CRYOBIOLOGY 31, 57–62 (1994)

Applications for Cryopreserved Blood Vessels in Pharmacological Research

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The development of drugs in cardiovascular research involves *in vitro* studies that are generally performed on freshly isolated blood vessels. Naturally, data obtained from human tissues would be the most accurate and predictable for human pharmacology. However, the main problems are that the supply of fresh human material is unpredictable and the tissue has a very short life span. Storage in physiologic salt solution at 4°C, the most common practice to preserve isolated blood vessels, induces rapid and progressive changes of physiologic and functional properties within a few days. Cryopreservation of isolated blood vessels at −70 to −196°C in a medium containing Me_2SO offers the prospect of virtually infinite storage of the material with the only damage being that associated with the freezing and thawing process. Despite certain changes, such as reductions in contractile forces and endothelial functions, the main biochemical properties, uptake mechanisms, and affinities of most agonists and antagonists have been shown to be well preserved after cryopreservation. Hence, this technique offers clear potential for ensuring the supply of vascular material for pharmacological studies.

The development of drugs in cardiovascular research involves *in vitro* studies that are generally performed on freshly isolated animal blood vessels. Naturally, data obtained from human tissue are the most accurate and predictable for human pharmacology. The problems with use of human vasculature are the irregularity of its supply and the quantity of material that can be utilized at one time as human vessels deteriorate rapidly after removal from the body. There is a need to define and develop reliable storage methods that will ensure a consistent supply of human vascular preparations for pharmacological studies. There are two possible ways to preserve vascular tissues for pharmacological research, namely (i) cold storage and (ii) cryopreservation.

COLD STORAGE OF VASCULAR TISSUE

Cold storage in physiologic salt solution at 2–6°C is the most common practice used to preserve isolated tissues for pharmacological studies. However, cold storage of isolate blood vessels induces several species- and time-dependent changes in their pharmacological characteristics. The most consistent cold storage-induced alteration is a progressive degeneration of nerve endings resulting in attenuated responses to transmural electrical stimulation (14, 15, 41) and/or in reduced neuronal norepinephrine (NE) content and [^{3}H]NE uptake (11, 13, 36, 42). Furthermore, prolonged cold storage leads to progressive morphological and functional deteriorations in endothelial cells (41). The contractile responsiveness to receptor-mediated stimuli of various blood vessels reflects both species differences and time dependency of the changes occurring during prolonged cold storage. Responses to stimulation of α-adrenoceptors and 5-HT receptors of peripheral canine arteries increase after storage for 24 h at 6°C (29), but these responses appear unchanged when stored for 3–7 days at 4°C (12, 37, 38). Similar findings have been reported for monkey peripheral arteries and bovine cor-

Received and accepted September 17, 1993.

0011-2240/94 $6.00

onary arteries after storage for 5–6 days at 4°C (14, 39). In contrast, the sensitivity to α-adrenoceptor stimulation of rabbit aortae has been shown to increase following storage for up to 7 days at 2–6°C but to decrease when stored for 10–14 days (3, 35, 36, 42). Contrary to the results of studies with receptor-mediated contractile stimuli, cold storage consistently attenuates vascular contractile responses to nonreceptor-mediated potassium chloride (35, 37–39, 42). Although there is a supersensitivity to NE in cold-stored rabbit aortae these vessels are less sensitive to calcium-induced contraction; i.e., they require a higher extracellular calcium concentration to develop the same tension as fresh aortae (3). Several observations support the contention that cold storage of vascular smooth muscle induces cell membrane depolarization, which seems to be due to inhibition of the electrogenic Na^+ pump (3, 13, 29). Indeed, after storage for 4 days at 2°C the resting cell membrane potential of guinea pig coronary arteries increases from −60 to −47 mV (15), indicating depolarization of the cell membrane.

Cold storage is a simple method of storing isolated blood vessels for a limited time period. However, rapid degeneration of nerve terminals, morphological and functional alterations of endothelial cells, and progressive depolarization of the smooth muscle cell membrane occur within a few days.

