conical 1.5-mL polypropene test tubes (10 000 $\times g$, 5 min), the

supernatant (250 μL) was removed and 15 μL of fresh preservation fluid (100m*M* sodium ethylenediaminetetraacetate [Na_2 EDTA], 10m*M* sodium metabisulfite [$Na_2S_2O_5$]) was added. After that, the vitreous fluid samples were stored in polypropene tubes at −70°C until analyzed.

The urine samples (A, B, and C) were collected in bottles already containing 250 μL of 10*M*(HCl) hydrochloric acid. After shaking, the total volume was noted and 100 μL of each was stored. The samples from the bladder (Sample D) were taken with similar syringes to the above during the preparation and 10 μL of 1*M* HCl was added to 100 μL of urine. The samples were then stored as before.

The vitreous fluid and urine samples were gently thawed on the day of analysis, after which the urine was centrifuged (10 000 ×*g*, 5 min). Two hundred microlitres of vitreous fluid and thirty microlitres of urine were taken for aluminum oxide (Al_2O_3) extraction of catecholamines.

Assay of Catecholamines

The catecholamines were extracted into Al_2O_3 using dihydroxybenzylamine (DHBA) and isopronaline (ISO) as internal standards. The extraction procedure was modified from the method of Eriksson and Persson [*5*]. The liquid chromatography-electrochemical (LC/EL) procedure as described by Taylor et al. [*6*] was used as the basis for the final assay of the catecholamines.

Statistical Methods

Student's *t* test was used to compare the results between groups. For a more critical test of statistical significance Bonferroni's *t* test was used, since this is better suited to the comparison of several groups [*7*].

Results

Catecholamines in the Vitreous Fluid (Tables 1 and 2 and Fig. 1)

Noradrenaline (NA) concentrations were more than 20 times higher in the cold exposure groups (1 and 2) than in the controls (Group 5), and were also high in the group with autolysis alone, the highest concentration of all being found in Group 2, with both exposure and

TABLE 1—*Catecholamines in guinea pig vitreous fluid.*[a]

Group	*n*	Noradrenaline, ng/mL	Adrenaline, ng/mL	Dopamine, ng/mL
1	5	44.2 ± 9.2	3.9 ± 1.5	3.6 ± 3.0
2	6	53.7 ± 15.8	4.4 ± 3.2	11.2 ± 5.8
3	6	13.4 ± 5.3	0.4 ± 0.2	7.1 ± 4.0
4	5	33.5 ± 7.7	1.8 ± 0.8	6.6 ± 2.0
5	6	2.0 ± 1.0	0.7 ± 0.5	5.3 ± 1.6

[a]The groups are:

1—exposure and freezing at −20°C, with thawing under cold tap water;
2—exposure and freezing at −20°C, thawing as in 1 and autolysis at +20°C for 6 h;
3—killed, frozen at −20°C for 6 h, and thawed as above;
4—killed, followed by autolysis at +20°C for 6 h; and
5—killed and sampled immediately.

TABLE 2—*Statistical analysis of the results of Table 1. The figures give the p values attached to student's t test and the asterisks the significance level of the more critical comparison with Bonferroni's t test.*[a]

Groups Compared	Noradrenaline	Adrenaline	Dopamine
1/2	0.2501	0.7339	0.0246
1/3	0.0005**	0.0057	0.1380
1/4	0.0820	0.0289	0.1123
1/5	0.0000***	0.0069	0.2986
2/3	0.0010**	0.0282	0.1868
2/4	0.0267	0.1038	0.1125
2/5	0.0005**	0.0373	0.0557
3/4	0.0016*	0.0139	0.7822
3/5	0.0030*	0.1782	0.2545
4/5	0.0007**	0.0336	0.2920

[a]* = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$. p = values of Student's t test.

