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Lowered Cold Tolerance in Cold-acclimated and Non-acclimated Guinea Pigs Treated with Diazepam

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Summary. The effects of the clinically most commonly used minor tranquilizer, diazepam, on the survival time and on the mechanism of death in non-acclimated and cold-acclimated guinea pigs in severe cold exposure (−20°C) were studied.

Cold acclimation for 2 months increased the average survival time from 4 h to 10 h. The lowest rectal temperature at death (14.6° C) was seen in the cold-acclimated animals. Diazepam at a dose of 5 or 15 mg/kg i.p. 30 min before the beginning of the exposure reduced dose-dependently the cold endurance of both cold-acclimated and non-acclimated guinea pigs.

The serum glucose and free fatty acid concentrations were low in the animals with the long survival time.

Histological studies of liver, kidney, and adrenal glands showed no specific changes. Exposure seemed to increase the frequency of contraction bands and to decrease focally the intensity of beta-hydroxybutyrate dehydrogenase reaction in the myocardium, which indicates a mild hypoxic lesion of the muscle cells.

Key words: Cold tolerance, diazepam - Hypothermia death - Diazepam, cold tolerance

Zusammenfassung. Die Wirkungen von Diazepam, einer der am meisten gebrauchten Tranquillantien, auf den Todesmechanismus in schwerer Kälte (−20°C), wurden an kältegewohnten und nicht gewohnten Meerschweinchen untersucht. Eine Kältegewöhnung von 2 Monaten erhöhte die durchschnittliche Überlebenszeit von 4 h bis 10 h. Die niedrigste rektale Temperatur (14.6°C) zum Zeitpunkt des Todes wurde bei den kältegewohnten Tieren gemessen.

Eine Diazepam-Dosis von 5 oder 15 mg/kg i.p. 30 min von dem Beginn der Kälteexponierung verringerte dosisabhängig die Kältetoleranz an sowohl den kältegewohnten als auch den nicht kältegewohnten Meerschweinchen. Die Konzentrationen der Glucose und der freien Fettsäuren im Serum waren

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niedrig bei Tieren mit langer Überlebenszeit. Histologische Untersuchungen von Leber, Nieren und Nebennieren zeigten keine spezifischen Veränderungen. Die Kälteexponierung schien die Frequenz der Kontraktionsbänder im Cytoplasma der Herzmuskelzellen zu erhöhen und lokal die Intensität der Reaktion der Beta-Hydroxybutyrat-Dehydrogenase im Myokard zu verringern, was auf eine leichte Hypoxie der Muskelzellen hinweist.

Schlüsselwörter: Kältetoleranz, Diazepam - Diazepam, Wirkung bei großer Kälte

Several drugs are known to affect thermoregulation, their effect on body temperature often depending on the ambient temperature. Psychotropic drugs, such as phenothiazines, incapacitate thermoregulation probably through their effects on the thermoregulatory center in hypothalamus. We have also found that reserpine pretreatment increases markedly the susceptibility of guinea pigs to cold stress, while propranolol sensitizes the animals only slightly (Hirvonen et al. 1976). Benzodiazepine are widely used drugs against anxiety and muscle rigidity and their toxicity is low. However, they have been found alone and/or with alcohol in accidental hypothermia deaths as a contributory factor (Irvine 1966; Wedin et al. 1979). These observations imply that benzodiazepines also could affect thermoregulation in man, as they apparently do in squirrel monkey, especially in the older ones (Clark and Lipton 1981).

Since for both theoretical interest and improvement of forensic pathology diagnostic a study of the effect of benzodiazepines on thermoregulation in cold exposure seemed worthwhile we decided to investigate the survival time as well as some metabolic parameters and the histological pictures of various organs after severe cold exposure of guinea pigs pretreated with diazepam, the most widely used benzodiazepine.

Material and Methods

Two groups of adult Dunkin-Hartley guinea pigs of both sexes (about 600 g b.wt.) were used. One group of animals (non-acclimated controls) was kept at room temperature (+ 20°C–+ 21° C) in a normal laboratory colony, and the other group (cold-acclimated) was kept at + 4° C for 2 months in individual plastic cages bedded with cutter chips. All animals received pelleted food (Hankkija Ltd., Helsinki, Finland), vegetables, and water ad libitum.

