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Investigation into cryopreservation of guinea pig colon

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INVESTIGATION INTO CRYOPRESERVATION OF GUINEA PIG COLON

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Ryoko Hirakawa

May 2004

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ABSTRACT

INVESTIGATION INTO CRYOPRESERVATION OF GUINEA-PIG COLON

by Ryoko Hirakawa

Cryopreservation may effectively maintain the viability of colon tissue for pharmacological study. The objectives of this study were to optimize cryopreservation conditions for guinea-pig colon and to investigate the effect of cryopreservation on its functional activity. Freezing with ten combinations of cryomedium showed that a cryomedium of 2.25 M DMSO and 0.25 M glucose gave the optimal contractile response and survival rate. For colon cryopreserved in this cryomedium, the contractile responses to a membrane depolarizing agent, muscarinic receptor agonists, a 5-HT₃ receptor antagonist and 5-HT₄ receptor agonists were measured. The contractile responses were reduced by damage of smooth muscle cells during cryopreservation, consistent with reduction of viable muscarinic, 5-HT₃ and 5-HT₄ receptors in their transduction mechanism. Nevertheless the affinities for most agonists and antagonist were preserved after cryopreservation. The present study shows that cryopreservation can preserve guinea-pig colon sufficiently for subsequent pharmacological experimentation.

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この論文は常に私を信じ、支え続けてくれている日本にいる両親と家族にささげたい。

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Introduction

Excitatory and inhibitory neurotransmitters in the myenteric plexus control the activity of the gastrointestinal tract. An integral excitatory neurotransmitter in the myenteric plexus that participates in the peristaltic reflex is serotonin (5-hydroxytryptamine or 5-HT). Over 90% of the 5-HT that is present in the body, is located in enterochromaffin cells in the gastrointestinal tract (Furness and Costa 1982). Seven major 5-HT-receptor subtypes, the majority of which have further subdivisions, have been identified: 5-HT₁, 5-HT₂, 5-HT₃, 5-HT₄, 5-HT₅, 5-HT₆, and 5-HT₇. The 5-HT₅, 5-HT₆, and 5-HT₇ receptors are distributed mainly throughout the limbic system. In the gastrointestinal system, 5-HT₃ and 5-HT₄ receptors are located on myenteric cholinergic neurons, and 5-HT₁ and 5-HT₂ receptors, on the longitudinal muscle of, for example, the colon (Woollard et al. 1994; Wardle and Snager 1993). Activation of these four receptors induces 5-HT-mediated muscle contraction (Woollard et al. 1994; Wardle and Snager 1993). The roles of 5-HT₃ and 5-HT₄ receptors in the gastrointestinal tract have been investigated extensively. The 5-HT₃ receptor, which forms a ligand-gated Na⁺-K⁺ ion channel, causes fast depolarization, which in turn releases acetylcholine (ACh) (Ito and Tamura 1995). The 5-HT₄ receptor also mediates contractions via promoting ACh release from myenteric neurons (Wardle and Sanger 1993). Whereas the 5-HT₃ receptor forms ligand-gated channels, the 5-HT₄ receptor promotes slow depolarization via coupling of G-protein to adenylyl cyclase for synthesizing the second messenger adenosine 3':5'-cyclic monophosphate (cyclic AMP).

The binding of ACh to a muscarinic receptor on the longitudinal muscle activates G-protein and increases calcium influx and activation of phospholipase C (PLC). This enzyme breaks down phosphatidylinositol bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol triphosphate (IP₃). DAG then activates protein kinase C (PKC) (Pollock et al. 1995) and opens smooth muscle calcium channels. IP₃ triggers the release of Ca²⁺ from the endoplasmic reticulum (Pollock et al. 1995; Somlyo and Himpens, 1989), and influx of Ca²⁺ via the dihydropyridine-sensitive L-type Ca²⁺ channel from the extracellular space (Mule et al. 2002). Intracellular Ca²⁺ then binds to calmodulin, and the Ca²⁺-calmodulin complex activates myosin filaments and cross-bridge cycling for muscle contraction.

Drugs that act on 5-HT receptors have an important role for the treatment of gastrointestinal motility disorders. The 5-HT₄ receptor agonist, tegaserod, for example, has proven useful for treating patients, particularly women, with constipation-predominant irritable bowel syndrome. On the other hand, a 5-HT₃ antagonist, alosetron, is useful for treating diarrhea. The development of such drugs in gastrointestinal research involves *in vitro* studies that are performed on freshly isolated colon muscle. The use of human tissues for human pharmacology assessment produces the most meaningful results. However, the irregularity of human tissue supply limits its availability for pharmacology studies.