CRYOPRESERVATION OF VASCULAR TISSUE

Freezing of vascular preparations without cryoprotectant(s) generally results in a complete loss of the contractile responsiveness. In comparative experiments with various agents known to possess cryoprotective activity, Me_2SO has been shown to be the most effective cryoprotectant for preservation of canine saphenous vein contractile functions (22, 23, 26, 27). Improvement of the post-thaw recovery of vascular function has been obtained when sucrose was added to Me_2SO-containing medium (25). Serum or other high-molecular-weight polymers are important for the preservation of function of many cell types, including preservation of endothelial cell function in canine and human coronary arteries (16, 17). However, other vessels such as pig pulmonary and circumflex coronary arteries do not require fetal calf serum (FCS) to maintain either smooth muscle contractility (Fig. 1) or endothelial cell function (Fig. 2). In addition to using the appropriate cryomedium, the optimal rate of cooling is important. The optimal rate of cooling for dif-

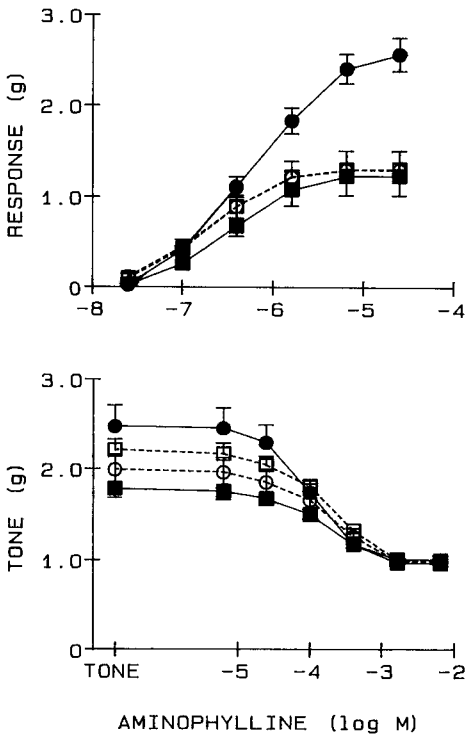

FIG. 1. Contractile responses to norepinephrine (top) and relaxant responses to aminophylline during stimulation with 10 n*M* U46619 (bottom) of rings from porcine pulmonary arteries without (●) and after cryopreservation at −196°C in fetal calf serum (FCS; ■), Krebs–Henseleit solution (KH; ○), and KH with 50% FCS (□), containing 1.8 *M* Me_2SO and 0.1 *M* sucrose as cryoprotectants. The effects are expressed in g; tone indicates the papaverine-induced relaxation in addition to the existing passive preload. The bars represent means ± SEM; for each point n = 8–12. Method according to Ref. 6.

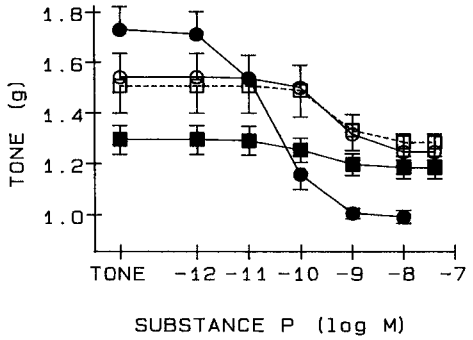

FIG. 2. Relaxant responses to substance P of rings from porcine circumflex coronary arteries stimulated with 3 μM $PGF_{2\alpha}$ without (●) and after cryopreservation at −196°C in fetal calf serum (FCS; ■), Krebs–Henseleit solution (KH; ○), and KH with 50% FCS (□), containing 1.8 *M* Me_2SO and 0.1 *M* sucrose as cryoprotectants. Changes in tone are expressed in g; the bars represent means ± SEM (for each point n = 14). Method according to Ref. 25.

ferent tissues may vary from 0.3 to 10°C per minute. Once a sample is cooled to about −70°C, it can be transferred into liquid nitrogen (−196°C) and stored there indefinitely until required for use.

After thawing, a stepwise dilution protocol is commonly used with isolated mammalian cells to avoid damage by osmotic shock. This procedure, however, does not improve the post-thaw recovery of canine saphenous veins and rat portal veins, which had been frozen in a cryomedium containing FCS and 1.8 *M* Me_2SO.

Therefore, during the freezing/thawing process, many factors such as cryoprotective additives, the rate of addition and removal of the cryoprotectant(s), and the rate of cooling and rewarming may influence the post-thaw recovery of cryopreserved tissues. Optimal combination of these factors for each tissue has to be derived by a process of trial and error (1, 31).

POST-THAW FUNCTIONAL RECOVERY OF ARTERIES

Responses to electric field stimulation of rabbit ear arteries that had been immersed in newborn calf serum containing 1.8 *M* Me_2SO and stored at −70°C were similar to the responses in fresh tissues, indicating that in these cryopreserved tissues the functional integrity of the adrenergic neurotransmission mechanism is unaltered following freezing (40). In arteries various hormones and neurotransmitters act via the release of endothelium-derived relaxing factor(s) (10). Endothelial cells contribute to the local control of vascular tone by releasing prostanoids and both endothelium-derived relaxing (EDRF) and contracting factors (9, 20). Well-maintained relaxant responses to acetylcholine of cryopreserved rabbit ear arteries (40) indicate preservation of endothelial function after cryopreservation. Furthermore, the endothelial cell function of porcine (25, 34; Fig. 2), canine (16) and human (17) coronary arteries can be successfully preserved when the arterial tissues are slowly frozen in a medium containing Me_2SO and stored at temperatures of −75 or −196°C.

