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Toxicity of cryoprotective agents at 30°

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The toxicity of four cryoprotective agents (glycerol, dimethylsulfoxide, dextran, magnesium ion) as a function of time was assessed at 30° on isolated rabbit atria. Glycerol, 2·0 M, in Ringer solution killed all atria in less than 30 min. Atria tolerated 2·1 M dimethylsulphoxide in Ringer for 1 hr, 0·7 M glycerol in Ringer for 4 hr, 6% (w/v) dextran in saline for 6 hr and $6{\cdot}6 \times 10^{-2}$ M magnesium chloride in Ringer for 8 hr. After washing out the cryoprotective agents the tissue was examined histologically and for its response to isoprenaline, MJ-1999 [4,2-(isopropylamino-10-hydroxyethyl)methanesulphonilide, a β-blocking agent] and isoprenaline and ouabain. These tests were the more sensitive indicators of functional integrity.

PREVIOUSLY Karow & Webb (1965), Karow, Webb & Stapp (1965) have shown the relation of concentration, time and temperature during hypothermia and freezing with known cryoprotectants (glycerol, dimethylsulfoxide, dextran and magnesium ions). The toxicity of these agents at concentrations normally used for cryoprotection as a function of time under normal laboratory conditions is now reported.

Experimental

Rabbits weighing approximately 2·0 kg were stunned by a blow on the head, quickly bled and their hearts removed. The hearts were immediately placed in oxygenated (95% O_2, 5% CO_2) Ringer solution (composition: mM NaCl 153·9, KCl 5·4, $CaCl_3$ 2·4, $NaHCO_3$ 16·8, dextrose 11, distilled water to 1 litre) and the left atria removed. The atria were carefully trimmed of peripheral tissue, tied to a Plexiglass holder containing two platinum electrodes and immersed in oxygenated Ringer solution maintained at 30° in an isolated muscle bath. One g tension was placed on each atrium and they were stimulated continuously throughout the entire experiment with a square wave of 5 msec duration (120 Hz, 5V). Contractions were measured with a (Grass) strain gauge and recorded on a (Sanborn) polygraph.

After allowing the muscle to equilibrate in the tissue bath for 1 hr, the Ringer solution was drained from the bath and a solution of one of the test agents was substituted. The agents tested were 0·7 M and 2·0 M glycerol, 2·1 M dimethylsulphoxide and $6{\cdot}6 \times 10^{-2}$ M $MgCl_2{\cdot}6H_2O$ made up in Ringer solution. Also tested was commercially prepared 6% (w/v) dextran (average *M* 70,000) in saline. The atria were incubated with a given agent for 0·5, 1, 2, 3, 6, 8, 10, 18, 20 hr) and then washed three times in Ringer solution. After incubating again in the Ringer solution for 1 hr following the washout of the test compounds, the responses of the tissue to three drugs were observed. The three drugs were: isoprenaline (10^{-6} M), MJ-1999 [4,2-(isopropylamino-10-hydroxyethyl)methane sulphonilide; a beta blocking agent] (10^{-3} M) and ouabain (10^{-5} M).

The procedure used was a follows. Atria were subjected to the isoprenaline for 20 min, after which they were washed three times. They were

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kept in drug-free Ringer solution for 20 min and then subjected to the MJ-1999 for 20 min. After this, isoprenaline was added to the bath for a further 20 min period and they were again washed three times and incubated for 20 min in Ringer solution. Finally, they were subjected to ouabain for 20 min. The atria were then removed and placed in formalin to await histological study. In tabulating the results, the criterion used for physiological normality was resumption of rhythmic contractions in response to the electrical stimulus during the 1 hr washout period following the administration of the cryoprotectant. Pharmacological normality was assumed when the inotropic response to isoprenaline was at least a 50% increase in amplitude of contraction over the control amplitude for those particular atria, a reduction in this response to less than 5% after treatment with MJ-1999, and a positive inotropic response to ouabain of at least 50%.

HISTOLOGICAL METHODS

These have been described in detail previously (Clower & Williams, 1966) and follow the techniques as presented by Lillie (1965). Complete cross sections were made through the atria in a frontal or longitudinal plane, or both.

Organs were fixed in 10% aqueous formaldehyde (U.S.P.) or in Lovdowsky's (formaldehyde, water, ethanol and acetic acid) solution. The latter was used in most instances at $-6°$ to $-12°$ to attain better fixation of glycogen. The periodic acid-Schiff's reagent method was used to show fibrosis, glycogen and recent myocardial necrosis. Von Kossa and alizarin techniques were used to demonstrate calcium, if present. These tissues were also stained with haematoxylin and eosin.

