Abnormalities of Volume Regulation and Membrane Integrity in Myocardial Tissue Slices After Early Ischemic Injury in the Dog

Effects of Mannitol, Polyethylene Glycol, and Propranolol

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The authors used an *in vitro* myocardial tissue slice technique to quantitate the transmural distribution of alterations in cell volume regulation and membrane integrity following early ischemic injury and to evaluate directly the effects of therapeutic interventions in a system not subject to influences of coronary blood flow. Left circumflex coronary occlusion was produced in 57 dogs for 30 or 60 minutes. After *in vitro* incubation in Krebs-Ringer-phosphate-succinate medium containing trace ^{14}C-inulin, typical values (ml H_2O/g dry weight) for control nonischemic myocardial slices were 3.68 ± 0.07 (SEM) for total tissue water, 2.67 ± 0.07 for inulin impermeable space, and 1.01 ± 0.04 for inulin diffusible space. Ischemic myocardial slices exhibited an impaired response to cold shock (0 C for 60 minutes) and rewarming (37 C for 60 minutes). After 60 minutes coronary occlusion, respective increases in total tissue water, inulin-impermeable space and inulin-diffusible space of ischemic slices were 25.5 ± 2.6%, 6.2 ± 4.9% and 84.4 ± 12.5% for papillary muscle, 22.2 ± 2.1%, 10.4 ± 4.2% and 52.5 ± 10.3% for subendocardium and 9.1 ± 1.5%, 7.2 ± 2.3% and 15.8 ± 5.5% for subepicardium. Significant but usually less marked alterations occurred after 30 minutes of coronary occlusion. Propranolol treatment *in vivo* (2 mg/kg) and/or *in vitro* (0.01 mg/ml medium) produced no significant changes in tissue water or inulin spaces of ischemic slices, compared with saline controls. Incubation in hyperosmolar mediums resulted in significant reductions in total tissue water and inulin-impermeable space with little change in inulin-diffusible space of both ischemic and control slices. Fifty milliosmolar polyethylene glycol (MW 6000) produced a greater reduction in tissue water and ultrastructural evidence of cell swelling than did either 40 or 100 milliosmolar mannitol (MW 182). The major effect of hyperosmolar incubation appeared to be a selective reduction in edema of cells with structurally intact membranes. Thus, *in vitro* studies with myocardial tissue slices provide evidence of widespread alterations of membrane integrity after 30–60 minutes of *in vivo* coronary artery occlusion. *In vitro* abnormalities of cell volume regulation can be partially reversed by direct osmotic effects on myocardial cells. (Am J Pathol 1981, 103:79–95)

PREVIOUS STUDIES have suggested that altered membrane permeability and associated derangements in fluid and electrolyte balance and cell volume regulation have an important role in the evolution of hypoxic and ischemic injury in the heart and other organs.[1-17] In the present study, we used an *in vitro* myocardial tissue slice technique to quantitate the transmural distribution of membrane alterations resulting from early experimental ischemic injury and to evaluate directly the effects of therapeutic interventions on injured muscle cells.[18]

The tissue slice technique has been used extensively for metabolic studies of many tissues[19] and has also been applied to the study of myocardial cell injury.[5-8] The technique represents a highly specialized model system involving *in vitro* manipulations of small segments of myocardium. However, the technique has the

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significant advantage for selected applications that alterations of muscle cells can be evaluated directly in a system not subject to influences of a functioning vasculature. With the tissue slice method, altered cell volume regulation and membrane permeability are measured by changes in tissue water, electrolyte abnormalities, and altered distribution of extracellular space markers, such as inulin, a polysaccharide with a molecular weight of 5000.[6]

The major objectives of the present study were: 1) to demonstrate the location and extent of membrane alterations following 30 and 60 minutes of experimental coronary occlusion and 2) to evaluate directly the cellular effects of interventions previously reported to exert protective effects on ischemic or hypoxic myocardium in the intact perfused heart. The agents selected were the beta adrenergic antagonist propranolol and two compounds, mannitol (MW 182) and polyethylene glycol (MW 6000), which are osmotically active because of a limited capacity to permeate normal cellular membranes. Previous studies have shown that elevation of serum osmolality by approximately 40 milliosmoles with mannitol exerts a protective effect on the extent of myocardial damage when the interval of ischemia is limited to one hour of coronary occlusion.[3,9,20,21] Hyperosmolar perfusion with polyethylene glycol or mannitol also has been shown to ameliorate certain manifestations of cell injury induced by hypoxia in isolated perfused hearts.[7] It has been pointed out, however, that studies of hyperosmolar agents in intact perfused hearts have not distinguished between effects on coronary blood flow and direct effects on muscle cells.[14] With regard to the protective effect of propranolol on myocardial ischemic injury, uncertainty also exists regarding the relative importance of global effects on myocardial oxygen consumption, effects on coronary vasculature, and direct effects on ischemic muscle cells.[22-24]

In the present study, an evaluation was made of the response of control and ischemic myocardial tissue slices to *in vitro* manipulation of the osmolality of the extracellular fluid with mannitol and polyethylene glycol, and the response of tissue slices also was studied after *in vivo* and/or *in vitro* administration of propranolol. Interventions were tested with the myocardial tissue slice technique based on the concept that the response of myocardial tissue slices would provide information regarding the severity and potential reversibility of ischemic membrane damage and that the *in vitro* response also would provide insight into the mechanisms of action of the agents through observation of their direct effects on myocardial cells.

Materials and Methods

Ischemia Model

Healthy adult mongrel dogs were anesthetized with an intravenous injection of sodium pentobarbital (30 mg/kg), intubated, and ventilated with room air with a Harvard respirator. Leads were attached for electrocardiographic monitoring of limb lead II. Following a left lateral thoracotomy, the proximal left circumflex coronary artery was permanently ligated with suture. After 30–60 minutes, the beating heat was excised and placed in ice cold saline for *in vitro* studies.

Groups Studied

Seventy dogs underwent permanent ligation of the left circumflex coronary artery and were used for five experimental groups (I–V) (Table 1). The study groups were limited to the 57 dogs that showed 1.0 mm or greater ST segment elevation in lead II of the electrocardiogram after a 2-minute test coronary occlusion (Group V) or at 15 minutes after permanent coronary occlusion (Groups I, II, III, and IV).

Further details of the protocol for Group V were as follows. Eighteen dogs were anesthetized with sodium pentobarbital (30 mg/kg) and 30 minutes later underwent a 2-minute temporary test occlusion of the circumflex coronary artery. Five of the 18 dogs were excluded from the study since these dogs did not manifest significant (at least 1.0 mm) ST-segment elevation at the end of the 2-minute test occlusion. The remaining 13 dogs were alternately divided into two subgroups, which were given bolus intravenous doses of propranolol (2 mg/kg) or an equal volume of saline, administered 15 minutes after the test coronary occlusion. Thirty minutes later, permanent ligation of the left circumflex coronary artery was performed. One propranolol-treated dog died after coronary occlusion. The other 12 dogs survived a 1-hour period of coronary occlusion prior to excision of the hearts. Thus, the original group of 18 dogs yielded 6 saline-treated hearts (Group Va) and 6 propranolol-treated hearts (Group Vb) for *in vitro* studies.

Preparation of Tissue Slices

After cooling for several minutes in ice-cold saline, the heart was removed from the saline, and the left ventricle was opened. A transmural block of ischemic tissue containing the posterior papillary muscle and a transmural block of control tissue containing the anterior papillary muscle were excised from the heart

and transferred to ice-cold media (Figure 1). The block of ischemic tissue was limited to the region of pallor identified on the endocardial surface and in the adjacent myocardium after excision of the block. Any pale damaged tissue in the control anterior block was excised, leaving only grossly normal muscle for preparation of control slices. The large blocks of anterior and posterior myocardium were divided into three zones: subepicardial free wall, subendocardial free wall, and papillary muscle. These blocks were then cut into thin tissue slices with razor blades by a free-hand technique.[5] The slices were cut parallel to the long axis of the heart and were 5–10 mm in length and approximately 1 mm or less in thickness. All slices were initially kept in ice-cold (0 C) medium in open beakers in an ice bath.

The basic medium for these studies was a modified Krebs-Ringer–phosphate (KRP) solution.[5] The solution was prepared with reagent-grade chemicals and contained in millimoles per liter the following ions: Na^+, 151.6; K^+, 4.8; Ca^{2+}, 1.3; Mg^{2+}, 1.2; sulfate, 1.2; phosphate, 15.6, and Cl^-, 127.7. In many experiments, the KRP medium was further modified by substitution of 20mM NaCl with 20mM sodium succinate (Sigma Chemical Company, St. Louis, Mo). Succinate was chosen as a substrate based on evidence that succinate can gain access to the interior of damaged cells and can support mitochondrial respiratory activity of such cells.[25–27]

Incubation of Tissue Slices

In the various studies, tissue slices were incubated under one or more of the following conditions: 0 C for 60 minutes, 0 C for 60 minutes followed by 37 C for 60 minutes or 37 C for 120 minutes. The cold (0 C) incubations were performed in open beakers maintained in an ice bath. For the warm (37 C) incubations, slices were placed in 25-ml Erlenmeyer flasks containing 10 ml of medium that had been equilibrated with 100% oxygen. The flasks were positioned on a Dubnoff shaker with the water bath at 37 C, agitated at a constant rate, and continuously gassed with 100% oxygen. In order to avoid evaporation of warm medium by dry gas, the oxygen was bubbled through a gas washing bottle before making contact with the medium.