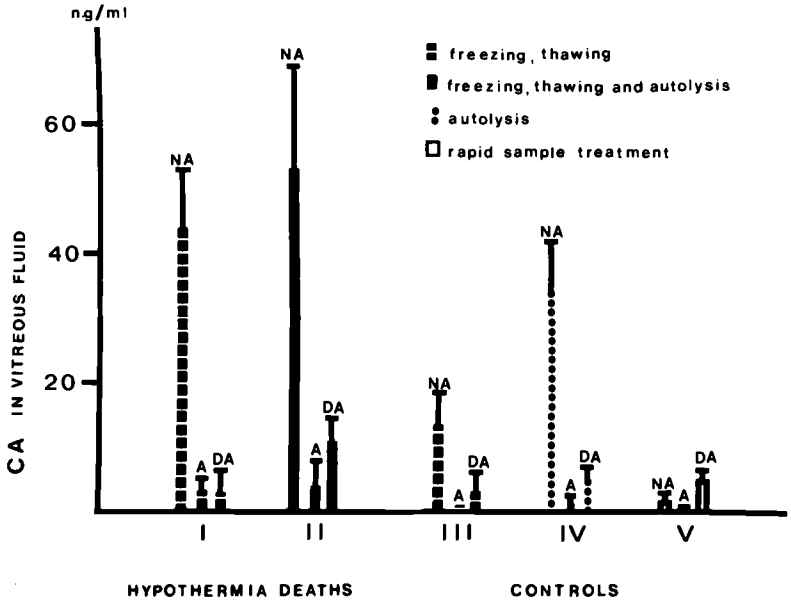

FIG. 1—*Catecholamines in the vitreous fluid of guinea pigs exposed to cold, killed, and frozen post-mortem, or subjected to autolysis without freezing. The Groups 1-5 are described in Table 1.*

autolysis. Freezing alone (Group 3) produced a sixfold increase in NA concentration. The differences between the hypothermia death group (1) and the sudden death-freezing-thawing group (3) were significant, but Group 1 did not differ from Groups 2 and 4, where autolysis was one factor. Group 2, with cold exposure and autolysis, differed from the sudden death autolysis group (4) ($p < 0.05$), the sudden death-freezing-thawing group (3) ($p = 0.001$), and the control group (5) ($p < 0.001$). The effect of autolysis was also evident in a

significant difference between Groups 3 and 4, sudden death-freezing-thawing versus sudden death-autolysis ($p < 0.05$).

Adrenaline (A) concentrations were also highest in the two hypothermia groups (1 and 2) and next highest in the autolysis group (4). The A concentration in the hypothermia group (1) differed significantly from those in the sudden death group (3) ($p < 0.01$) and the sudden death-autolysis group (4) ($p < 0.05$) and from the controls, Group 5 ($p < 0.01$). The differences between Groups 2 and 3, and between Groups 2 and 5, were also significant, indicating the increasing effect of both hypothermia and autolysis. The effect of autolysis was also seen in the significant differences between Groups 3 and 4, and Groups 4 and 5 ($p < 0.05$).

Dopamine (DA) concentrations were highest in the hypothermia death and autolysis group (2), but the lowest value was found in the hypothermia death group (1). The standard deviation was high in all groups, and the only significant difference ($p < 0.05$) was between the hypothermia groups (1 and 2).

Catecholamines in the Urine (Table 3 and Fig. 2)

The urine concentrations of catecholamines at each step in the exposure and in each experiment are presented in Table 3. Sample A is the average value for 18 h before exposure, and Sample D is withdrawn from the bladder after the whole procedure. The concentrations of noradrenaline and adrenaline in the four animals in the two cold exposure groups from which Sample D could be obtained were many times greater than the control values (A). Freezing and thawing (Group 3) and autolysis (Group 4) had a clear elevating effect on urine NA concentration ($p < 0.05$), but this was not as obvious as in the cold exposure groups (1 and 2). Dopamine also tended to be higher in Sample D than in Sample A (without statistical significance). Postmortem treatment did not alter the concentration of adrenaline.

Samples B and C were collected during cold exposure in Groups 1 and 2, in which the exposure was similar but the postmortem treatment differed (see **Materials and Methods**).

The combined results from these cold exposure groups are presented as one column in Table 3 and Fig. 2. The average concentration of noradrenaline had increased from 19.4 ± 6.9 to 84.5 ± 53.1 ng/mL (t test, $p < 0.01$) during the first 6 h of exposure. The increase in average NA concentration during the whole exposure was fivefold, to 109.9 ± 57.3 ng/mL. The final average concentration of NA measured in four animals was 203.4 ± 178.3 ng/mL, which is ten times higher than the base value, but the difference was not statistically significant because of small number of samples and great variation. The average excretion of NA rose from 50.1 ± 18.9 to 157.3 ± 111.1 ng/h during the first 6 h (t test, $p < 0.01$).

