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13. ABSTRACT *(Maximum 200 words)*

A hibernation induction trigger (HIT) molecule derived from the plasma of deeply hibernating woodchucks exerts profound effects mimicking a hibernation-like state when infused I.C.V. or I.V. in primates. The profound opiate-like behavioral and physiological depression occurring shortly after the infusion of the HIT-containing albumin fraction include hypothermia, bradycardia, long-term hypophagia and markedly depressed renal function. All of the aforementioned effects are reversed or retarded by the infusion of the opiate antagonists, naloxone and naltrexone. Such evidence enforces our hypothesis that the HIT molecule is not specific for hibernators, but that it may initiate its action through specific opioid receptor(s). We have shown that only DADLE, a *delta* class of opioid, could induce hibernation in summer-active ground squirrels (the traditional bioassay) in a fashion similar to those injected with HIT. Such evidence indicates that this *delta* opioid mimics the action of the HIT molecule and may be intimately involved in natural winter and summer-induced hibernation. Recently, we have developed a rapid *in vitro* bioassay utilizing the criteria of DNA and protein synthesis inhibition to monitor the metabolic inhibitory activity of hibernating plasma, and plasma fractions as well as the metabolic effects of the *delta* opioids DADLE and [D-Pen^{2,5}]-Enkephalin (DPDPE). We have also demonstrated the profound effects of HIT and DADLE on markedly extending effective tissue preservation time of a multiorgan autoperfusion system from baseline levels of less than 15 hours to up to 45 hours and permitting successful lung transplantation after 24 hours of preservation time following HIT infusions.

Our most recent analytical and preparative SDS-PAGE gels studies indicate that the HIT-active fraction may be identified with a 72,000 kD protein band closely associated with albumin and only occurring in winter hibernating plasma fractions. The aforementioned studies indicate that the clinical potential of a purified, characterized HIT molecule, and even the *delta* opioids, may be vast.

14. SUBJECT TERMS Hibernation Induction Trigger (HIT), Woodchucks, Delta Opioids (D-Ala^2-Leu^5-Enkephalin & [D-Pen^{2,5}]-Enkephalin--DPDPE), Multiorgan Autoperfusion System, Metabolic Inhibition (3H-Thymidine uptake, 14C-Leucine uptake)

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Peter R. Oeltgen, Ph.D. 12-7-93

PI Signature Date

TABLE OF CONTENTS

TABLES

FIGURES

INTRODUCTION

I. FURTHER DEVELOPMENTS IN THE CHARACTERIZATION OF THE OPIATE-LIKE HIBERNATION INDUCTION TRIGGER (HIT) MOLECULE(S)

Our previous work indicates that a Hibernation Induction Trigger (HIT) molecule derived from the plasma of deeply hibernating woodchucks may act as an endogenous opioid. When infused I.C.V. or I.V. into primates, it exerts profound effects mimicking a hibernation-like state. These effects, which include hypothermia, bradycardia, long-term hypophagia, and markedly depressed renal function, are all reversed or retarded by the infusion of the opiate antagonists naloxone or naltrexone,[1, 2] and Bruce, *et al.*,[3] have shown that continuous naloxone infusion will effectively block natural winter hibernation in ground squirrels. The HIT-containing-albumin fraction utilized in the aforementioned primate studies was obtained by passing heparinized plasma obtained from deeply hibernating woodchucks through an affinity column using Affi Gel Blue as the chromatography matrix. Furthermore, we have shown that only the *delta* receptor agonist D-Ala^2-Leu^5-Enkephalin (DADLE) is able to induce hibernation in summer-active ground squirrels (the traditional bioassay) in a manner similar to those injected with HIT[4]. Such evidence suggests that this *delta* opioid mimics the action of the HIT molecule and may be intimately involved in natural winter and summer-induced hibernation.

In an effort to further characterize this potent opioid-like peptide molecule, we utilized the following techniques: flat bed isoelectric focusing (IEF), SDS-PAGE, and two dimensional IEF and SDS-PAGE. For IEF, the lyophilized plasma albumin fraction was dissolved in a sample solution containing urea, methanol, and decyl sodium sulfate (DSS), and focused on a pH 4.0-6.5 IEF gel (PAG-Plate, Pharmacia). SDS-PAGE samples were dissolved in a buffer containing

Tris, Acetic Acid, SDS, and mercaptoethanol run either horizontally on a discontinuous 8-18% gradient gel (ExcelGel, Pharmacia) or vertically on the Hoeffer SE500. Two-dimensional characterization involved running a pH 4-7 Immobiline IEF strip gel (Dry Strip, Pharmacia) for the first dimension, followed by horizontal SDS-PAGE (ExcelGel, Pharmacia) in the second dimension.