Results

The results are summarized in Table 1 and Fig. 1. The most toxic agent at 30° was 2·0 M glycerol and the least toxic was the magnesium ion. The pharmacological test was more sensitive in defining tissue functional integrity than the physiological test. Of the three drugs used, ouabain appeared to be the most sensitive to changes in the functional integrity of the atrial tissue. Although in many instances, the muscle responded normally to isoprenaline, no positive inotropic response could be elicited by ouabain. In fact, in some instances in which the criteria of physiological responses were not met, i.e., muscle did not exhibit sustained rhythmic contractions in response to electrical stimulation during cryoprotective washout, the administration of isoprenaline stimulated the muscle to contract. In such instances, the response to ouabain was abnormal. The most obvious effect of treating heart muscle with a cryoprotective agent was the cessation of cardiac activity. This effect was demonstrated for each cryoprotective agent, but was much less rapid with 0·7 M glycerol than for any of the other agents used (Fig. 2). This shows a noticeable difference in the contractile response of the atria to the various agents. The pattern for a given cryoprotectant was reproducible and typical for that agent. Glycerol (0·7 M), dimethylsulphoxide,

TABLE 1. TOXICITY OF CRYOPROTECTANTS AT 30° ON STIMULATED RABBIT ATRIA

Cryoprotective agent	Time (hr)	Tissues which contracted after treatment	Tissues with normal pharmacological responses
Ringer	2	5/5	5/5
	20	8/8	8/8
Glycerol (0·7 M)	1	4/4	4/4
	2	2/2	2/2
	4	6/6	6/6
	6	7/10	4/10
	8	10/12	6/12
	10	6/10	3/10
	12	3/10	0/10
Glycerol (2·0 M)	0·5	0/10	0/10
Dimethylsulphoxide (2·1 M)	0·5	11/11	11/11
	1	9/9	6/9
	2	3/5	2/5
	3	2/8	0/8
Dextran (6% w/v)	1	9/9	9/9
	6	12/12	11/12
	8	2/9	1/9
$MgCl_2 \cdot 6H_2O$ ($6{\cdot}6 \times 10^{-2}$ M)	1	9/9	9/9
	6	4/4	4/4
	8	8/8	4/8
	12	4/12	2/12
	16	0/16	0/10

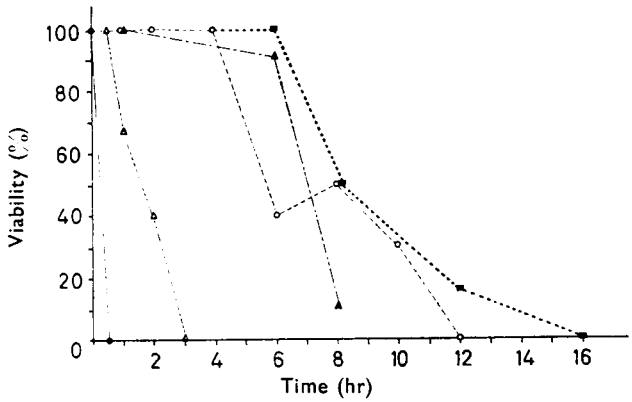

FIG. 1. Comparative toxicity of cryoprotective agents at 30° on electrically stimulated rabbit atria. Ordinate represents percent of atria viable according to pharmacological criteria. ○, 0·7 M glycerol. ●, 2·0 M glycerol. △, 2·1 M dimethylsulphoxide. ▲, 6% w/v dextran. ■, $6{\cdot}6 \times 10^{-2}$ M $MgCl_2$.

magnesium and dextran all resulted in a curvilinear decrease in contraction. However, the rate of change was obviously more rapid with dimethylsulphoxide or magnesium than with either 0·7 M glycerol or dextran. Glycerol (2·0 M) caused a response that was entirely different to that produced by the other agents. With 0·7 M glycerol, atria contracted for as long as 12 hr, but with diminished contractile force. Contractions ceased within 90 min when atria were tested with dimethylsulphoxide, dextran or magnesium.