Contractile responses to receptor-mediated stimuli are generally diminished in cryopreserved arteries but vary considerably from tissue to tissue and are dependent on the cryopreservation procedure employed. In animal experiments good preservation, i.e., a post-thaw contractile force development of about 60 to 80% of the maximum achieved in fresh tissue, has been obtained following cryopreservation of rabbit ear arteries (40), canine basilar (26) and coronary arteries (16), and porcine pulmonary and coronary arteries (25; Figs. 1 and 2). A reduction of the post-thaw contractility to less than 50% of fresh tissue indicates severe damage of the smooth muscle cell function in larger arterial blood vessels such as rat aorta (23) and canine saphenous arteries (25). The contractile responses of cryopreserved human arteries also show considerable variations with a maximum response of 20–30% in human coronary arteries (17), 40% in postmortem human basilar arteries (23), and 75% in human pulmonary arteries (6). The variation in these data ap-

pears to be dependent largely on the agonists, cryomedia, and the freezing/thawing procedures employed. Similar results have been found for the endothelium-independent relaxant responses to isoproterenol in human coronary arteries (17) and for various potassium channel activators in human pulmonary arteries (6). However, in most studies there is a significant correlation between drug potencies for various agonists and antagonists in fresh and cryopreserved arterial tissues (24), suggesting that the results of drug testing using cryopreserved arterial tissues would be representative of fresh arterial tissues.

POST-THAW FUNCTIONAL RECOVERY OF VEINS

Slow freezing of venous tissue in a Me_2SO-containing medium and storage at -196°C do not modify the activity of tissue transaminases (43), monoamine oxidase (26), the soluble guanylate cyclase system (8), or protein synthesis (2, 18). Furthermore, under these conditions the adrenergic nerve endings appear to be well preserved, as indicated by an unaltered absolute tritium overflow during sustained electrical stimulation from frozen/thawed canine saphenous vein strips following preincubation with [^{3}H]NE (27).

Although the endothelial cells of cryopreserved saphenous veins from both canine and human retain their basic morphologic structure and fibrinolytic activity (2, 7, 19, 21, 32), an increased production of prostanoids after thawing of cryopreserved canine saphenous veins has been reported (8). Human veins that had been rapidly frozen showed similar features and after thawing, both tissue enzymes and PGI_2 synthesis were found to be significantly enhanced (19, 30), suggesting that the process of cryopreservation has modified the metabolic characteristics of the venous endothelial cells.

As observed with arteries, the contractile responses of cryopreserved canine veins to receptor-mediated stimuli are generally diminished. Compared to fresh canine veins, the maximal contractile responses to epinephrine, norepinephrine, and tryptamine were 50–75%, whereas those to KCl, 5-HT, and α_2-adrenoceptor stimulation were only about 30–40% (2, 4, 5, 8, 25, 26, 43). In human peripheral veins optimal post-thaw functional recovery was obtained with tissues stored at -196°C using a Krebs–Henseleit solution containing 1.8 *M* Me_2SO and 0.1 *M* sucrose. These vessels showed maximal post-thaw contractile responses to norepinephrine and 5-HT of about 60–70% of fresh veins (33). Following cryopreservation, endothelium-independent relaxant responses in precontracted ($PGF_{2\alpha}$) canine saphenous veins to isoprenaline, ADP, and nitric oxide were maintained. The response to calcium ionophore A23187 was also intact; however, thrombin failed to relax cryopreserved veins. This further suggests that the process of freezing/thawing modified aspects of endothelial cell function (8). Nevertheless, the sensitivities of fresh and cryopreserved canine (5, 8, 26) and human (27, 28) veins for various relaxant agonists and antagonists were similar. Such results indicate that, in spite of some functional modifications, cryopreserved venous tissues are suitable for screening of drug responses.

CONCLUSION

Cryopreservation in media containing Me_2SO at -196°C offers the prospect of virtually indefinite storage of isolated blood vessels. Despite certain problems, such as reduced contractile forces and overall endothelial cell functions, the main biochemical properties, uptake mechanisms, and affinities for most agonists and antagonists have been shown to be well preserved after thawing. The results indicate that cryopreserved vascular tissues are suitable for pharmacological studies.

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