Cryopreservation, a process for long-term storage of living tissue, makes human tissues readily available for pharmacological research applications. Cryopreserved human vascular and airway smooth muscle has been investigated for use in

pharmacological studies (Ellis and Müller-Schweinitzer 1991; Müller-Schweinitzer 1994; Müller-Schweinitzer and Fozard 1997; Müller-Schweinitzer et al. 1998; Ruddle et al. 2000). Cryopreservation has been shown to preserve the activity of monoamine oxidase (Müller-Schweinitzer and Tapparelli 1986), endogenous prostaglandin synthesis (Müller-Schweinitzer and Tapparelli 1986), adrenergic nerve function (Thompson et al. 1989), and endothelial function in vascular smooth muscle (Thompson et al. 1989). However, cryopreservation seems to reduce contractile force by altering transmembrane and intracellular signal transduction. Freezing injuries activate the dihydropyridine-sensitive Ca^{2+} channel, which enhances Ca^{2+} influx in human internal mammary arteries (Müller-Schweinitzer et al. 1998; 2000). Elevated basal intracellular Ca^{2+} levels attenuate the contractile response to additional influx of Ca^{2+} (Müller-Schweinitzer et al. 2000).

Unfortunately, no single procedure of cryopreservation applies to all tissues (Pegg 1985). The main problem with cryopreservation is cell damage during freezing and thawing. If the freezing rate is too high, small ice crystals form within the cell and damage the cell membrane. On the other hand, if the freezing rate is too slow, the ice crystals form outside the cell and injure the cell by osmotic shock (Mazur 1977). Therefore, thawing and freezing rates need to be optimal to minimize cell damage. Adding a cryoprotective agent to the cryomedia minimizes cell injury. The two most widely used cryoprotective agents are dimethyl sulfoxide (DMSO) and glucose. DMSO is a permeating agent that enters the cell to replace water (Pegg 1985). Glucose is a nonpermeating agent that stabilizes the cell volume by retaining more water (Mazur 1977). Magnesium sulfate also has been used to stabilize cell volume (Pegg and Gallant

1977). However, long-term exposure to high concentrations of cryoprotectants can be toxic (Fathy et al. 1984). Therefore, the concentration of cryoprotectants needs to be high enough to minimize cell damage, but low enough to prevent toxic effects.

Another factor that may improve the viability of colon, and potentially other gastrointestinal tissues, is the K^+ concentration in the dissecting medium. Unpublished work at Theravance Inc. showed that increasing the K^+ concentration of the dissecting medium improved the viability of tracheal smooth muscle. A high extracellular concentration of K^+ improves viability by making smooth muscle more excitable.

Since human tissue was difficult to obtain due to cost and regulatory issues, guinea-pig tissue, commonly used for gastrointestinal pharmacology studies, was the tissue of choice herein. The objectives of this study were to 1) optimize cryopreservation conditions for guinea-pig colon and 2) investigate the effect of cryopreservation on its functional activity by comparison with freshly dissected tissue. In the first set of experiments, the efficacy of cryopreservation of guinea-pig colon was investigated by varying the following: 1) concentration of cryoprotectants DMSO, glucose and $MgSO_4$ in the cryomedium, 2) extracellular K^+ concentration, and 3) freezing and thawing rate. The second set of experiments investigated the functional activity of colon cryopreserved with the optimal conditions. This experiment involved the measurement of the contractile responses evoked by muscarinic, 5-HT₃ and 5-HT₄ receptor activation, and by direct smooth muscle cell activation.

Materials and Methods

Optimization of cryopreservation of colon

Male Dunkin Hartley guinea-pigs, in a protocol approved by Institutional Animal Care and Use Committees (IACUC) at San Jose State University and Theravance Inc., were euthanized by CO₂ asphyxiation. A 20 cm segment of the distal colon was removed and placed in Krebs-Henseleit solution (composition mM: D-glucose, 11.0; MgSO₄, 1.2; KH₂PO₄, 1.0; KCl, 4.8; NaCl, 118.0; NaHCO₃, 25.0). Fecal matter was removed and the distal colon was cut into 2 cm sections. Müller-Schweinitzers' (1998) cryopreservation procedure for pulmonary tissue was applied here for colon. Briefly, 3-5 segments were placed in liquid nitrogen storage ampoules filled with 1.5 ml of Krebs-Henseleit solution containing 1.8 M DMSO and 0.1 M glucose as cryoprotecting agents. Following equilibration for 10-30 minutes at room temperature, the samples were placed in a polystyrene box and frozen at -80°C for 1.5-3 hours. Samples were then stored in liquid nitrogen (-196°C) for 1-2 weeks. Upon removal, samples were placed on dry ice for 10 minutes, exposed to room temperature (25°C) for 6 minutes and kept in a 40°C water bath for 4 minutes.

The first experience showed that Müller-Schweinitzers' (1998) conditions failed to preserve colon viability. Therefore, the alternation of DMSO and glucose concentration in cryomedium was tested to improve colon viability. The following nine variations of cryomedium, with Krebs-Henseleit solution as a base, were tested: 2.0 M DMSO with either 0.15 M, 0.25 M, 0.3 M glucose; 2.25 M DMSO with either 0.15 M, 0.25 M, 0.3 M glucose; 2.5 M DMSO with either 0.15 M, 0.25 M, 0.3 M glucose.

When the optimized concentration of DMSO and glucose was determined, the concentration was used as a control condition for the following optimization studies to improve colon viability. First, the distal colon was dissected in Krebs-Henseleit solution with a high concentration of KCl (48 mM) to investigate the influence of extracellular K^+ concentration. Second, either, 3 mM or 10 mM of $MgSO_4$ was tested to optimize the concentration of $MgSO_4$ in the cryomedium. Third, the samples were placed in a freezer at $0^\circ C$ for 2 hours before they were transferred to a freezer maintained at $-80^\circ C$, to optimize the freezing rate. Fourth, the samples were placed on dry ice for 5 minutes, after removal from the liquid nitrogen, to optimize the thawing rate. Finally, the most effective condition for each of the variable measured above was combined into one procedure.