Space Determinations

For determination of inulin-diffusible space, a trace quantity of ^{14}C-hydroxymethyl-inulin (Amersham/Searle Corporation) was added to the medium. In experiments involving both cold and warm incubations, the inulin was added only to the final incubation medium.

In each experiment, three slices were used for calculation of total tissue water from wet weight and dry weight measurements, and three additional slices were used for calculation of inulin-diffusible space, based upon wet weight and ^{14}C-inulin measurements. After completion of the incubation, the six slices were removed from the incubating flasks, blotted on Whatman No. 41 low-ash filter paper, and weighed on a Mettler analytical balance to the fifth decimal place to obtain wet weight. For dry weight determination, three of the six slices were placed into separate glass scintillation vials, which were loosely capped and dried overnight in a 105 C oven. After cooling, the dried slices were removed with forceps and weighed on the Mettler balance to the fifth decimal place. The other three slices were used for liquid scintillation counting. These slices were placed in separate glass scintillation vials containing 1 ml of Soluene 400 (Packard) for digestion of the tissue. For determination of the inulin concentration of the medium, three separate 0.1-ml aliquots of medium were placed in vials containing 1 ml of Soluene 400. The next day 15 ml of acidified (9:1 with 0.5 N HCl) Insta-Gel (Packard) was added to the vials containing tissue digest or medium. The vials were mechanically shaken and counted for ^{14}C on a Packard Tri-Carb scintillation counter.

We prepared a standard quenching curve by adding aliquots of acetone to preparations of a ^{14}C standard (New England Nuclear) made up in a solution of Soluene and Insta-Gel. In addition, evaluation was made of the relative amounts of quenching resulting from the medium and tissue slices. Aliquots of medium and 50, 100, and 150 mg of myocardium were added to vials containing 1 ml Soluene. The next day 15 ml of acidified Insta-Gel containing a known amount of ^{14}C standard was added. These samples were then counted. The results showed that medium and tissue samples had comparable counting efficiencies.

Total tissue water (TTW) was calculated from the wet weight (WW) and dry weight (DW) of the tissue slices according to the formula:

$$\text{TTW} = \frac{\text{WW} - \text{DW}}{\text{DW}} = \text{g (ml) of water per g dry tissue}$$

The percentage of water in the slices was calculated as follows:

$$\%\,H_2O = \frac{\text{WW} - \text{DW}}{\text{WW}} \times 100 \text{ or from } \frac{\text{TTW}}{\text{TTW} + 1} \times 100$$

Inulin diffusible space (IDS) was calculated as milliliters of inulin-diffusible water per gram of dry weight. This calculation was based on the assumptions that the inulin-diffusible water was in equilibrium with the medium and, therefore, that the concentration of ^{14}C-inulin was identical in the medium and in the inulin-diffusible water of the slice.[5] Dry weight and inulin-diffusible space could not be determined from the same slices, since it was not possible to dissolve slices for liquid scintillation counting after the slices were dried. Therefore, the dry weight for the IDS calculations was assumed from the wet weight of the slices and the TTW of other slices from the same incubation flask. The theoretical dry weight of the slices was derived by the formula

$$DW = \frac{WW}{TTW + 1}$$

IDS in gram (ml) per gram dry tissue was calculated as follows:

$$IDS = \frac{\text{inulin counts per minute per slice}}{DW \times \text{inulin counts per minute per ml of medium}}$$

The inulin impermeable space (IIS) was calculated by the formula:

$$IIS = TTW - IDS = \text{g (ml) per g dry tissue}$$

In Vitro Effects of Agents

The osmotically active agents, mannitol and polyethylene glycol (Carbowax 6000, Union Carbide, Sigma Chemical Company) were added in varying concentrations to an isosmolar (300 mOsm) KRP-succinate medium. In other experiments, propranolol was added to KRP-sucinate medium at a concentration of 0.01 mg/ml.[28,29] Control and ischemic slices were then incubated in control medium and in medium containing mannitol, polyethylene glycol, or propranolol.

Morphologic Studies

In selected experiments, samples were obtained from control and ischemic regions directly after excision of the heart or after incubation of tissue slices. Samples were cut from center strips of the tissue slices. Samples were fixed in phosphate-buffered 3% glutaraldehyde solution, washed, postfixed in phosphate-buffered 1% osmium, dehydrated through a graded series of alcohols and propylene oxide, and embedded in Epon-Araldite. Epoxy sections were cut, stained with toluidine blue, and examined by light microscopy. For electron microscopy, ultrathin sections were stained with uranyl acetate and lead citrate and examined and photographed with a JEOL 100C electron microscope.

Electron-microscopic determination of the percentage of swollen muscle cells was performed on samples from two experiments in Group IV. Two blocks were picked at random for each subgroup in the two experiments, yielding a total of four sections for each category. The sections were coded, mixed together, and examined in a random fashion. A total of approximately 200 cell profiles (50 per section) for each category were counted.

Statistical Analysis

Statistical analysis of the data was initially performed using paired or group *t* tests. If the results suggested differences in the groups, the data were reanalyzed by analysis of variance procedures to confirm the findings. The Neuman-Keuls multiple range test was then applied to determine specific differences in the groups at the 0.05 significance level.[30]

Results

Tissue Water and Morphology Prior to Incubation

With tissue slices obtained from dogs in Group I and prepared directly after excision of the heart without *in vitro* incubation, ischemic papillary muscle, subendocardium, and subepicardium exhibited small (6–11%) but significant increases in total tissue water, compared with control papillary muscle (Figures 2 and 3). After 60 minutes of permanent coronary occlusion, muscle cells from the ischemic zone exhibited variable degrees of damage and cell swelling (Figures 4 and 5). Severely damaged cells exhibited marked clumping of nuclear chromatin, edema, glycogen depletion, and multiple amorphous matrix (flocculent) and linear (lamellar) mitochondrial densities, which are characteristic of established irreversible injury (Figure 4).[2,25,31,32] Other cells showed less severe damage, characterized by edema, glycogen depletion, and clearing of the mitochondrial matrix without the presence of amorphous matrix or linear mitochondrial densities (Figure 5). The most severely damaged muscle cells were located in the papillary muscle and subendocardium. Cell swelling was generally mild, but some muscle cells with more prominent swelling were noted (Figure 4).

In Vitro Response of Control Tissue Slices

In Group I dogs (n = 15), which were subjected to 60 minutes coronary occlusion, the response of con-

Table 1—Experimental Protocols for Five Groups of Dogs

Group I (n = 15 dogs)
In vivo left circumflex coronary artery (LCCA) ligation for 60 minutes.

In vitro incubation of control and ischemic tissue slices in isosmolar (300 mOsm) Krebs-Ringer-phosphate (KRP) medium at different incubation temperatures.

Group II (n = 10 dogs)
In vivo LCCA ligation for 30 minutes.

In vitro incubation of control and ischemic tissue slices in isosmolar (300 mOsm) KRP-succinate medium, 40 mOsm mannitol medium (MW 182) and 100 mOsm mannitol medium.

Group III (n = 8 dogs)
In vivo LCCA ligation for 60 minutes.

In vitro incubation of control and ischemic slices in isosmolar KRP-succinate medium and 40 mOsm mannitol medium.

Group IV (n = 6 dogs)
In vivo LCCA ligation for 60 minutes.

In vitro incubation of control and ischemic tissue slices in isosmolar KRP-succinate medium, 100 mOsm mannitol medium and 50 mOsm polyethylene glycol medium (MW 6000).

Group V (n = 12 dogs)
In vivo LCCA ligation for 60 minutes.
Group Va (n = 6 dogs)—pretreatment with normal saline.
Group Vb (n = 6 dogs)—pretreatment with propranolol (2 mg/kg).

In vitro incubation of control and ischemic tissue slices in isosmolar KRP-succinate medium with and without propranolol (0.01 mg/ml medium).

trol anterior papillary muscle slices was evaluated under a variety of *in vitro* incubation conditions (Figure 2). After incubation at 37 C for 120 minutes in oxygenated isosmolar KRP medium, total tissue water was 3.88±0.06 (SEM) g (ml) H_2O per gram dry weight. This value did not differ significantly from the total tissue water (3.87±0.05) of slices that were not incubated but prepared directly after excision of the heart. After incubation at 37 C for 120 minutes, inulin-impermeable space was 2.65±0.07, and inulin-diffusible space was 1.23±0.05 g (ml) H_2O per gram dry weight. After incubation at 0 C for 60 minutes, total tissue water increased by 35% and inulin-impermeable space by 52.5%, but inulin-diffusible space was unchanged. After incubation at 0 C for 60 minutes followed by incubation at 37 C for 60 minutes, total tissue water and inulin-impermeable space returned to or close to values obtained after incubation at 37 C for 120 minutes.