SDS-PAGE separations of the summer-active affinity chromatography fraction revealed an intensely staining band with an approximate molecular weight of 70 kD. This band separated slightly below that of a lightly staining affinity chromatography fraction from winter plasma and may in fact represent a unique protein fraction occurring only in summer-active plasma and was much lighter in the winter fractionation. Separation of the winter-fraction showed a number of more intensely staining protein bands; moreover, we observed a band in both SDS-PAGE and two-dimensional separations with a M.W. of approximately 72 kD (although this M.W. determination is not fixed) that did not occur in the summer fraction. There is a great likelihood that this protein band may represent the purified HIT-active component. IEF separation of the summer-active fraction showed protein bands with a pI of approximately 4.99 and 5.06 that were not present in the winter-hibernating fraction. While differentiation of the bands was difficult, it appears that the winter fraction revealed a band at roughly pI 4.35, which was not present in the summer-active fraction.

MATERIALS AND METHODS

1. Collection of Plasma Fractions from Woodchucks.

   Plasma for these experiments was obtained from winter-hibernating and summer-active woodchucks weighing 3.0 to 5.0 kg. Hibernating woodchucks are maintained in a temperature- and humidity-controlled hibernaculum at 4-6°C, their heart rate is 1 or 2

beats per minute, and they have 1 or 2 respirations per minute. Summer-active woodchucks are anesthetized with ketamine and rompum.

2. Analytical Techniques Employed in Isolating and Characterizing the HIT Molecule(s)

A. Affinity Chromatography

The albumin fraction, which has been previously shown to contain Hibernation Induction Trigger (HIT) molecule(s), was obtained by passing heparinized plasma through an affinity chromatography column using Affi-gel Blue as the chromatography matrix following a procedure described by Travis *et. al.*[5] The plasma is pumped onto the gel and the column is eluted with 0.02M Na_2HPO_4 for absorption of the sample. The albumin fraction is removed from the column by desorbing it with 1.4M NaCl dissolved in the 0.02M Na_2HPO_4. This fraction is then desalted by placing it in Spectrapor dialysis tubing (3,500 molecular weight cut off), which is immersed in deionized water. The desalted fraction is then frozen and lyophilized.

B. Isoelectric Focusing (IEF)

Separation of a protein by its isoelectric point (pI = a physical constant of that protein) has become a widely utilized technique for protein purification. Flatbed IEF was performed on a Pharmacia Multiplior II utilizing modifications of methods described by Haglund[6] and Righetti[7].

Lyophilized material from summer-active woodchucks (SAWP) and winter-hibernating woodchucks (HWP) is solubilized in a solution containing urea, decyl sodium sulfate, and methanol. Microliter aliquots of the dissolved fractions are then placed at the cathode end of a pH 4-6.5 PAG plate and focused at 2000 volts,

25mAmps, and 25 Watts for five hours. After fixing in an solution of trichloracetic and sulfosalicylic acids, the gels are stained in a CoomassieBlue (R-250) solution and then destained in several changes of an ethanol, and acetic acid mixture. SAWP and HWP gels were compared for differences.

C. SDS-PAGE

In SDS-PAGE proteins are dissociated into their components by heating them to 100°C in the presence of 2-mercaptoethanol and sodium dodecyl sulphate (SDS). This reduces the interchain and intrachain disulfide bridges and causes the molecules to conform to a rod-like shape[8, 9]. While the diameter of the rod remains constant at about 18 angstroms, the length varies in proportion to the molecular weight of the specific peptide. This allows for molecular sieving according to the weight of the molecule. The weight range of the resolution varies according to the gel's acrylamide concentration and the amount of cross-linking. In our experiments the lyophilized albumin fraction was dissolved in a sample buffer containing tris, acetic acid, 2-mercaptoethanol, SDS, and bromphenol (as a marker for the front of the migrating proteins). After being heated to 95ÁC for three minutes, the samples were placed on sample application strips located at the cathodic side of the gel (Pharmacia ExcelGel SDS 8-18% gradient). The runs were made for approximately 80 minutes at 600 V, 50 mA, and 30 W. The peptides were fixed in an ethanol and acetic acid solution. As in IEF, the gels were stained in Coomassie Blue R-250 and then destained in another ethanol and acetic acid solution. Approximate molecular weights were determined via comparison with the migration distance of known standards run with the samples. Discontinuous vertical SDS-PAGE was also performed using a Hoeffer SE500 System. The vertrical gel is cast as a homogenous 10% tricone - SDS gel after the method of Schagger and von Jagow[10].