Organ bath study

The tissues were thawed with the procedure above and placed into 10 ml of Krebs-Henseleit solution. The tissue was slipped over a glass rod, and the surface of the tissue was cut longitudinally with a blade. The longitudinal muscle with its myenteric plexus was carefully peeled off. This muscle strip was mounted vertically in a 10 ml organ bath with Krebs-Henseleit solution at $37^\circ C$ and then gassed constantly with 95% O_2 and 5% CO_2 . The organ bath contained methysergide ($1\ \mu M$) to block $5-HT_1$ and $5-HT_2$ receptors. It also contained cocaine ($30\ \mu M$) to block neuronal 5-HT uptake, and indomethacin ($3\ \mu M$) to inhibit endogenous prostaglandin production. During 2-3 hours of equilibration, the solution in the organ bath chamber was changed every 15 minutes. Contractile responses were measured isometrically with a resting tension of 500 mg.

Cryopreservation impact on smooth muscle and 5-HT₄ receptors

Ondansetron (1 μ M) was added to the modified Krebs-Henseleit solution throughout the experiment to block 5-HT₃ receptors. In each tissue, two non-cumulative concentration response curves for agonists were constructed by adding half-log increasing concentrations of agonist at 15 minutes time intervals. The first curve was constructed to 5-HT and served as a control. A second curve was then constructed either to 5-HT, as a time control, or test agonists. One hour separated the two curves. Agonist was left in the bath for 4 minutes or until the maximum response was observed.

Cryopreservation impact on 5-HT₃ receptors

Piboserod (1 μ M) was added to the modified Krebs-Henseleit solution throughout the experiment to block 5-HT₄ receptors. A non-cumulative concentration response curve to 5-HT was constructed by adding half-log increasing concentrations of 5-HT at 15 minutes time intervals. After the first concentration response curve was obtained, ondansetron (3 μ M) was added to the bath medium. After one hour, the second concentration response curve to 5-HT was constructed.

Data analysis

Data was collected with a MP 100 data acquisition system using AcknowledgeTM Waveform Acquisition and Analysis Software (Biopac System, Inc., Santa Barbara, CA). Concentration-response curves were analyzed through iterative curve fitting to a logistic equation with PrismTM (Graph Pad, Inc., San Diego, CA). The E_{max} (maximal response) and pEC₅₀ value (negative logarithm of the agonist concentrations that produced 50% of the E_{max}) were calculated based on this analysis. Antagonist affinity (pK_B value) was

calculated using the following formula: $pK_B = -\log [\text{antagonist}] + \log (\text{dose ratio} - 1)$ (Furchgott 1966). Data is shown as means $\pm$ SEM. Statistical analysis of data was done by the unpaired t-test. A P-value <0.05 was considered to be significant.

Drugs

Ondansetron, prucalopride, cisapride, TS-951, piboserod and tegaserod were synthesized at Theravance Inc., South San Francisco, California., USA. Methysergide, 5-hydroxytryptamine creatinine sulphate (5-HT), cocaine and indomethacin were purchased from Sigma Chemical Co., St. Louis, Missouri., USA.

Results

Optimization of colon cryopreservation

The first experiment determined the extent to which ten different cryomedium mixtures preserved the contractile activity of colon. The maximal contractile responses to 5-HT in the cryopreserved tissues were lower than those for freshly dissected tissues. A contractile force of 100 mg, which is the lowest force that can be analyzed accurately, was used to define a tissue as having survived cryopreservation. The highest survival rate (57%) occurred for tissue cryopreserved with 2.25 M DMSO and 0.25 M glucose (Table 1). This survival rate considerably exceeded the 11% survival rate of tissues cryopreserved with 1.8 M DMSO and 0.1 M glucose, the mixture that was found to be optimal for vascular and pulmonary tissues (Müller-Schweinitzer and Ellis 1992; Müller-Schweinitzer 1998). The pEC_{50} value of 5-HT in the tissues that were cryopreserved in 2.0 M DMSO / 0.25 M glucose and 2.25 M DMSO / 0.1 M DMSO was diminished. On the other hand, the tissues that were cryopreserved in 2.25 M DMSO and 0.25 M glucose produced a pEC_{50} value for 5-HT (7.21 ± 0.49), which was similar to that observed in freshly prepared tissues (7.18 ± 0.30). With acceptable responses for 5-HT maximal contractile response, survival rate and pEC_{50} , the combination of 2.25 M DMSO and 0.25 M glucose produced the best viability (Table 1). Therefore, all colon tissues in later experiments were cryopreserved in 2.25 M DMSO and 0.25 M glucose.

A second series of experiments investigated the influence of varying the extracellular K^+ concentration. The tissues dissected in Krebs-Henseleit solution with an original concentration of KCl (4.8 mM) were associated with a maximal contractile

response, pEC_{50} , and survival rate that were much higher than for cryopreserved tissues initially dissected in a higher KCl level solution (48 mM; Table 2). Therefore, the control concentration of KCl (4.8 mM) was used through the study.