The animals of both groups were divided into three subgroups. Controls received saline by i.p. injection, the other two groups received diazepam (Diapam inject, Orion Ltd., Helsinki), by the same route, either 5 mg/kg or 15 mg/kg. Thirty minutes after the injections half of the subgroup animals were exposed to severe cold (– 20°C) and kept there until death, the other animals were kept at room temperature and killed by a blow on the head.

Rectal Temperature

The rectal temperature of the animals in the cold chamber (– 20°C) was monitored with an electrical thermometer (YSI Model 42 sc, Tele-Thermometer, Honeywell). Each animal was kept in its cage until death, which was established by observing cardiac arrest with an ECG-monitor (Olli 201, Ollituote Oy, Helsinki). The terminal temperature was also recorded.

Free Fatty Acids (FFA)

FFA in the serum were measured colorimetrically according to the method of Laurell and Tibbling (1967).

Glucose

Glucose in the serum was measured with the colorimetric method of Hyvärinen and Nikkilä (1972).

Histological Studies

Formaline-fixed and fresh-frozen tissue samples were studied histologically. Samples of heart, liver, kidney, and adrenal gland were fixed in neutral 4% formaldehyde solution for 1 week and processed automatically into paraffin blocks and cut at 10 μm. The following stainings were applied: hematoxylin-eosin (HE) on all tissues and acid fuchsin, Heidenhein's iron hematoxylin (HIH), and phosphotungstic acid hematoxylin (PTAH) on heart muscle for demonstration of dyscoloration in the early lesions. The methods are principally the same as those described by Pearse (1970).

The fresh-frozen samples were taken from heart, liver, kidney, and adrenal gland. Sections were cut in a cryostat at 10 μm and stained with Oil Red O for lipids; in the heart beta-hydroxybutyrate dehydrogenase and cytochrome oxidase enzymes were also demonstrated (Barka and Anderson 1965). Special attention was paid to the myocardium to find out early lesions, such as dyscoloration, fragmentation, and contraction bands in the myofibers, and decrease of enzyme reactions. Accumulation of lipids in the liver and kidney and depletion of lipids in the adrenal glands were registered.

Statistics

Student's *t*-test was used for statistical analysis.

Results

The cold-acclimated guinea pigs *endured severe cold* longer than their corresponding controls (Table 1). The cold-acclimated animals without diazepam treatment survived longest, and they were also able to maintain their *rectal temperature* constant for about 3 h. Even after that the lowering of the temperature was slower than in the other groups (Fig. 1), and their rectal temperature at death was lower than that of the non-acclimated controls (Table 1). Diazepam at a dose of 15 mg/kg shortened clearly the survival time in both non-acclimated and cold-

Table 1. Survival time at −20°C and rectal temperatures at death of guinea pigs treated with diazepam, means ± SD (*n*). Student's *t*-test

Treatment	Non-acclimated			Cold-acclimated		
	(*n*)	Survival time (min)	*T* rectal (°C)	(*n*)	Survival time (min)	*T* rectal (°C)
Saline (controls)	(8)	269 ± 98	17.8 ± 1.4	(7)	610 ± 295[b]	14.6 ± 3.2
Diazepam 5 mg/kg	(11)	190 ± 77	16.8 ± 1.7	(10)	347 ± 204[a] *	16.2 ± 2.8
Diazepam 15 mg/kg	(9)	140 ± 42 **	17.3 ± 1.4	(10)	202 ± 74[a] **	17.2 ± 1.3

[a] $P < 0.05$ and [b] $P < 0.01$ as compared to the corresponding non-acclimated group
* = $P < 0.05$ and ** = $P < 0.01$ as compared to the corresponding saline group

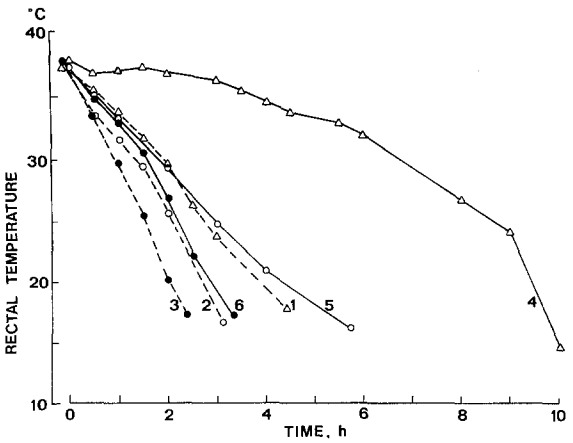

Fig. 1. The fall of the rectal temperature and survival time of diazepam-pretreated guinea pigs at −20°C. The statistical significance is given in Table 1. The symbols for the curves: *1* = non-acclimated controls; *2* = non-acclimated, treated with 5 mg/kg diazepam; *3* = non-acclimated, treated with 15 mg/kg diazepam; *4* = cold-acclimated controls; *5* = cold-acclimated, treated with 5 mg/kg diazepam; *6* = cold-acclimated, treated with 15 mg/kg diazepam

acclimated animals, whereas a dose of 5 mg/kg reduced the cold tolerance significantly only in the cold-acclimated animals.