The average concentration of adrenaline had increased from 10.0 ± 5.1 to 97.0 ± 170.0 ng/mL (n.s.) during the first 6 h of exposure to an average of 213.2 ± 168.7 ng/mL during the last 5 h (from 6 h to death) (t test, $p < 0.01$). The final average concentration (D) was 414.2 ± 293.4 ng/mL, which is 41 times higher than the base value, although the small number of samples and great variation rendered the result statistically nonsignificant. The average excretion of adrenaline rose by a factor of 4 during the first 6 h, from 25.9 ± 16.4 to 102.0 ± 134.5 ng/h ($p < 0.05$).

The average concentration of dopamine had doubled during the first 6 h (from 28.9 ± 14.7 to 53.7 ± 30.1 ng/mL), but had returned to close to the base value by the end of the exposure. A slight increase in excretion was observed during the first 6 h of exposure.

Individual variation in urine catecholamines in response to cold exposure was marked. The average total catecholamine (NA and A) concentration rose above 100 ng/mL in five of the eleven animals after 6 h of exposure. The highest value was 670 ng/mL, while the lowest was only 35 ng/mL. The average total catecholamine (CA) during the last 5 h from 6 h to death reached 100 ng/mL in all the animals.

TABLE 3—*Urine catecholamine concentrations (ng/mL) collected before and during cold exposure, and withdrawn from the bladder after the postmortem treatment.*[a]

	GROUPS					
	1	2	1 + 2	3	4	5
			NORADRENALINE			
A	22.6 ± 8.1 $n = 6$	16.2 ± 3.8 $n = 6$	19.4 ± 6.9 $n = 12$ $p < 0.005$	28.1 ± 8.4 $n = 6$	12.2 ± 11.7 $n = 5$	17.6 ± 14.6 $n = 6$
B	N.S.	N.S.	84.5 ± 53.1 $n = 11$ $p < 0.05$	$p < 0.001$	$p < 0.05$	N.S.
C			109.9 ± 57.3 $n = 10$			
D	288.9 ± 240.6 $n = 2$	118.0 ± 90.7 $n = 2$		53.5 ± 26.5 $n = 6$	55.5 ± 28.3 $n = 4$	36.9 ± 25.3 $n = 4$
			ADRENALINE			
A	9.3 ± 3.2 $n = 4$	10.7 ± 6.9 $n = 4$	10.0 ± 5.1 $n = 8$ N.S.	8.8 ± 5.4 $n = 6$	17.5 ± 30.3 $n = 5$	5.8 ± 4.2 $n = 6$
B	N.S.	N.S.	97.0 ± 170.0 $n = 11$ $p < 0.01$	$p < 0.01$ N.S.	N.S.	N.S.

C			213.2 ± 168.7 $n = 10$				
D	586.7 ± 350.1 $n = 2$	241.8 ± 129.8 $n = 2$			7.8 ± 3.8 $n = 6$	7.4 ± 6.4 $n = 4$	10.4 ± 6.1 $n = 4$
				DOPAMINE			
A	31.6 ± 19.3 $n = 6$	26.1 ± 9.3 $n = 6$	28.9 ± 14.7 $n = 12$ $p < 0.01$		33.2 ± 20.8 $n = 6$	12.9 ± 17.0 $n = 5$	18.6 ± 19.5 $n = 6$
B	N.S.	N.S.	53.7 ± 30.1 $n = 11$ $p < 0.05$	N.S.	N.S.	N.S.	N.S.
C			30.9 ± 16.79 $n = 10$				
D	39.8 ± 54.0 $n = 3$	27.3 ± 35.4 $n = 2$			53.8 ± 46.8 $n = 6$	25.5 ± 45.0 $n = 4$	32.5 ± 29.5 $n = 4$

[a]A = average basic value in +20°C.
B = average basic value of the first 6-h exposure in −20°C.
C = average value of the exposure from 6 h to death in −20°C.
D = value in the bladder after the whole procedure.
The experimental groups (1-5) are explained in Table 1.
The combined results from the two equal exposure groups are presented in Column 1 + 2.