A third series of experiments tested the effects on the survival rate of varying the concentration of $MgSO_4$ in the cryomedium. Literature indicates that the $MgSO_4$ concentration can change the survival rate (Matheny et al. 1969; Pegg and Gallant 1977). However, the highest level, 10 mM $MgSO_4$, abolished the viability of colon tissues. The maximal contractile response in a cryomedium with 3 mM $MgSO_4$ (405 ± 42.1 mg) was comparable to the maximal contractile response with the original concentration of 1.2 mM $MgSO_4$ (438 ± 90.3 mg; Table 2). There was no significant difference between the pEC_{50} value of 5-HT in a cryomedium with the original concentration of 1.2 mM $MgSO_4$ (7.21 ± 0.49), with 3 mM $MgSO_4$ (7.35 ± 0.27), and the pEC_{50} value in fresh tissue, (7.18 ± 0.30 ; Table 2). A cryomedium with 3 mM $MgSO_4$ improved the survival rate of the colon slightly more (63 %) than did the original concentration of 1m M $MgSO_4$ (57%; Table 2). Therefore, the optimal concentration of $MgSO_4$ in cryomedium appeared to be 3 mM.

In the fourth set of experiments, the influence on tissue survival of slower freezing was investigated. The slower freezing procedure required placing the samples in a freezer maintained at $0^{\circ}C$ for 2 hours before they were transferred to the $-80^{\circ}C$ freezer. The control procedure required a direct transfer to the $-80^{\circ}C$ freezer. The slower freezing procedure was associated with a markedly smaller maximal contractile response compared to that produced by the original freezing procedure, from 438 ± 90.3 to $229 \pm$

21.2 mg (Table 2). The pEC_{50} value of 5-HT in the original procedure (7.21 ± 0.49) was closer to the pEC_{50} value of 5-HT in fresh tissue (7.18 ± 0.30), than in a slower freezing procedure (7.96 ± 0.21 ; Table 2). However, the slower freezing procedure improved the survival rate to 63%, compared to that in the control condition, 57% (Table 2). Therefore, the slower freezing procedure appeared to be more optimal than the original freezing procedure.

In addition, an optimal thawing protocol was investigated. The faster thawing rate required placing the samples on dry ice for 5 minutes after the samples were removed from liquid nitrogen. The control thawing rate required placing the samples on dry ice for 10 minutes after removal from liquid nitrogen. The E_{max} diminished to 189 ± 164 mg, compared to that produced by the control thawing rate procedure (438 ± 90.3 mg; Table 2). However, the survival rate increased to 71% with the faster thawing procedure, compared to that in the control thawing procedure (57%; Table 2). Therefore, the faster thawing procedure appeared to be more optimal than the original thawing procedure.

Finally, all optimized cryopreservation conditions were combined. Accordingly, the colon was dissected in Krebs-Henseleit solution containing normal KCl concentration (4.8 mM). The tissues were placed in a cryomedium containing Krebs-Henseleit solution with 2.5 M DMSO, 0.25 M glucose and 3 mM $MgSO_4$. The slower freezing rate (Table 2) and faster thawing rate (Table 2) were applied. This procedure markedly lowered maximal contractile response (215 ± 20.7 mg) and survival rate (47%) compared to the control procedure (438 ± 90.3 mg, 57%; Table 2). The pEC_{50} value of 5-HT in the

control procedure (7.21 ± 0.49) was closer to its pEC_{50} value in fresh tissue (7.18 ± 0.30), although lower than that when all conditions were optimized (7.61 ± 0.19 ; Table 1 and Table 2). Based on the lower maximal response and survival rate, the anticipated “optimal” combination was discontinued. Instead, experiments to characterize the pharmacology of the cryopreserved colon relied on the concentration of cryoprotectants (2.25 M DMSO and 0.25 M glucose), as tissue viability was deemed acceptable.

Cryopreservation impact on smooth muscle pharmacology

The maximal contractile force in response to 1 M KCl diminished to 36% in cryopreserved tissues (727 ± 103 mg) compared to fresh tissues (2016 ± 170 mg; Table 3 and Figure 1). The maximal response for carbachol (CCh) also diminished to 29 % in the cryopreserved colon (949 ± 72.2 mg) compared to that produced by fresh tissues (3258 ± 121 mg; Table 3 and Figure 1). Moreover, the maximal response for acetylcholine (ACh) was diminished to 25 % in cryopreserved tissues (663 ± 30.3 mg) compared to that produced by fresh tissues (2656 ± 90.3 mg; Table 3 and Figure 1). The calculated pEC_{50} value in cryopreserved tissues for CCh and ACh (6.8 ± 0.13 , 6.8 ± 0.11) were relatively close to each pEC_{50} in fresh tissues (7.0 ± 0.14 , 6.3 ± 0.22 ; Table 3). The calculated pEC_{50} value of KCl significantly increased in cryopreserved tissues (Table 3).