The data indicate that: 1) control slices respond to cold shock by developing cell swelling (increase in total water and inulin-impermeable space without an increase in inulin-diffusible space), and 2) control slices have the capacity to reverse cell swelling induced by cold shock when the slices are subsequently incubated at 37 C.

Comparison of Control and Ischemic Tissue Slices Incubated in Isosmolar Media

Following incubation of ischemic slices from Group I dogs at 37 C for 120 minutes, total tissue water was increased above control slice values by 24.0% in ischemic papillary muscle slices and 25.8% in subendocardial slices and was not increased in ischemic subepicardial slices (Figures 2 and 3). Inulin-diffusible space was increased by 100% in ischemic papillary muscle slices, 97.6% in subendocardial slices and 28.2% in subepicardial slices. Values for inulin-impermeable space of ischemic and control slices were similar. Following incubation at 0 C for 60 minutes, ischemic papillary muscle, subendocardial and subepicardial slices exhibited greater increases in total tissue

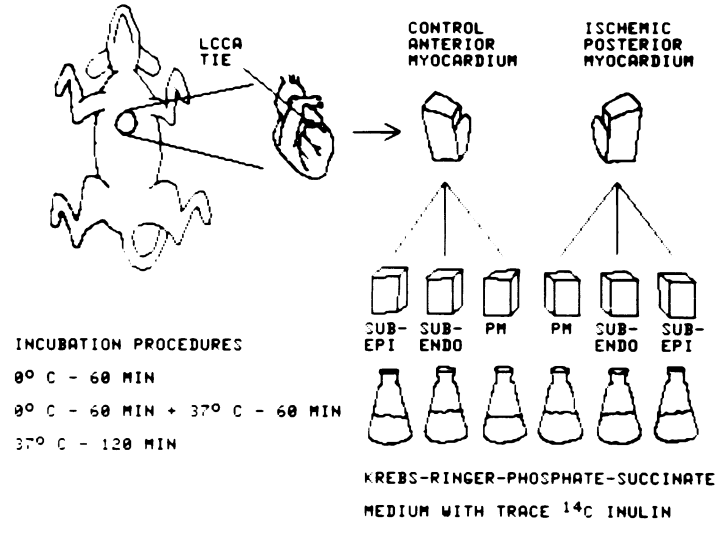


Figure 1—The illustration shows general features of the experimental protocol. *In vivo* myocardial ischemia is produced in dogs by permanent ligation of the left circumflex coronary artery for 30 to 60 minutes. The heart is then excised, and transmural blocks of control anterior and ischemic posterior myocardium are removed. Thin myocardial tissue slices are cut free-hand with razor blades from the papillary muscle, subendocardial free wall, and subepicardial free wall of the control and ischemic regions. The slices are incubated *in vitro* in Krebs-Ringer-phosphate (KRP) or KRP-succinate media containing trace ^{14}C-inulin. Incubation conditions consist of 37 C for 120 minutes, O C for 60 minutes, or O C for 60 minutes plus 37 C for 60 minutes. Following incubation, total tissue water, inulin-diffusible space and inulin-impermeable space are determined.

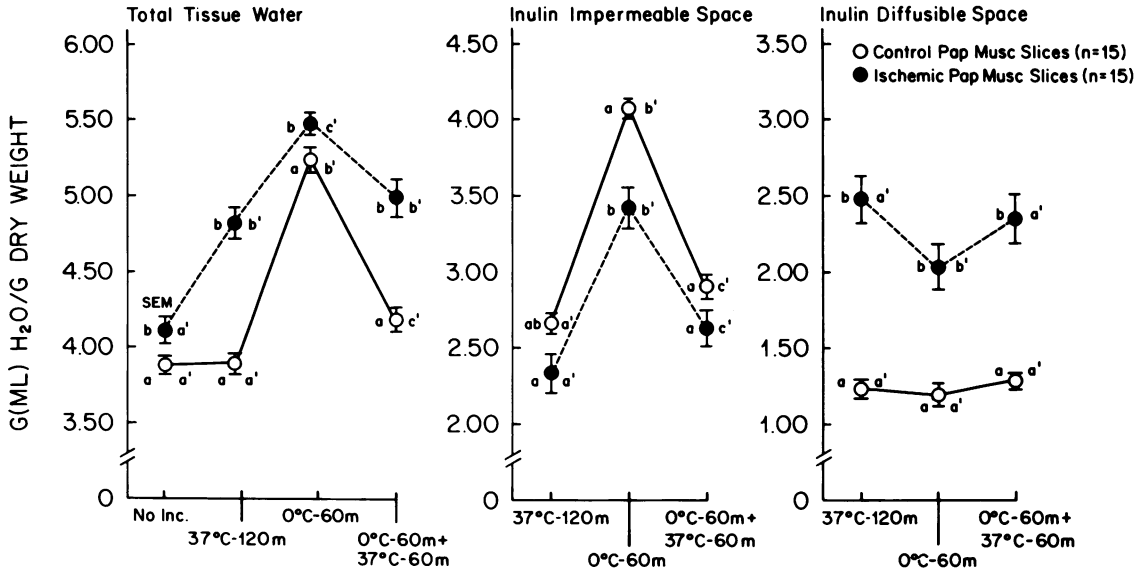


Figure 2—Response to various experimental conditions (no incubation or incubation at 37 C for 120 minutes, O C for 60 minutes, or O C for 60 minutes plus 37 C for 60 minutes) of control and ischemic papillary muscle tissue slices from dogs after 60 minutes coronary occlusion (Group I). Statistical significance of the data shown in Figures 2 and 3 was evaluated in the same three-way analysis of variance (dog as a random effect and slice group and experimental condition as fixed effects). Since the analysis showed significant interactions between slice groups and experimental conditions, separate comparisons were made of the response of the different slice groups for each experimental condition (vertical comparisons of slice groups in Figures 2 and 3 indicated by the a, b, c series) and of the response of the same slice group to the four experimental conditions (horizontal comparisons indicated by the a', b', c' series) using Newman-Keuls multiple range testing to determine specific differences in the groups.[30] For each analysis, all data points with different letters are significantly different, and those with the same letter are not significantly different. In response to cold shock and rewarming, control papillary muscle slices exhibit reversible increases in total tissue water and inulin-impermeable space without change in inulin-diffusible space. Ischemic papillary muscle slices show an abnormal response, characterized by elevated total tissue water and inulin-diffusible space, under all experimental conditions tested.

water than control slices. The increases in total tissue water were associated with prominent elevations in inulin-diffusible space, while inulin-impermeable space was below or at the level of control slices. After incubation at 0 C for 60 minutes followed by incubation at 37 C for 60 minutes, the total tissue water of all ischemic slices and the inulin diffusible space of ischemic papillary muscle and subendocardial slices remained elevated above values for control slices. These results indicate that the ischemic slices exhibited im-