Serum FFA Concentrations (Table 2)

Serum FFA concentrations were significantly increased in the non-acclimated groups exposed to frost, and they were also higher, but not significantly, in the cold-acclimated animals after frost. There were no significant differences in the FFA levels between the cold-acclimated and non-acclimated animals either at room temperature or in the cold. Neither diazepam dosis as such altered the FFA values.

Serum Glucose Concentrations (Table 3)

Serum glucose concentrations were markedly lowered after exposure to −20°C as compared to the non-exposed animals. In the exposed groups, the concentrations were generally the lower the longer were their survival times in the frost.

Histological Findings (Table 4)

In the *hearts* dyscoloration (Fig. 2) was seen in all groups at the frequency of about 2/3, but animals that had received the higher dosis of diazepam and were exposed to frost only showed dyscoloration in about half of the preparations. *Contraction bands* (Fig. 3) were also seen in all groups, but less in non-exposed animals. *Fragmentation* of the cardiac muscle cells was less in the exposed animals treated with the higher doses of diazepam. Mild, spotty decrease of the cardiac enzyme *beta-hydroxybutyrate dehydrogenase* reaction was observed in most animals with the exception of diazepam-treated non-exposed groups. *Cytochrome oxidase* reaction was less sensitive and remained unchanged in most samples (not shown in Table 4).

Table 2. Concentrations of free fatty acids in serum (mmol/l) at death in guinea pigs treated with diazepam, means ± SD (*n*). Student's *t*-test

Treatment	Non-acclimated				Cold-acclimated			
	(*n*)	at +20°C	(*n*)	at −20°C	(*n*)	at +20°C	(*n*)	at −20°C
Saline (controls)	(5)	0.39 ± 0.21	(8)	1.02 ± 0.44 *	(5)	0.56 ± 0.11	(5)	1.04 ± 0.79
Diazepam 5 mg/kg	(5)	0.69 ± 0.10	(9)	0.95 ± 0.18 *	(7)	0.69 ± 0.21	(10)	1.00 ± 0.49
Diazepam 15 mg/kg	(4)	0.53 ± 0.11	(9)	1.15 ± 0.24 ***	(5)	0.71 ± 0.35	(10)	1.01 ± 0.45

* = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ as compared to the corresponding group at +20°C

Table 3. Concentrations of glucose in serum (mmol/l) at death in guinea pigs treated with diazepam, means ± SD (*n*). Student's *t*-test

Treatment	Non-acclimated				Cold-acclimated			
	(*n*)	at +20°C	at −20°C	(*n*)	(*n*)	at +20°C	at −20°C	(*n*)
Saline (controls)	(5)	10.3 ± 2.8 ← *** →	2.4 ± 1.4[a]	(7)	(5)	10.7 ± 1.4 ← *** →	1.1 ± 0.3 ↓	(6)
Diazepam 5 mg/kg	(5)	12.4 ± 3.1 ← *** →	3.3 ± 1.3[b]	(10)	(4)	10.1 ± 1.1 ← *** →	1.9 ± 0.8 ↓*	(9)
Diazepam 15 mg/kg	(5)	10.2 ± 2.6 ← *** →	3.7 ± 1.3	(9)	(5)	11.9 ± 2.0 ← *** →	3.8 ± 1.6 ↓ ** ↓ **	(10)

[a] $P < 0.05$; [b] $P < 0.01$ as compared to the corresponding cold-acclimated group

Table 4. Histological changes in *myocardium* of guinea pigs treated with diazepam. The figures are means of the changes observed with various stainings from the total numbers of hearts studied