Cryopreservation impact on 5-HT₄ receptors

The maximal contractile responses of prucalopride, cisapride, TS-951 and tegaserod in fresh tissue were 95%, 85%, 64% and 23%, respectively, of the contractile response to 5-HT in fresh tissue (Table 4). The contractile force evoked by 5-HT was diminished to 44% in cryopreserved tissues (281 ± 26.9 mg) compared to fresh tissues

(631 ± 56.5 mg; Table 6 and figure 2). The pEC_{50} value for 5-HT in cryopreserved tissues (7.5 ± 0.18) decreased slightly compared to that produced by fresh tissues (7.9 ± 0.19 ; Table 6). The maximal response for 5-HT, prucalopride, cisapride, TS-951 and tegaserod, in cryopreserved tissues were reduced by 56%, 69%, 82%, 76% and 75% of their contractile force in fresh tissues (Table 5). In comparing cryopreserved and fresh tissues, the pEC_{50} values for prucalopride, cisapride, TS-951, and tegaserod did not differ significantly (Table 6).

Cryopreservation impact on 5-HT₃ receptors

The 5-HT₃ antagonist, ondansetron (3 μ M) produced a parallel and rightward shift of the concentration response curves to 5-HT (Figure 3). The pK_B values of ondansetron were 6.6 ± 0.2 in fresh tissues and 7.0 ± 0.2 in cryopreserved tissues.

Discussion

Optimization of colon cryopreservation

This research affirms that a single cryopreservation procedure may not be appropriate for all tissues (Müller-Schweinitzer 1998). The concentration of DMSO and glucose (1.8 M DMSO and 0.1 M glucose) that effectively cryopreserved human pulmonary tissues was associated with an unacceptably low survival rate (11%) for guinea-pig colon. It is possible that guinea-pig colon requires higher concentrations of cryoprotectants to lower the risk of intracellular ice formation and raise the survival rate. However, long term exposure to high concentrations of cryoprotectants increases toxicity for tissues (Fathy et al. 1984). Therefore, optimal concentrations need to be high enough to prevent the formation of ice crystals and low enough to avoid damaging tissue due to toxic effects. This study suggests that the optimal concentrations of cryoprotectants for guinea-pig colon are 2.25 M DMSO and 0.25 M glucose.

The other critical factors for cryopreservation are the freezing and thawing rate. Improper freezing damages tissues by thermal and osmotic shock (Pegg 1985). Fortunately, the freezing rate can be optimized by varying the concentration of cryoprotectants in the cryomedium. A high concentration of cryoprotectants, which prevents cell injury at low temperature, permits a slower freezing rate and increased survival rate (Pegg 1985). Most likely, a slower freezing rate requires a rapid thawing rate (Pegg 1985) to limit the growth of ice crystals. However, thawing too rapidly leads to mechanical stress-inducing fractures (Pegg 1985). The present study showed that for

the guinea-pig colon tissue with a slow freezing rate or a fast thawing rate, the survival rate increased.

The concentration of extracellular potassium affects the resting membrane potential of smooth muscle cells. A high extracellular concentration of K^+ makes smooth muscle cells more excitable. Likewise, unpublished experiments at Theravance Inc. showed that increasing the K^+ concentration of the dissecting medium improved the viability of tracheal smooth muscle. However, the higher K^+ concentration of the dissecting medium in the present study did not improve the viability of colonic smooth muscle after cryopreservation. Indeed, high K^+ promoted a lower E_{max} to 5-HT. The colonic tissues may be more sensitive than vascular tissues to high concentrations of extracellular K^+ , which may have damaged colonic smooth muscle and/or nerves.

Magnesium sulfate stabilizes cell volume during short term preservation (Pegg and Gallant 1977). However, too high a concentration of $MgSO_4$ damages the cell directly and during freezing. This explains the finding in this study that the highest concentration of $MgSO_4$ (10 mM) did not permit survival of any tissue. Hence, 3 mM $MgSO_4$ was optimal for viability of colonic smooth muscle after preservation.

Finally, all of the optimized conditions, including the slow freezing rate, fast thawing rate, 2.25 M DMSO, 0.25 M glucose and 3 mM $MgSO_4$ in the cryomedium were combined. Unexpectedly, this combination markedly reduced viability for survival rate and for 5-HT maximal contractile response compared to the control conditions, including the original freezing and thawing rate, 2.25 M DMSO, 0.25 M glucose and 1 mM $MgSO_4$. The reason for this apparent anomaly is unclear. It may have occurred as a result of

controlling freezing and thawing rates manually rather than by programmable machine. Theoretically, the combination of a slower freezing rate and faster thawing rate increases the survival rate (Pegg 1985). However, the unavailability of a programmable machine caused a lack of precise and gradual changes in these rates. When both improper freezing and thawing rates were applied the tissues were damaged more than when either a slow freezing or fast thawing rate. This damage eventually decreases the survival rate. Programmable machines for controlling the rate of freezing and thawing have been applied for cryopreservation (Ruddles et al. 2000; Müller-Schweinitzer et al. 2000). It is also possible that at the high MgSO_4 concentration (3 mM), the precise freezing and thawing rates are more critical.