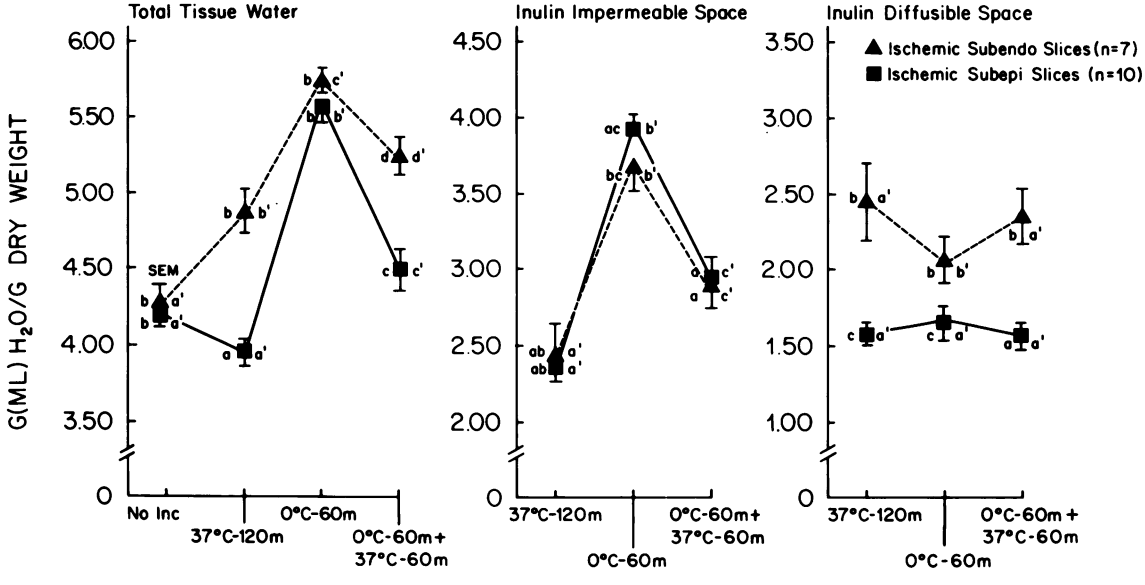
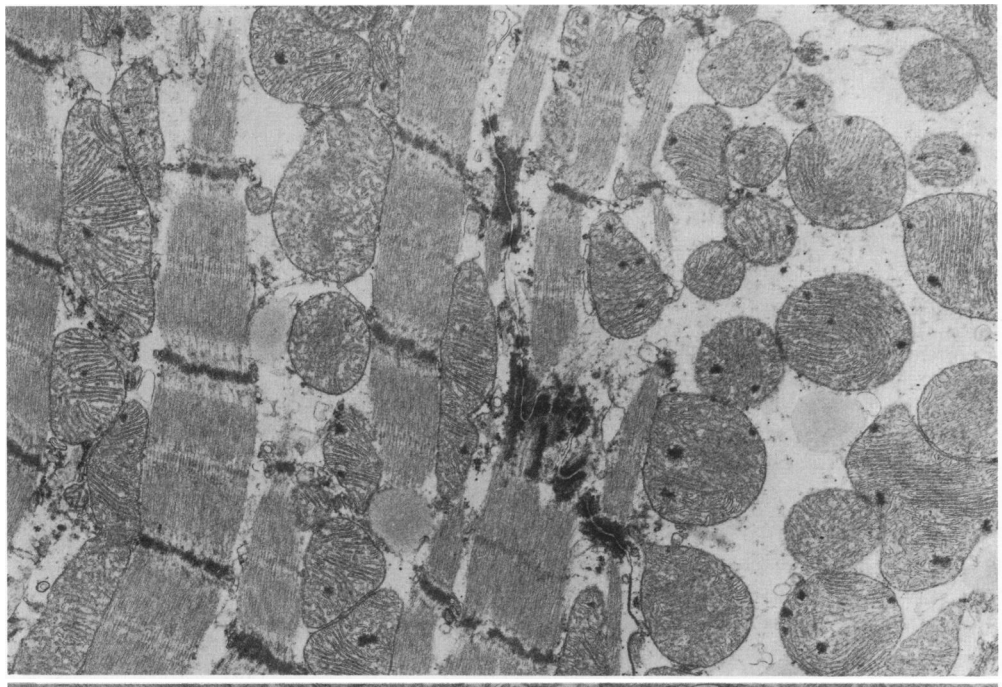


Figure 3—Response to various experimental conditions of ischemic subendocardial and subepicardial slices from dogs after 60 minutes of coronary occlusion (Group I). Statistical analysis of the data is described in the legend to Figure 2. Ischemic subendocardial slices show a response similar to ischemic papillary muscle slices (Figure 2). Ischemic subepicardial slices exhibit less marked but usually significant changes in total tissue water and inulin-diffusible space, compared with control slices (see Figure 2).



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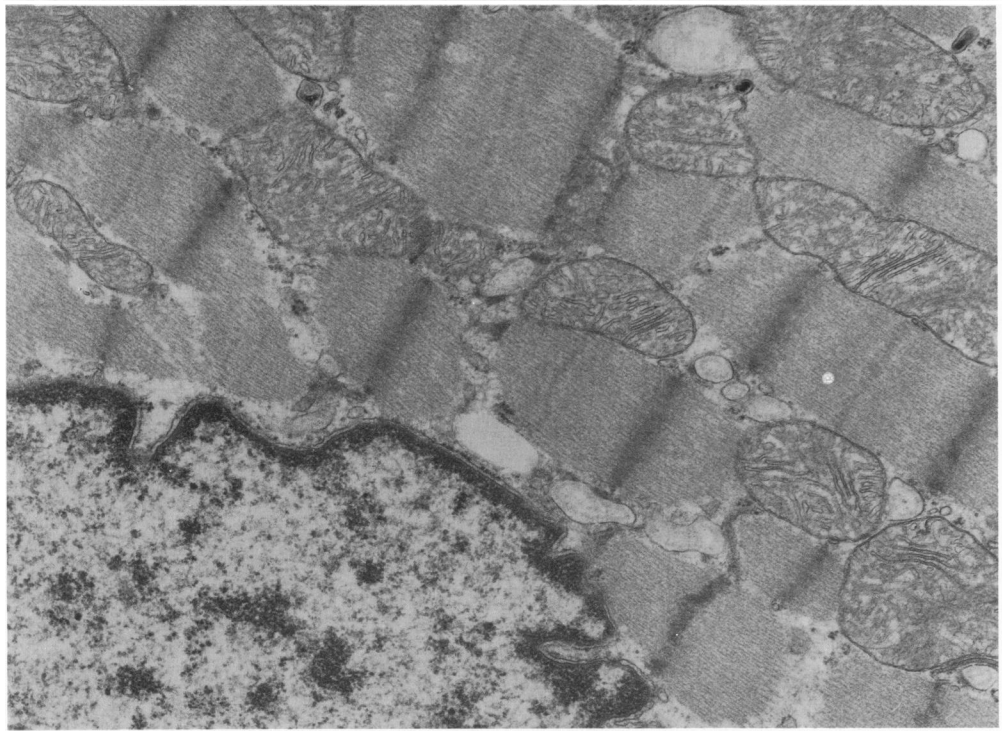

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Figures 4 and 5—Ultrastructural alterations of muscle cells from the subendocardium of the ischemic zone of a dog subjected to 60 minutes of coronary occlusion *in vivo*. Samples were prepared after *in vivo* ischemia without *in vitro* incubation. **Figure 4**—Severely damaged muscle cells show total glycogen depletion and mitochondria with amorphous matrix (flocculent) densities indicative of established irreversible injury. One muscle cell shows mild edema characterized by slight separation of the organelles. The other muscle cell shows more prominent edema. (× 10,000) **Figure 5**—A muscle cell with less severe injury exhibits glycogen depletion, slight edema, and mitochondria with clear matrices but no amorphous matrix densities. (× 17,800)

Table 2—Water Content and Inulin Spaces of Control and Ischemic Tissue Slices After Cold (0 C) Plus Warm (37 C) Incubations (60 Minutes Each) in Isosmolar Krebs-Ringer-Phosphate-Succinate Medium

	Control slices			Ischemic slices		
Categories	TTW	IIS	IDS	TTW	IIS	IDS
Group II: 30-minute coronary occlusion (n = 10)						
Papillary muscle slices	3.93 ± 0.09	2.91 ± 0.05	1.02 ± 0.05	4.43 ± 0.09*	3.20 ± 0.07*	1.23 ± 0.06*
Subendocardial slices	3.93 ± 0.12	2.92 ± 0.09	1.01 ± 0.06	4.40 ± 0.11*	2.94 ± 0.07	1.46 ± 0.08*
Subepicardial slices	4.00 ± 0.11	2.99 ± 0.07	1.02 ± 0.07	4.19 ± 0.10*	2.94 ± 0.06	1.25 ± 0.08*
Groups III, IV, Va: 60-minute coronary occlusion (n = 20)						
Papillary muscle slices	3.68 ± 0.07	2.67 ± 0.07	1.01 ± 0.04	4.59 ± 0.06*	2.78 ± 0.07*	1.81 ± 0.09*
Subenodcardial slices	3.66 ± 0.06	2.54 ± 0.07	1.12 ± 0.05	4.53 ± 0.07*	2.74 ± 0.09*	1.80 ± 0.12*
Subepicardial slices	3.77 ± 0.06	2.66 ± 0.07	1.11 ± 0.03	4.11 ± 0.07*	2.83 ± 0.06*	1.28 ± 0.06

Note: Data are presented as mean ± standard error of the mean. Units are g (ml) H_2O per g dry weight.
* Ischemic value is significantly different from control value (Newman-Keuls test).
Abbreviations: TTW = total tissue water; IIS = inulin-impermeable space; IDS = inulin-diffusible space.

paired volume regulation in response to cold shock and subsequent incubation at 37 C. Total tissue water, however, was lower after warm only and cold plus warm incubations than after cold incubation alone, indicating partial reversal of cold-induced swelling by ischemic tissue slices. The differences in total tissue water of ischemic tissue slices after various incubation conditions were accompanied by directionally similar but greater differences in inulin-impermeable space. The differences in inulin-impermeable space, however, may have been accentuated by differences in inulin-diffusible space, which appeared to be related in part to different incubation times (60 minutes versus 120 minutes).