Treatment	Non-acclimated								Cold-acclimated							
	at +20°C				at −20°C				at +20°C				at −20°C			
	D	C	F	E	D	C	F	E	D	C	F	E	D	C	F	E
Saline (controls)	3/5	1/5	4/5	5/5	8/8	8/8	6/8	8/8	4/5	1/5	3/5	5/5	6/8	6/8	6/8	6/8
Diazepam 5 mg/kg	4/5	1/5	3/5	4/5	8/11	11/11	8/11	11/11	3/5	3/5	2/5	0/5	7/9	7/9	6/9	6/9
Diazepam 15 mg/kg	5/5	5/5	3/5	2/5	5/10	6/10	4/10	9/10	4/5	3/5	4/5	1/5	5/10	9/10	2/10	9/10

D, dyscoloration; C, contraction bands; F, fragmentation; E, enzyme (HBD) decline

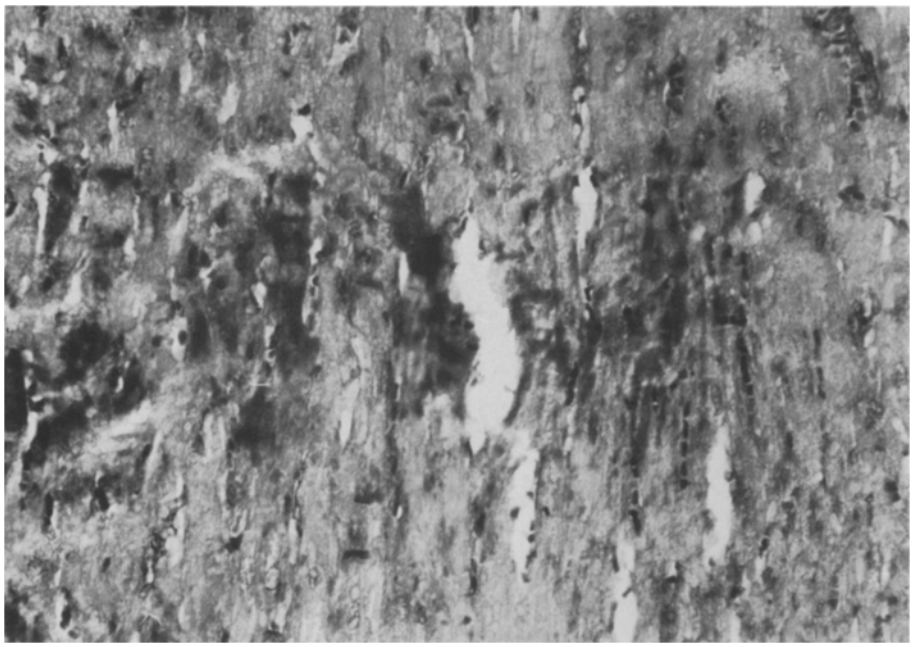

Fig. 2. Myocardium from a non-acclimated guinea-pig which had received 5 mg diazepam and died in the cold. Dyscoloration of the myofibers and edema. Acid fuchsin, ×115

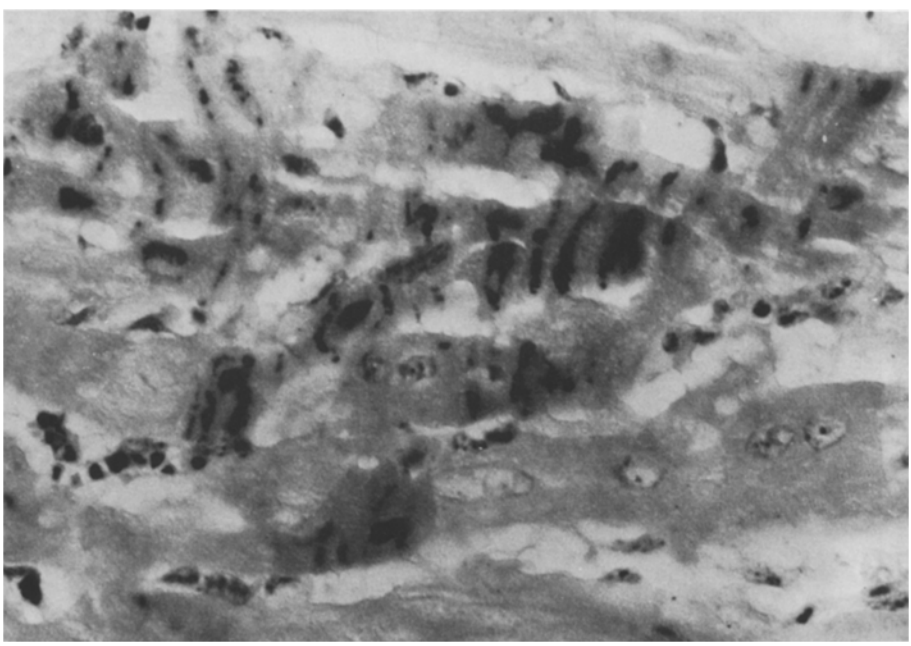

Fig. 3. Myocardium from another non-acclimated guinea pig which had received 5 mg diazepam and died in the cold. Interfiber edema and contraction bands in the muscle cells. HIH, ×290