Cryopreservation impact on smooth muscle pharmacology

The impact of cryopreservation on the physiology of colonic smooth muscle was investigated with a contractile agonist, potassium chloride (KCl). The cryopreserved tissue produced only 37% of the maximal contractile response to KCl compared to that in fresh tissues. This is similar to cryopreserved human internal mammary arteries, where the contractile response to KCl is 40% of the fresh tissue contractile response (Müller-Schweinitzer 2000). KCl induces non-receptor mediated smooth muscle contraction by depolarizing the membrane, which causes Ca^{2+} influx through voltage gated Ca^{2+} channels (Pollock et al. 1995). Loss of K^+ -induced contraction can be explained by cryoinjury elevating basal intracellular Ca^{2+} , which attenuates the contractile response potentially induced by the additional influx of Ca^{2+} (Müller-Schweinitzer et al. 2000).

Contraction mediated by the muscarinic receptor was investigated with the muscarinic agonist carbachol (CCh), and the endogenous ligand, acetylcholine (ACh). CCh and ACh activate muscarinic receptors and increase the intracellular level of Ca^{2+} via Ca^{2+} influx through L-type Ca^{2+} channels (Ashrani et al., 1997) and Ca^{2+} release from intracellular calcium stores (Tanovic et al., 2000). The contractile responses of cryopreserved tissues to CCh and ACh were reduced by 71% and 75% respectively, compared to the 63% shown with KCl. This result may suggest that the reduction of the contractile response was due mainly to an enhanced Ca^{2+} influx through the voltage gated Ca^{2+} channels in the damaged tissue. However, contractile responses to CCh and ACh showed a slightly greater decrement than for KCl (Table 3). This difference may represent alteration of the receptor-operated Ca^{2+} release from intracellular calcium stores and/or damage of muscarinic receptors on the smooth muscle. In addition, the pEC_{50} values in cryopreserved tissues for CCh and ACh (6.8 ± 0.13 , 6.8 ± 0.11) were relatively close to each pEC_{50} in fresh tissues (7.0 ± 0.14 , 6.3 ± 0.22). This indicates that the affinities for both muscarinic receptor agonists were preserved after cryopreservation.

Cryopreservation impact on 5-HT₄ receptors

The 5-HT₁, 5-HT₂ and 5-HT₃ receptors were blocked with methysergide and ondansetron to investigate, in isolation, the effect of cryopreservation on the 5-HT₄ receptors mediating contraction. The 5-HT₄ receptor evokes contraction by promoting release of ACh from enteric cholinergic motor neurons (Wardle and Sanger 1993). Therefore, the intact 5-HT₄ receptor contractile response found in cryopreserved tissues suggests that at least some neurons releasing ACh also survived after cryopreservation.

A reduction of the contractile response to 5-HT after cryopreservation and a slight reduction of pEC_{50} also occurred in coronary artery smooth muscle (Schoeffter and Müller-Schweinitzer 1990). All contractile responses of the 5-HT full agonist, prucalopride, and partial agonists, cisapride, TS-951, and tegaserod were reduced, when compared to the contractile force they evoked in fresh tissue. The percent response for each compound relative to 5-HT responses in fresh colonic tissues is comparable to previous studies of TS-951 (Suzuki et al. 2001), cisapride (Suzuki et al. 2001), tegaserod (Theravance Inc. unpublished data), and prucalopride (Briejer et al. 2001). However, the reduction of percent response to the partial agonists was slightly greater than to the full agonists (Table 5). A full agonist requires activation of fewer receptors than partial agonists for a given response. Therefore, loss of receptors during cryopreservation would be expected to have a more significant effect on activity of a partial agonist. In addition, the pEC_{50} was not significantly different between fresh and cryopreserved tissue. As for the muscarinic agonists, affinities for the 5-HT₄ receptor agonists seem to have been preserved. Perhaps receptor loss rather than damage to the 5-HT₄ receptor-dependent transduction mechanism better explains the present results. The reduction of a 5-HT₄ receptor-mediated contraction could also be explained by alteration of the Ca²⁺-coupled contraction mechanism and muscarinic receptor mechanisms, as well as by a reduction of 5-HT₄ receptors and cholinergic neurons.

Cryopreservation impact on 5-HT₃ receptors

Similar to 5-HT₄ receptors, 5-HT₃ receptor, a ligand-gated ion channel, activation via 5-HT also causes release of ACh, which in turn induces muscle contraction (Ito and

Tamura 1995). In the present experiment, 5-HT₁, 5-HT₂ and 5-HT₄ receptors were antagonized by methysergide and piboserod to permit, in isolation, the study of 5-HT₃ receptor-mediated contraction after cryopreservation. The 5-HT₃ receptor antagonist, ondansetron, inhibits the nerve mediated contraction of the isolated longitudinal muscle-myenteric plexus (Woollard et al. 1994). The pK_B values from use of ondansetron in this study were calculated to compare antagonist affinity in fresh and cryopreserved tissues. Both the pK_B in fresh tissue (6.7 ± 0.2) and in cryopreserved tissue (7.0 ± 0.2) herein were relatively close to Yamano and Miyake's (1996) pK_B in fresh tissue (7.0 ± 0.1). The finding that ondansetron has similar affinities in cryopreserved tissue compared to fresh tissue suggests that 5-HT₃ receptors, like muscarinic and 5-HT₄ receptors, were preserved after cryopreservation.