Table 2 and Figure 6 present data obtained without interventions for control and ischemic slices from dogs in Groups II, III, IV, and Va (no propranolol *in vivo* or *in vitro*). All slices were incubated at 0 C for 60 minutes followed by incubation at 37 C for 60 minutes in isosmolar (300 mOsm) modified KRP medium with 20 mM succinate. The interval of *in vivo* coronary occlusion was 30 minutes for Group II (n = 10) and 60 minutes for Groups II-Va (n = 20). The results indicate that elevated total tissue water and inulin-diffusible space occurred after 30 and 60 minutes of myocardial ischemia in papillary muscle, subendocardial, and subepicardial slices, and that the inulin-impermeable space of ischemic slices was generally increased

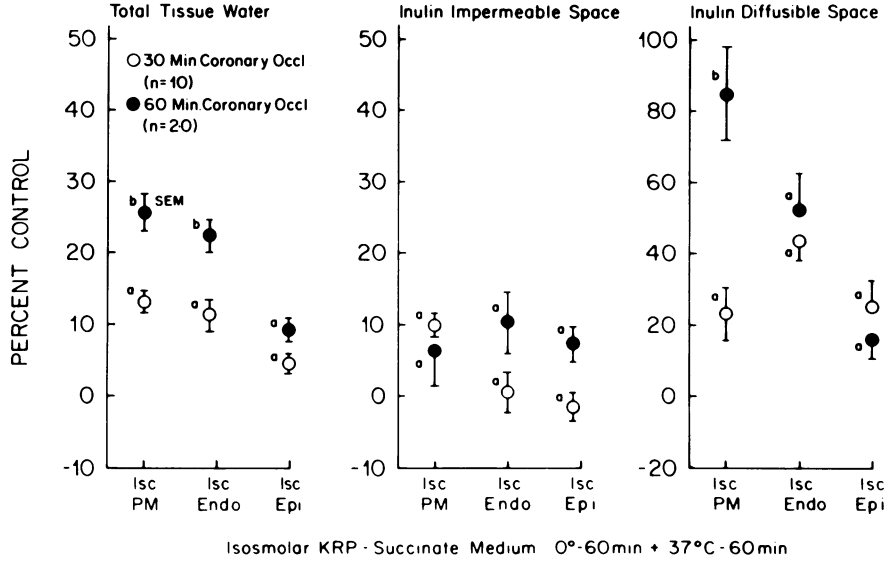


Figure 6—Comparison of percent changes from control slice values observed for ischemic myocardial tissue slices after 30 minutes coronary occlusion (Group II) and after 60 minutes of coronary occlusion (Groups III, IV, Va combined). Statistical significance was evaluated by a three-way analysis of variance (slice group and duration of coronary occlusion as fixed effects and dog as a random effect nested within groups) followed by Newman-Keuls multiple-range testing to determine specific differences in the groups.[30] Data points for the 30-minute and 60-minute groups with different letter designations (a, b) are significantly different, and those with the same letter designations are not significantly different. The percent increase in total tissue water is significantly greater in ischemic papillary muscle (PM) and subendocardial (Endo) slices but not in subepicardial (Epi) slices after 60 minutes of coronary occlusion, compared with 30 minutes of coronary occlusion. The increase in total tissue water is accompanied by a marked increase in the inulin-diffusible space of the papillary muscle slices.

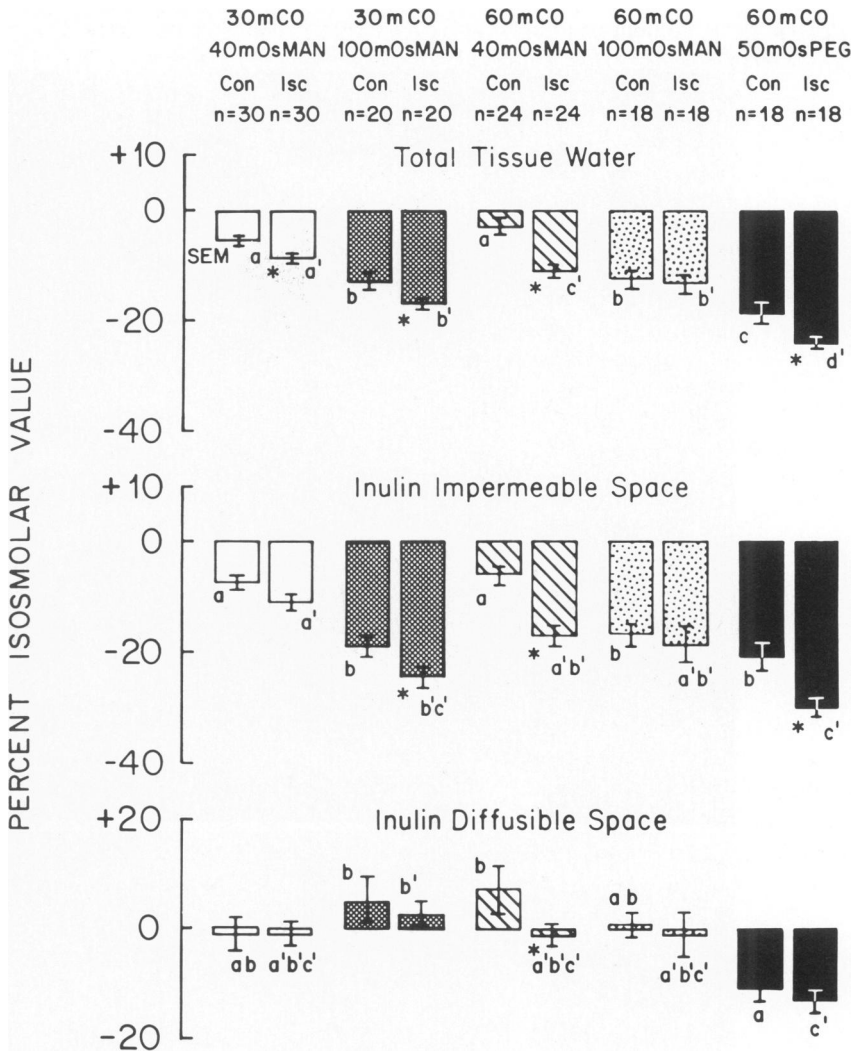
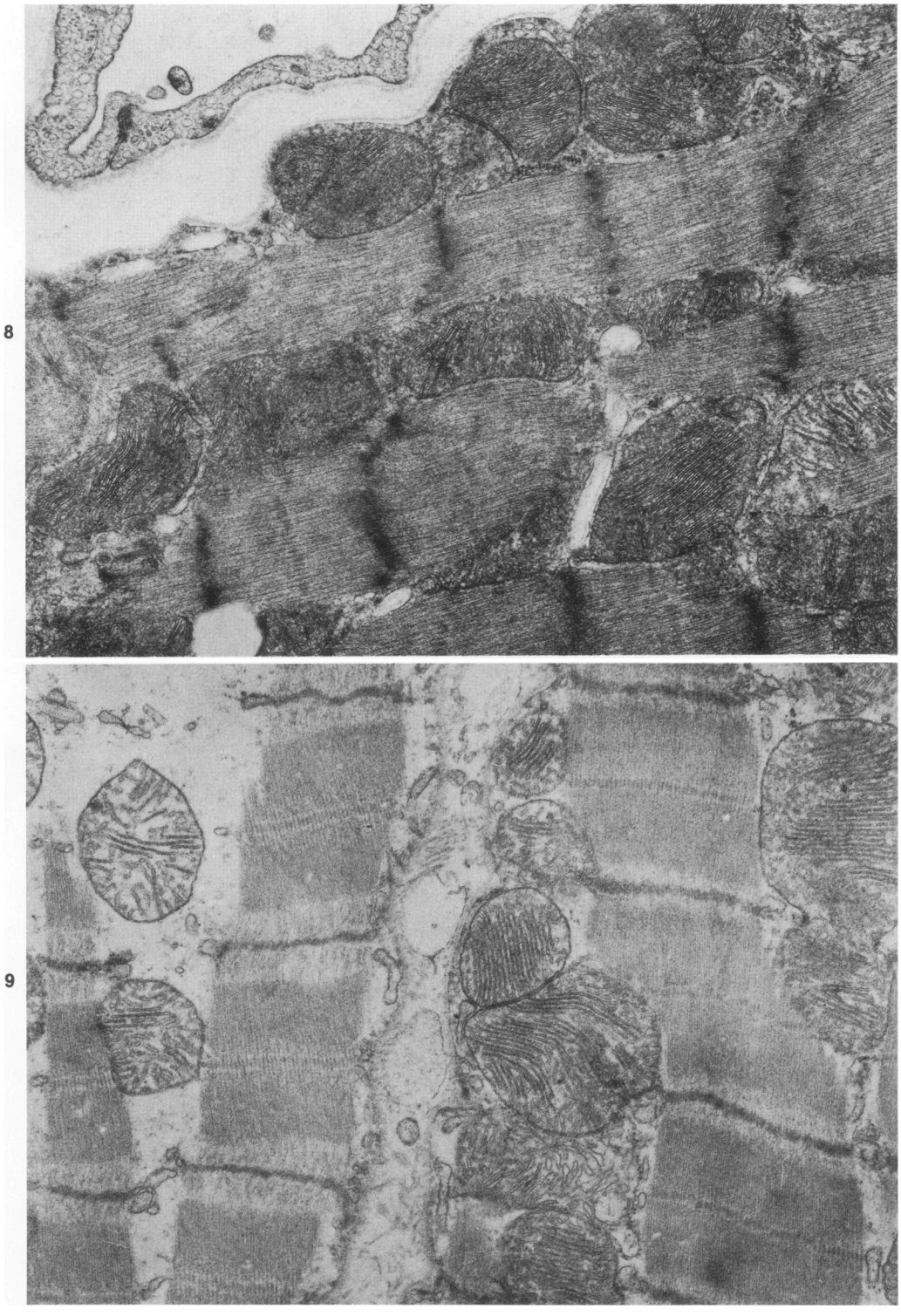