In the *liver* and in the *kidneys hyperemia* was found in animals exposed to frost, but not in non-exposed animals. Diazepam did not modify these findings. *Neutral lipids* were present in all livers and in most kidneys, and no differences could be observed between various groups. No tubular degeneration was found in the kidneys.

In the *adrenal glands local lipid depletion* occurred frequently, but it could not be interpreted as a regular phenomenon of cold stress.

Discussion

The present results confirm our earlier findings (Hirvonen et al. 1976) that cold-acclimation increases the survival time of guinea pigs in severe cold exposure. In the present experiment, it was about doubled in the acclimated animals up to even 10 h. The average rectal temperature at death in the acclimated animals that lived longer was lower than in the non-acclimated ones, which is apparently a feature of improved resistance to hypothermia.

Diazepam pretreatment shortened moderately the survival time in both non-acclimated and cold-acclimated guinea pigs dose-dependently. The rectal temperatures at death in cold-acclimated groups correlated inversely with the survival times, but the differences were not as marked as the differences in the survival times. In non-acclimated animals no such correlation was observed.

On the basis of the present results we conclude that diazepam reduces the endurance of both non-acclimated and cold-acclimated guinea pigs to severe cold exposure. Its effect is probably of central origin and due to disturbed temperature regulation. Diazepam receptors are also found in hypothalamus, and the drug is known to reduce catecholamine excretion from adrenal medulla and other sympathetic nervous functions (Haefely 1980). These observations agree with the findings in man (Hirvonen 1979) that central nervous depressants are contributory factors in accidental death caused by exposure to cold.

Phenothiazines are potent drugs, which can abolish the thermoregulatory function which makes animals poikilothermic (Borbely and Loepfe-Hinkkanen 1979). Both hypothermic and hyperthermic responses have been seen, depending on the ambient temperature. Even therapeutic doses can be a risk factor especially with elderly persons. Tricyclic antidepressants also affect body temperature, which is proportional to the phenothiazine-like or sedative activity of the compounds. Against this background the benzodiazepine effect on thermoregulation is understandable.

In our experiments the guinea pigs were not very sensitive to diazepam, but this species is also more tolerant to phenothiazines than, e.g., mouse (Borbely and Loepfe-Hinkkanen 1979). In man diazepam does not cause hypothermia alone, but with lithium a synergistic hypothermic effect has been reported in one patient (Naylor and McHarg 1977).

We have found earlier that an adrenergic beta-receptor blocking agent, propranolol, moderately decreases body temperature in guinea pigs exposed to frost (−20°C) (Hirvonen et al. 1976), as did diazepam in the present experiments. Whether there is a common mechanism, e.g. via inhibition of the sympathico-adrenal system by drugs, remains to be elucidated.

If the shivering response to cold is attenuated, tolerance to cold, especially in non-acclimated animals, is diminished. Diazepam is a potent muscle relaxant, and thus its mode of action in cold exposure could also, at least partially, be the prevention of shivering. Microscopic changes in the tissues were scarce in spite of the many hours of hypothermia. Beta-hydroxybutyrate dehydrogenase reaction revealed mild signs of hypoxia in the myocardium. The decrease of the intensity of this enzyme reaction was, however, only one grade, which indicates a reversible lesion. Proof of a hypoxic lesion is also the release of creatine phosphokinase into blood, which has been found to occur in guinea pigs during hypothermia (Hirvonen et al. 1980). Other mild morphological changes, such as fragmentation and contraction bands in the myocardium, were slightly more frequent in the exposed animals than in the controls. Other organs were only hyperemic which supports the old observation of the mild effect of low temperature on various tissues.

In conclusion, our results agree with the earlier findings that centrally acting drugs, even those with weak central depressing effects and otherwise low toxicity, such as diazepam, reduce in moderate doses the survival time of animals in severe cold. The results thus partly explain the occasional finding of benzodiazepines in accidental hypothermia deaths.

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