Conclusions

An optimized concentration of cryoprotectants in cryomedium for colon tissue (2.25 M DMSO and 0.25 M glucose) increased the survival of 5-HT-mediated functional activity after cryopreservation compared to the other combinations tested. However, muscle contraction after cryopreservation decreased compared to contractility of fresh tissue. Nevertheless, the presence of 5-HT contractile responses via 5-HT₃ and 5-HT₄ receptor activation showed that cholinergic nerves of the myenteric plexus are intact and viable after cryopreservation. The slightly greater loss of the contractile response with partial 5-HT₄ agonists, than with full 5-HT₄ agonists, after cryopreservation suggests that cryopreservation reduces the number of 5-HT₄ receptors or the efficacy of the receptor.

transduction mechanism. Further study of the cryopreservation effect on the smooth muscle components and transmembrane signal transduction units is needed.

Even though the colonic contractile responses are likely to have been reduced mainly by damage to the smooth muscle and partially by the reduction of viable muscarinic, 5-HT₃ and 5-HT₄ receptors, the affinities for most agonists and antagonists were preserved after cryopreservation. Although not evaluated in this study, it is hoped that the optimized conditions for cryopreservation of guinea-pig colon will be similar to those for human colon, and that cryopreserved human colon will be useful for investigation of 5-HT₃ and 5-HT₄ pharmacology.

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Table 1 Comparison of survival rate and contractile activity of fresh and cryopreserved colon: Optimization of DMSO and glucose concentration in cryomedium.

Cryomedium	Survival Rate (%)	5-HT E_{max} (mg)	pEC_{50}	n
1.8 M DMSO/0.1 M Glucose	11	314 ± 24.9	7.64 ± 0.21	8
2.0 M DMSO/0.1 M Glucose	50	$162 \pm 17.6^*$	6.67 ± 0.25	8
/0.15 M Glucose	21	$155 \pm 17.9^{***}$	6.51 ± 0.28	8
/0.25 M Glucose	16	582 ± 120	5.12 ± 0.27	8
2.25 M DMSO/0.1 M Glucose	40	$237 \pm 15.0^*$	5.82 ± 0.13	8
/0.15 M Glucose	54	$283 \pm 29.6^{**}$	7.55 ± 0.28	8
/0.25 M Glucose	57	438 ± 90.3	7.21 ± 0.49	8
2.5 M DMSO/0.1 M Glucose	11	$99.5 \pm 5.77^{**}$	7.60 ± 0.15	8
/0.15 M Glucose	50	$315 \pm 29.4^*$	7.94 ± 0.25	8
/0.25 M Glucose	25	212 ± 33.6	7.56 ± 0.43	8
Fresh		684 ± 61.2	7.18 ± 0.30	8

E_{max} =maximal contractile response. pEC_{50} =negative logarithm of the molar concentration producing 50% of E_{max} . Values are means $\pm$ S.E.M. Differences from fresh control significant at * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 2 Comparison of survival rate and contractile activity of cryopreserved colon with modified condition: effect of extracellular K^+ , optimization of cooling and thawing rate and concentration of $MgSO_4$, and effect of all optimized conditions.

	Survival Rate (%)	5-HT E_{max} (mg)	pEC ₅₀	n
Control (2.25 M DMSO/0.25 M Glucose) ¹	57	438 ± 90.3	7.21 ± 0.49	8
High KCl (48 mM)	47	293 ± 46.1	6.80 ± 0.42	8
Slower Freezing Rate ²	63	229 ± 212	7.96 ± 0.21	8
Faster Thawing Rate ³	71	189 ± 164	7.33 ± 0.16	8
3 mM Mg SO ₄	63	405 ± 42.1	7.35 ± 0.27	8
10 mM Mg SO ₄	0	--	--	8
Optimized conditions ⁴	47	215 ± 20.7	7.61 ± 0.19	8

¹ Control condition includes that dissection in Krebs-Henseleit solution with a normal concentration of KCl (4.8 mM), use of 2.25 M DMSO, 0.25 M glucose and 1.2 mM $MgSO_4$ in cryomedium, the freezing procedure directly transferring to the -80°C freezer, thawing procedure placing the samples on dry ice for 10 minutes after they were removed from the liquid nitrogen.

² The slower freezing procedure required placing the samples in a freezer maintained at 0°C for 2 hr before they were transferred to the -80°C freezer.

³ The faster thawing rate required placing samples on dry ice for 5 minutes after the samples were removed from the liquid nitrogen.

⁴ Slower freezing and faster thawing rate, 3 mM $MgSO_4$ 2.25 M DMSO and 0.25 M glucose were used in the condition.

Values are means ± S.E.M.

Table 3 Parameters for contractile response to KCl, CCh and ACh on fresh and cryopreserved longitudinal muscle-myenteric plexus of guinea-pig colon.

	E_{max} (mg)		pEC_{50}	
	Fresh Colon (n=4)	Cryopreserved Colon (n=4)	Fresh Colon (n=4)	Cryopreserved Colon (n=4)
KCl	2016 $\pm$ 170	727 $\pm$ 103*	1.0 $\pm$ 0.33	1.7 $\pm$ 0.25*
CCh	3258 $\pm$ 121	949 $\pm$ 72.2***	7.0 $\pm$ 0.14	6.8 $\pm$ 0.13
ACh	2656 $\pm$ 90.3	663 $\pm$ 30.3**	6.3 $\pm$ 0.22	6.8 $\pm$ 0.11

E_{max} =maximal contractile response. pEC_{50} =negative logarithm of the molar concentration producing 50% of E_{max} . Values are means $\pm$ S.E.M.