Figure 7—Percent change from isosmolar values produced by incubation (0 C for 60 minutes plus 37 C for 60 minutes) of control and ischemic slices in hyperosmolar Krebs-Ringer-phosphate-succinate mediums. Slices were obtained from dogs after 30-minute coronary occlusion (30M CO) (Group II) and 60-minute coronary occlusion (60M CO) (Groups III and IV). Mediums were made hyperosmolar by the addition of 40 mOsm or 100 mOsm mannitol (MAN) (MW 182) or 50 mOsm polyethylene glycol (PEG) (MW 6000). Data for slices of papillary muscle, subendocardium, and subepicardium were combined. Statistical significance was evaluated by a three way analysis of variance (occlusion/intervention groups and control versus ischemic slice groups as fixed effects and dog as a random effect nested within groups) followed by Newman-Keuls multiple range testing to determine specific differences in the groups.[30] The asterisks (*) indicate significant differences between control (Con) and ischemic (Isc) slices after incubation in a certain type of hyperosmolar medium. Statistical comparisons of the response of control slices to different hyperosmolar media are indicated by the a, b, c series, and the response of ischemic slices by the a′, b′, c′ series. For each analysis, all groups sharing the same letter designation are not significantly different. Each group shows prominent reductions in total tissue water and inulin-impermeable space of ischemic as well as control slices, with little change in inulin-diffusible space. The greatest effects are observed with 50 mOsm polyethylene glycol.

after 60 minutes coronary occlusion (Table 2). The percentage of increase in total tissue water was significantly greater in ischemic papillary muscle and subendocardial slices but not in subepicardial slices after 60 minutes coronary occlusion compared to 30 minutes coronary occlusion (Figure 6). The increase in total tissue water was accompanied by a marked increase in the inulin-diffusible space of the ischemic papillary muscle slices after 60 minutes coronary occlusion. (Figure 6)

Effect of Osmotically Active Agents

Figure 7 presents the findings related to *in vitro* incubation of control and ischemic slices in various hyperosmolar media, with the results expressed as percent change from the isosmolar values for each experimental group. Osmolarity of the medium was increased by the addition of 40 or 100 mOsm mannitol (MW 182) or 50 mOsm polyethylene glycol (MW 6000). Data for slices of papillary muscle, subendocardium, and subepicardium were combined, since similar changes occurred in slices from all regions. For both control and ischemic slices, the hyperosmolar agents reduced total tissue water as a result of major change in inulin-impermeable space, while little change occurred in inulin diffusible space. The reductions were significantly greater for ischemic than for control slices with the exception of 100 mOsm mannitol incubation after 60 minutes coronary occlusion. After 30 and 60 minutes coronary occlusion, 100 mOsm mannitol produced greater reductions in total tissue water of control and ischemic slices than did 40 mOsm mannitol. However, 50mOsm polyethylene glycol produced greater reductions in total tissue water of control and ischemic slices than either 40 or 100 mOsm mannitol. Differences in inulin-impermeable space were less distinct because



Figures 8 and 9—Ultrastructural features of control and ischemic myocardial tissue slices after incubation in isosmolar KRP-succinate medium at 0 C for 60 minutes and 37 C for 60 minutes. **Figure 8**—Muscle cell from a control myocardial tissue slice exhibits intact ultrastructure. (× 18,450) **Figure 9**—After 60 minutes of *in vivo* ischemia and *in vitro* incubation, muscle cells from ischemic papillary muscle slice exhibit mild to marked cytoplasmic and mitochondrial edema and swelling. (× 10,420)

of the influence of slight differences in inulin-diffusible space.

Electron Microscopy of Tissue Slices After Isosmolar and Hyperosmolar Incubations

Electron-microscopic examination of control slices following isosmolar incubation showed that the ultrastructure of most muscle cells was generally intact, although the mitochondria frequently exhibited clearing of the matrix and slight swelling (Figure 8). Some control muscle cells also exhibited generalized cytoplasmic swelling. Ischemic slices showed large numbers of damaged muscle cells, which exhibited variable degrees of cellular and organellar edema and swelling with and without structural defects in the plasma membrane (Figure 9). Although many muscle cells had prominent edema, some muscle cells showed little evidence of edema. No obvious change was noted in the size of the interstitial space between ischemic and control slices.

Ultrastructural examination revealed a mixed population of muscle cells in ischemic slices after incubation with mannitol or polyethylene glycol (Figures 10–12). Some muscle cells showed little or no cell or organelle swelling, others had minimal cytoplasmic edema but did show mitochondrial swelling, and others exhibited marked cytoplasmic and organellar edema. The most severely swollen cells frequently exhibited prominent disruption of the plasma membrane, whereas the cells with mild or no edema had anatomically intact cell membranes.

Table 3 presents results of quantitative electron microscopic determinations of the percent swollen muscle cells in control and ischemic papillary muscle slices after isosmolar and hyperosmolar incubations. The criteria for cell swelling consisted of separation of intracellular organelles and separation of the sarcolemma from adjacent organelles. Ischemic slices incubated in isosmolar medium exhibited a significantly higher percentage (67.8%) of swollen cells, compared with control slices (24.6%). A significant reduction in the percentage of swollen cells was detected in ischemic slices incubated in 50 mOsm polyethylene glycol medium (44.1%) but not in ischemic slices incubated in 100 mOsm mannitol medium (60.9%). No striking differences in other features of cell injury were observed in the ischemic slices incubated in isosmolar and hyperosmolar media.

Effect of Propranolol

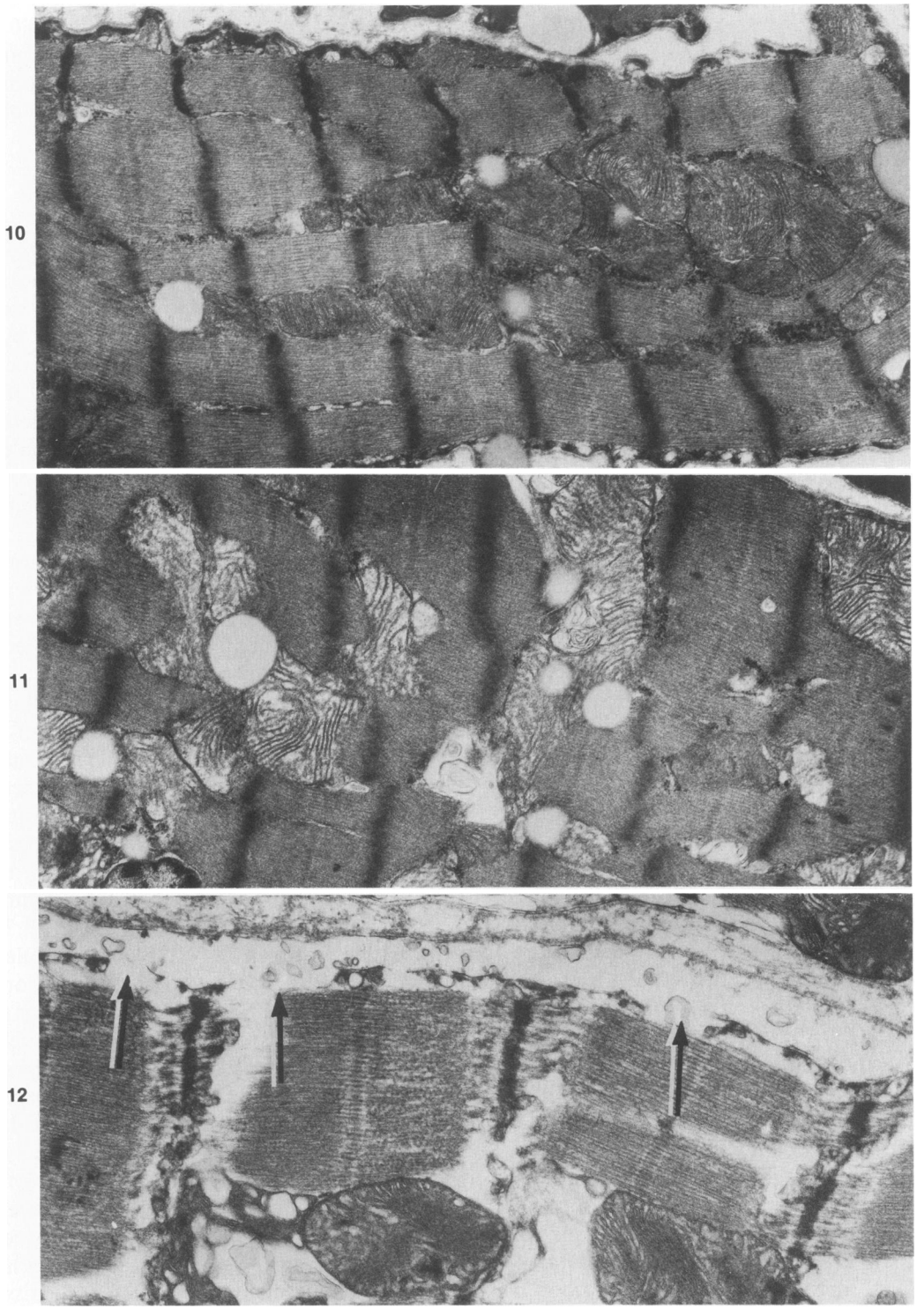
Significant elevations (group *t* test) of total water and inulin space occurred after 60 minutes of coronary occlusion with or without propranolol administered *in vivo* (2 mg/kg) and/or *in vitro* (0.01 mg/ml medium (Table 4). There were no significant differences in the values for ischemic and nonischemic slices between the control and propranolol groups (group *t* tests).