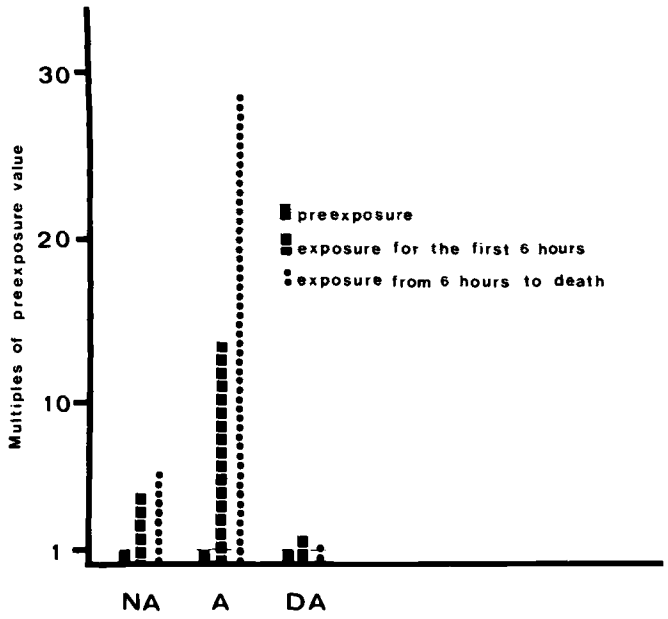

FIG. 2—*Effect of cold exposure (−20°C) on guinea pig urine NA, A, and DA concentrations. The preexposure value is taken as 1 and the other values expressed as multiples of this. Combined results for Groups 1 and 2.*

Discussion

Vitreous noradrenaline and adrenaline concentrations were elevated in both of the hypothermia groups, the values being highest in Group 2 where hypothermia was followed by autolysis. Autolysis alone also caused high concentrations of NA, probably the effect of diffusion of NA from the nerve endings in the retina over the intervening 6 h. This effect was not seen with the A concentration, which suggests that the final A content of the vitreous fluid is mainly derived from the blood, but a conclusion such as that cannot be made for the NA content. Thus an A assay could better be used in cases which have been left in a warm atmosphere after hypothermia death. Such case should be interpreted with care, however, since there was considerable individual variation within the group. Postmortem freezing and thawing without autolysis did not have such a marked effect. This observation further emphasizes the care needed in interpreting the results of postmortem biochemical assays when autolysis has taken place, even in a fairly resistant organ such as the eye and when the autolysis period has been as short as 3 h [*8*].

Elevated glucose values in vitreous fluid have also been observed in hypothermia deaths, averaging 82 versus 37 mg/100 mL in other deaths, but the individual variation was great [*3*]. In an experimental setting, chilling and freezing of the eyes of sheep prevented the fall of glucose content and the rise in lactic acid content of the vitreous fluid, but storage at room temperature for 6 h reduced these values [*9*].

NA and A excretion into the urine began to increase during the first hours of exposure and the concentrations at death were many times greater than the base values. The excretion of A rose more rapidly, an observation which agrees with that made in cardiac operations using slight hypothermia, a body temperature of 34 to 35°C [*10*]. The fact that autolysis did not increase the concentration significantly speaks in favor of using the urine sample as the primary evidence in hypothermia deaths. The CA findings in the urine support our earlier ob-

servation in cases of accidental hypothermia, where combined A and NA was more than 100 ng/mL. According to the present experiments, it would take 4 to 8 h to reach that concentration, which gives some basis for estimating the duration of exposure.

Dopamine (DA) values in the vitreous fluid tended to be low in the hypothermia death group (1), but variation was great in those animals which had been frozen and thawed after death (Groups 1, 2, and 3). Autolysis alone seemed to have a small elevating effect, but together with cold exposure and freezing in Group 2 it caused the highest concentration.

The DA concentration in the urine rose during the first 6 h of exposure, but decreased later. DA may well be used more completely for the synthesis of A and NA during the later stages of exposure. The final concentration was nevertheless higher than the base value in four of the groups.

The exact effect of autolysis on urinary CAs is difficult to determine, since it was impossible to get good samples in all cases. It is unlikely, however that the results of the A and NA measurements could have been disturbed by postmortem diffusion through the bladder wall, since the average concentrations were 41 and 10 times higher than the base value, respectively.

In conclusion, biochemical data such as elevated concentrations of CAs and glucose in the vitreous fluid can be useful for the diagnosis of hypothermia deaths if morphological signs are limited or lacking. Measurements of CA concentrations in the urine are more recommendable and A and NA should be measured separately, since the data concerning A could be related to hypothermia with greater certainty. Freezing and rapid thawing alone did not greatly affect the NA values in the vitreous fluid, but the following diffusion as a result of autolysis rendered the results unreliable.

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