Differences from fresh control significant at * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 1 Concentration response curves for KCl, CCh and ACh on fresh (closed symbols) and cryopreserved (open symbols) guinea-pig colon. Data are expressed as means $\pm$ S.E.M.; for each curve $n=4$.

Table 4 Maximal responses to 5-HT, prucalopride, cisapride, TS-951 and tegaserod in fresh and cryopreserved longitudinal muscle-myenteric plexus of guinea-pig colon. Data are expressed as a percentage of the 5-HT maximum response (100%) in fresh tissue.

	E _{max} (%)	
	Fresh Colon (n=4)	Cryopreserved Colon (n=4)
5-HT	100 ± 8.95	44 ± 4.41
Prucalopride	95 ± 19.2	30 ± 5.35
Cisapride	85 ± 17.7	16 ± 2.15
TS-951	64 ± 8.52	16 ± 3.38
Tegaserod	23 ± 3.00	6 ± 6.30

E_{max} =maximal contractile response. Values are means ±S.E.M.

Table 5 Reduction of the maximal responses to 5-HT, prucalopride, cisapride, TS-951 and tegaserod expressed in cryopreserved longitudinal muscle-myenteric plexus of guinea-pig colon relative to their own maximum response (100 %).

	Reduction of $E_{\max}$ (%)
	Cryopreserved Colon (n=4)
5-HT	56 ± 7.61
Prucalopride	69 ± 14.6
Cisapride	82 ± 6.34
TS-951	76 ± 9.50
Tegaserod	75 ± 8.08

$E_{\max}$ =maximal contractile response. Values are means ± S.E.M.

Table 6 Parameters for contractile response to 5-HT, prucalopride, cisapride, TS-951 and tegaserod on fresh and cryopreserved longitudinal muscle-myenteric plexus of guinea-pig colon.

	E_{max} (mg)		pEC_{50}	
	Fresh Colon (n=4)	Cryopreserved Colon (n=4)	Fresh Colon (n=4)	Cryopreserved Colon (n=4)
5-HT	631 ± 56.5	281 ± 26.9*	7.9 ± 0.19	7.5 ± 0.18
Prucalopride	602 ± 121	190 ± 33.8	7.7 ± 0.45	7.8 ± 0.32
Cisapride	515 ± 80.9	94.8 ± 12.6*	7.6 ± 0.28	7.6 ± 0.23
TS-951	406 ± 53.8	101 ± 20.1*	8.3 ± 0.30	8.1 ± 0.42
Tegaserod	156 ± 21.2	39.8 ± 5.48	7.8 ± 0.29	8.3 ± 0.33

E_{max} = maximal contractile response. pEC_{50} = negative logarithm of the molar concentration producing 50% of E_{max} . Values are means ± S.E.M.

Differences from fresh control significant at * $P < 0.05$.

Figure 2 Concentration response curves for 5-HT, prucalopride, cisapride, TS-951 and tegaserod on fresh (closed symbols) and cryopreserved (open symbols) guinea-pig colon. Data are expressed as means $\pm$ S.E.M.; for each curve n=4.

Figure 3 Concentration-response curves to 5-HT in the fresh (**top**) longitudinal muscle-myenteric plexus of guinea-pig colon (n=4) in the absence (*solid square*) or presence (*solid triangle*) of 5-HT₃ antagonist, ondansetron. In the cryopreserved (**bottom**) colon (n=4) in the absence (*empty square*) or presence (*empty triangle*) of 5-HT₃ antagonist, ondansetron. Ondansetron (3 μ M) was incubated with the colon for 1 hr prior to the addition of each concentration of 5-HT. Experiments were carried out in the presence of 5-HT₄ antagonist, piboserod (1 μ M). Each point represents the mean $\pm$ S.E.M.

San Jose State University
Institutional Animal Care and Use Committee

LETTER OF OFFICIAL PROTOCOL REVIEW

Date: February 17, 2003

Dear Dr. Hyde,

The animal care and use portion of your research proposal indicated below was reviewed by the Institutional Animal Care and Use Committee (IACUC). The status of your proposal is as follows:

Principal Investigator(s): Robert Hyde, Ryoko Hirakawa

Protocol #: 826

**Title: Investigation of Functional Activity of Guinea Pig Colon After
Cyropreservation.**

The application was approved without modification by the IACUC.

Approval date: February 14, 2003 * Expiration Date: February 6, 2005

The IACUC must be informed in writing of any proposed changes to the approved protocol outline and approval must be granted in writing by the IACUC before any change is instituted. If you wish to continue the approved outline beyond the expiration date, it is recommended that you resubmit a renewal application by December 2004.

The protocol number (#826) may only be used by the principal investigator and participants included on the approved application form. The protocol number will be required on grant and contract proposals to fund the project. Please notify the UAC office to request the transfer of live animals to or from this approved protocol. If you have any questions, feel free to contact me at extension 4-4929.

Larry Young, RVT, LATg
IACUC Coordinator

Cc: UAC Office
Department Chair