Discussion

The data obtained from an *in vitro* myocardial tissue slice technique have provided quantitative information regarding the severity and extent of ischemic membrane damage produced by 30–60 minutes of experimental canine coronary occlusion. The data also have characterized certain effects of osmotically active agents and propranolol on ischemic myocardial tissue. The information obtained was based on the differential response of control and ischemic slices to *in vitro* incubation in balanced salt solutions.

In the present study, control values for total water and inulin spaces were comparable to those reported by others for myocardial tissue slices.[5,6] The absolute values for control and ischemic slices were lower when incubations were performed in KRP medium containing succinate than in KRP medium. This result could be due to a beneficial effect of the succinate on mitochondrial function,[25] but an additional factor is that the initial experiments were performed with KRP medium and later experiments with KRP–succinate medium. The control values for tissue slices incubated *in vitro* were higher than those reported for intact cardiac muscle. For example, Polimeni and Al-Sadir reported a total tissue water of 3.34–3.35, extracellular water of 0.77–0.78, and cellular water of 2.56–2.58 (ml H_2O/g dry weight) for normal rat myocardium.[33] The differences between intact cardiac muscle and tissue slices are probably related to the presence of damaged cells on the cut edges as well as some expansion of the extracellular space of the slices.[5,34] Another possible factor is that the damaged surface cells of the tissue slices could exert adverse effects on adjacent cells by means of electrolyte shifts across nexal junctions. These problems, however, are not limited to myocardial slices, since many cell types have nexal junctions.[35] These considerations have not precluded the successful application of the tissue slice technique for metabolic studies of many tissues.[19]

Slices of control nonischemic myocardium exhibited evidence of normal cell volume regulation in response to cold shock and rewarming. This response of control slices was characterized by an increase in the total tissue water and inulin-impermeable space



Figures 10, 11 and 12—Ultrastructural features of muscle cells from ischemic papillary muscle slices after incubation with 50 mOsm polyethylene glycol (Group IV). Qualitatively similar findings were observed with mannitol. **Figure 10**—This muscle cell with intact plasma membrane is free of cytoplasmic or mitochondrial swelling. (× 20,000) **Figure 11**—This muscle cell lacks generalized cytoplasmic edema but has mitochondria that show some swelling and clearing of the matrix. (× 15,000) **Figure 12**—A muscle cell with prominent swelling exhibits large defects in the plasma membrane (*arrows*). (× 17,310)

Table 3—Electron Microscopic Analysis of Muscle Cell Swelling in Control and Ischemic Papillary Muscle Tissue Slices Incubated at 0 C and 37 C (60 Minutes Each) in Isosmolar or Hyperosmolar Media After 60 Minutes of Coronary Occlusion (Group IV)

Categories	Number (%) swollen muscle cells
1. Control slices: Isosmolar medium	50/203 (24.6%)[a]
2. Ischemic slices: Isosmolar medium	141/208 (67.8%)[b]
3. Ischemic slices: 100 mOsm mannitol medium	126/207 (60.9%)[b]
4. Ischemic slices: 50 mOsm polyethylene glycol medium	93/211 (44.1%)[c]

Note: Superscript letters (a, b, c) indicate the results of chi-square statistical analysis. Categories 1 and 4 are significantly different from all other categories, and categories 2 and 3 are not significantly different from each other at the $\alpha = 0.05$ significance level.

during cold incubation and a return of these parameters toward baseline values upon rewarming (Figures 2 and 3). The inulin-diffusible space remained constant during both cold and warm incubations. Since inulin appears to be excluded from normal cells,[5,34,36] the results are consistent with cell swelling during cold-induced inhibition of energy metabolism followed by a return to normal cell volume during rewarming. Cold-induced swelling of myocardial tissue slices also is accompanied by an increase in Na^+ and decrease in K^+, and these electrolyte changes are reversed upon rewarming.[5,6,19] Swelling of control tissue slices also can be induced by incubation with ouabain.[19,35] The electrolyte changes and response to ouabain suggest a major role for Na^+-K^+ ATPase in normal cell volume regulation.[19] Under certain experimental conditions, however, an apparent dissociation has been demonstrated between cell volume regulation and Na^+-K^+ exchange pump activity of myocardial tissue slices.[38]

Ischemic slices exhibited increased total tissue water and inulin-diffusible space under all *in vitro* conditions tested, including incubation at 37 C for 120 minutes as well as after cold plus warm incubations. Abnormalities in total tissue water and inulin spaces showed a transmural distribution after 30 minutes of coronary occlusion and became more severe in the papillary muscle and subendocardium after 60 minutes of coronary occlusion (Table 2, Figure 6). The response of ischemic slices is interpreted as indicating defective cell volume regulation, cell swelling, and increased plasma membrane permeability due to membrane damage induced by ischemia *in vivo*. The defective cell volume regulation and cell swelling were manifested by the increase in total tissue water and the increased membrane permeability by the increase in inulin-diffusible space. Morphologic evidence of extensive cell damage and swelling in ischemic slices supported this interpretation and tended to negate other theoretical interpretations of the inulin space data. In addition, other workers have shown that alterations of total tissue water and inulin-diffusible space of ischemic slices are accompanied by alterations in electrolytes (marked increase in Na^+ and marked decrease in K^+) and in the content and metabolism of high-energy phosphates.[6,8]

The *in vitro* tissue slice technique does not provide information regarding the degree of cell swelling that may exist *in vivo* or in isolated heart preparations. Rather, the technique appears to provide evidence of membrane damage, which becomes manifest by cell swelling and abnormal inulin permeability when the damaged tissue is exposed to an infinite volume of

Table 4—Effect of Propranolol Administered *in Vivo* and/or *in Vitro* on Total Issue Water (TTW), Inulin-Diffusible Space (IDS), and Inulin-Impermeable Space (IIS) of Tissue Slices From All Control and Ischemic Regions (Papillary Muscle, Subendocardium, and Subepicardium) of Dogs After 60 Minutes of Coronary Occlusion (Group V)

	TTW		IIS		IDS	
Categories	Control	Ischemic	Control	Ischemic	Control	Ischemic
Group Va: Dogs treated with saline KRP-succinate medium	3.59 ± 0.05	4.31 ± 0.08 ($P < 0.001$)	2.41 ± 0.06	2.86 ± 0.09 ($P < 0.001$)	1.19 ± 0.04	1.45 ± 0.10 ($P < 0.05$)
Medium with propranolol (0.01 mg/ml)	3.61 ± 0.05	4.30 ± 0.10 ($P < 0.001$)	2.34 ± 0.06	2.71 ± 0.10 ($P < 0.01$)	1.27 ± 0.03	1.59 ± 0.12 ($P < 0.02$)
Group Vb: Dogs treated with propranolol (2 mg/kg) *in vivo* KRP-succinate medium	3.57 ± 0.04	4.23 ± 0.10 ($P < 0.001$)	2.43 ± 0.05	2.83 ± 0.07 ($P < 0.001$)	1.14 ± 0.03	1.40 ± 0.06 ($P < 0.001$)
Medium with propranolol (0.01 mg/ml)	3.64 ± 0.06	4.12 ± 0.12 ($P < 0.01$)	2.46 ± 0.08	2.77 ± 0.12 ($P < 0.05$)	1.18 ± 0.04	1.35 ± 0.05 ($P < 0.02$)

Note: 1) Data are presented as mean values of 18 samples ± standard error of the mean. Units are g (ml) H_2O per g dry weight. Data for slices of papillary muscle, subendocardium, and subepicardium are combined. 2) All slices were incubated at 0 C for 60 minutes, followed by 37 C for 60 minutes. Both incubations were performed in isosmolar KRP-succinate medium. 3) Statistical comparisons (group *t* tests) of the control and ischemic values are shown.

extracellular fluid. In this respect, *in vitro* incubation of tissue slices is similar to the effect produced by reperfusion of ischemic myocardium *in vivo*.