At the onset of the washout of the cryoprotectant, a difference in the behaviour of the glycerol-treated tissue was observed. Atria treated with either 0·7 or 2·0 M glycerol exhibited at this point a marked contracture which was transient with atria treated with the lower concentration, but

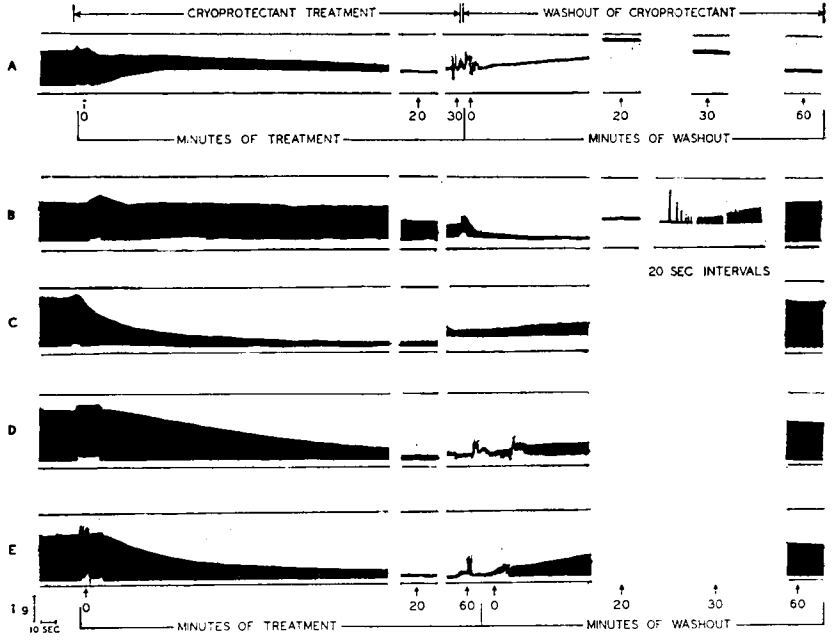

FIG. 2. Recordings illustrating effects of cryoprotective agents on rabbit atria at 30°. Panel A is typical recording obtained with 2·0 M glycerol; Panel B, 0·7 M glycerol; Panel C, 2·1 M dimethylsulphoxide; Panel D, 6% (w/v) dextran; and Panel E, $6{\cdot}6 \times 10^{-2}$M $MgCl_2$.

sustained with the higher concentration. Atria treated with 2·0 M glycerol showed no contractile activity at the end of the washout period, whereas, atria treated with 0·7 M glycerol frequently resumed contraction in the last 30 min of the washout. Tissues treated with magnesium, dextran or dimethylsulphoxide contracted within 5 min after the onset of washout.

HISTOLOGICAL RESULTS

Most atria showed no evidence of damage that would be detrimental to cardiac function. There was no necrosis, calcification, inflammation or oedema. The atrial cardiac muscle contained a rich supply or source of glycogen which implied that the muscle was healthy. However, in several instances, there was fatty infiltration of the cardiac muscle of atria from all groups, but not to the extent which would cause damage or possible abnormal cardiac (atrial) function. Also, there were areas of fibrosis within the atrial walls; these probably were spontaneous (old) changes. Three atria (one control, one from 2·0 M glycerol group and one from dimethylsulphoxide group), had severe necrosis and inflammation of the muscle. These inflammatory changes must have been pre-experimental since no source of inflammatory cells can be maintained after death. The necrotic changes probably were pre-experimental, but could have been due to the experimental procedure.

Discussion

In the present experiments, magnesium was the least toxic agent. Twenty min at −20° appears to be the maximum time heart tissue can be frozen and still survive (Smith, 1957; Karow, Webb & Stapp, 1965). Since dimethylsulphoxide and 2·0 M glycerol produced their toxic effect within the initial 20 min period of exposure, this imposes limitations on their use for tissue freezing. The other agents might also be toxic within this time period at freezing temperatures due to an increased sensitivity of some tissue constituent(s) (Almond, Anido & others 1966).

The results serve to emphasize the need for careful selection of cryoprotective agents. For many tissues, 2·0 M glycerol is an excellent cryoprotectant, but for heart muscle this concentration of glycerol is toxic (Levy, Richards & Persidsky, 1962; Karow & Webb, 1965). On the other hand, 0·7 M glycerol is relatively innocuous, but its cryoprotective ability for cardiac muscle has not been demonstrated. The pharmacological evaluation detected abnormal tissue responses even before they were detected physiologically. This suggests that the toxic manifestations of the cryoprotective agents are due to alteration of biomolecules, such as drug receptors.

The rapid loss of contractile ability of cardiac tissue treated with the cryoprotective agents at 30° is comparable to the reduction in contractile ability of intestinal and uterine smooth muscle subjected at normothermia to concentrations greater than 1·0% of dimethylsulphoxide, glycerol, methyl formamide, methyl acetamide or dimethyl acetamide. As reported by Farrant (1964) each of these cryoprotectants reduced the responses of these smooth muscle preparations to standard test doses of acetylcholine, histamine or nicotine.

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