Results of the *in vitro* tissue slice studies are consistent with evidence from other studies that important membrane alterations develop early in the course of ischemic and hypoxic myocardial injury. This evidence includes decreased activity of Na^+-K^+-ATPase in the canine heart after one to two hours of permanent or temporary ischemic injury[10,14] and cardiac muscle cell swelling and electrolyte alterations, including calcium accumulation, following temporary coronary occlusion in the dog.[4,25,32] In other studies with isolated cat papillary muscles and perfused rat hearts, hypoxia, with and without substrate deprivation, resulted in morphologic evidence of swelling of muscle cells and mitochondria[7,39] and abnormal membrane permeability to ionic lanthanum.[15,16] Pine et al, however, reported that isolated rat papillary muscles failed to develop an increased myocardial water content after 60 minutes of hypoxia or total metabolic blockade with hypoxia plus inhibition of glycolysis.[40] Variation in response of different isolated muscle preparations to hypoxia and other components of the ischemic process may be related to species differences and to differences in severity of injury produced in each study. The basis for membrane alterations in ischemic and hypoxic myocardium is under active investigation, one area of major interest involving accelerated phospholipid degradation.[11,41,42]

The response of ischemic tissue slices to incubation with osmotically active agents indicated that hyperosmolar incubation retarded the characteristic *in vitro* swelling of ischemic tissue slices. Polyethylene glycol (MW 6000) and mannitol (MW 182) consistently produced reductions in total water and inulin-impermeable space of ischemic as well as control slices after 30 or 60 minutes of coronary occlusion without producing major change in the inulin-diffusible space (Figure 7). The reductions in total tissue water and inulin-impermeable space occurred in tissue slices from all ischemic regions, including the maximally ischemic papillary muscles. The morphologic findings, including the quantitative cell counts (Table 3), were consistent with the water and inulin space measurements. Muscle cells exhibited variable degrees of damage in samples obtained from the ischemic zone prior to *in vitro* incubation (Figures 4 and 5). Following isosmolar incubation (Figures 8 and 9), ischemic tissue slices showed muscle cells with prominent edema and structural defects in the plasma membranes as well as muscle cells with ultrastructually intact plasma membranes and variable degrees of cytoplasmic and organellar edema and swelling.

Some of the effect produced by hyperosmolar incubation on ischemic tissue slices could be explained by a reduction in water content of a subpopulation of normal cells and by retarded swelling of mitochondria and other organelles of cells with severe plasma membrane damage. Nevertheless, the major difference between ischemic slices incubated in isosmolar and hyperosmolar media appeared to be a reduction in severity of cytoplasmic and mitochondrial edema of muscle cells with ultrastructurally intact plasma membranes (Table 3, Figures 10–12). The greater response to 50 mOsm polyethylene glycol than to 40 or 100 mOsm mannitol could be explained either on the basis of a greater reduction of water content of the same cell population or by a more widespread effect of the larger molecular weight agent on cells with intermediate as well as milder forms of plasma membrane damage. Although electron microscopy did not reveal a major difference in the percent swollen cells in ischemic slices after incubation with 100 mOsm mannitol, this agent produced significant reductions in the measured water content of the slices.

The patterns of response of ischemic slices to cold shock and rewarming (Figures 2 and 3) and to hyperosmolar incubation (Figure 7) support the concept that early ischemic injury results in a mixed population of muscle cells with variable degrees of plasma membrane damage. According to this view, muscle cells with severe plasma membrane damage would be characterized by abnormal permeability to inulin and lack of responsiveness to osmotically active agents. Other muscle cells with less severe plasma membrane damage would be characterized by *in vitro* cell swelling without inulin permeability and by responsiveness to osmotically active agents. It also follows that the inulin-impermeable space of ischemic slices would be composed of a smaller number of cells with increased water content, compared with control slices. This interpretation implies that ischemic cellular and membrane injury does not progress uniformly, even in a discrete microanatomic region, but that the rate of progression varies on an individual cellular basis. Similar findings have been observed with hypoxic injury in an isolated cat papillary muscle preparation.[15,16]

The *in vitro* response of ischemic tissue slices to hyperosmolar incubation has provided insight into the beneficial effects of osmotically active agents on ischemic injury in intact animals[3,9,12,13,20,21,43–45] and on hypoxic injury in isolated muscle preparations.[7,39] During relatively short periods of ischemic injury in dogs, moderate elevation of serum osmolality by

40–50 mOsm with mannitol has been shown to improve total and collateral coronary blood flow, improve ventricular function, modify the severity of the reduced reflow phenomenon, retard cell swelling, and reduce myocardial infarct size.[3,9,12,13,20,21,43–45] In an isolated heart preparation subjected to hypoxia and substrate deprivation, perfusion with mannitol or polyethylene glycol retarded explosive cell swelling and creatine kinase release induced by reoxygenation; polyethylene glycol had a greater beneficial effect than mannitol.[7] The degree of hyperosmolarity appears critical because of the physiologic importance of cell volume regulation,[19] and 40–50 mOsm appears to be an optimal degree of hyperosmolarity *in vivo*.[3,9,12,13,20,21,43–45] In intact dog models, however, the beneficial effects of hyperosmolar mannitol were shown to be temporary, since reduced infarct size and improved coronary blood flow were observed after 60 minutes but not after 90 minutes of temporary coronary occlusion and reflow or after prolonged permanent coronary occlusion.[21,45,46] There also is evidence that long-term administration of hyperosmolar sugars may be detrimental to myocardial cells.[47]

Studies in isolated intact hearts have suggested that the temporary beneficial effects of hyperosmolar mannitol on cell injury and coronary blood flow are not related to a prevention of vascular compression, since ischemia-induced swelling of muscle cells is not severe enough to produce significant capillary compression.[14] The beneficial effects of hyperosmolarity more likely are exerted by direct effects on cardiac muscle cells and/or by direct effects on the coronary vasculature.[9,14,44] Hyperosmolar mannitol has been shown to exert a vasodilatory effect on isolated coronary arteries.[48,49] The present study has shown that osmotically active agents also can act directly to retard the swelling of ischemic muscle cells. Thus, the beneficial effects of hyperosmolar agents *in vivo* appear to be due to dual effects on coronary vasculature and cardiac muscle cells.

The myocardial tissue slice technique also was employed to evaluate the potential of the beta adrenergic blocking agent propranolol to protect against membrane damage induced by ischemia. No significant change, however, was observed in the measured parameters of volume regulation and membrane damage of ischemic slices following *in vivo* administration of 2 mg/kg of propranolol prior to coronary occlusion and/or incubation of ischemic tissue slices with 0.01 mg/ml of propranolol. The doses of propranolol used in this study have been shown to exert beta adrenergic blockade *in vivo*[24] and to have marked pharmacologic effects *in vitro*.[28,29] Peter et al reported that propranolol failed to exert a beneficial effect on regional creatine kinase depletion after coronary occlusion[50]; however, total infarct size was not measured. Several other *in vivo* studies have shown that propranolol reduces the severity of muscle cell injury, vascular damage, and infarct size after one to several hours of coronary occlusion.[22,23,24] The present study, however, has failed to demonstrate a direct protective effect of propranolol on membrane damage in ischemic myocardial tissue slices.

In summary, the results of *in vitro* experiments with myocardial tissue slices indicate that periods of 30–60 minutes of coronary occlusion in the dog result in significant membrane damage to cardiac muscle cells. The membrane damage induced *in vivo* is manifest *in vitro* by impaired cell volume regulation, cell swelling, and increased membrane permeability when muscle cells are exposed to an infinite volume of extracellular fluid. The distribution of membrane damage is transmural but is more extensive and severe in the papillary muscle and subendocardial free wall than in the subepicardium. The severity of membrane damage appears to be variable rather than uniform. The findings suggest that muscle cells with severe cellular and membrane damage admit inulin (MW 5000), which is excluded by normal cells, and these cells apparently fail to respond to incubation with the osmotically active agents, mannitol (MW 182) and polyethylene glycol (MW 6000), which normally have a predominantly extracellular distribution. The findings also suggest that muscle cells with less damaged and structurally intact plasma membranes develop abnormal cell swelling but remain impermeable to inulin and shrink upon incubation with osmotically active agents. The latter muscle cells may represent a population of reversibly injured cells that may be potentially salvagable by appropriate therapeutic interventions. Under the experimental conditions utilized, however, a protective effect of propranolol on parameters of membrane injury was not demonstrated.

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