D. Two-Dimensional SDS-PAGE

In two-dimensional separations, isoelectric focusing (IEF) is performed followed by SDS-PAGE. The affinity fractions of the plasma from summer-active and winter-hibernating woodchucks is applied to an IEF strip gel with an immobilized pH gradient (Pharmacia Immobiline Dry Strip, pH 4-7). After electrofocusing, the gel strip is equilibrated for approximately 20 minutes in a buffer containing Tris, SDS, glycerol, DTT, and urea. The strip is then placed on the cathodic side of an SDS gel (Pharmacia ExcelGel SDS 8-18) and electrophoresed for roughly 90 minutes at 600 volts, 50 mAmps, and 30 watts. The SDS gel is fixed in an ethanol and acetic acid solution to precipitate the proteins. It is then stained by coomassie blue mixture or silver stain and destained over approximately 24 hours in another ethanol and acetic acid mixture.

RESULTS

Comparison of the IEF gels of the albumin-raction of HWP (Fig 1) and SAWP (Fig 2) reveals a more intense band of protein deposition on the pH range of 4.50 to 4.70 (cf., Figs 1 & 2). The SAWP gel, however, shows clearer band formation from pH 4.99 to 5.06 (cf., Figs. 1 & 2). Both of the gels smeared around the pI of serum albumin, which is 4.5 (Figs 1 & 2).

SDS-PAGE comparison of summer-active woodchuck plasma (SAWP), hibernating woodchuck plasma (HWP), and spring active woodchuck plasma (SpAWP) shows the seasonal variations in the protein concentrations of the plasma albumin fractions (Fig 3). However, it is noteworthy that the HWP (lanes 9-14) and SpAWP (lanes 15-20) both show clearer bands in the range just below 66 kD. The SpAWP appears to have a distinct protein just above 66 kD.

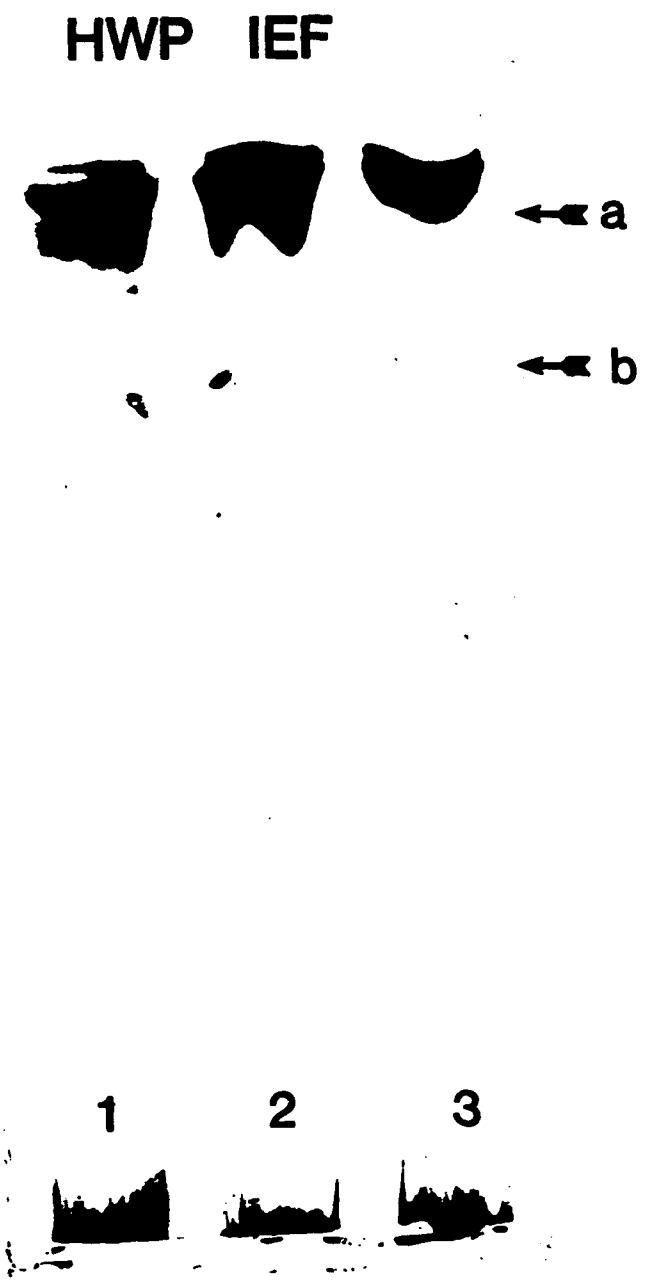

Figure 1: Isoelectric focusing of 1.0 mg of HWP (all three lanes) (a) Indicate pH ≡ 4.5; (b) Indicates pH ≡ 5.0.

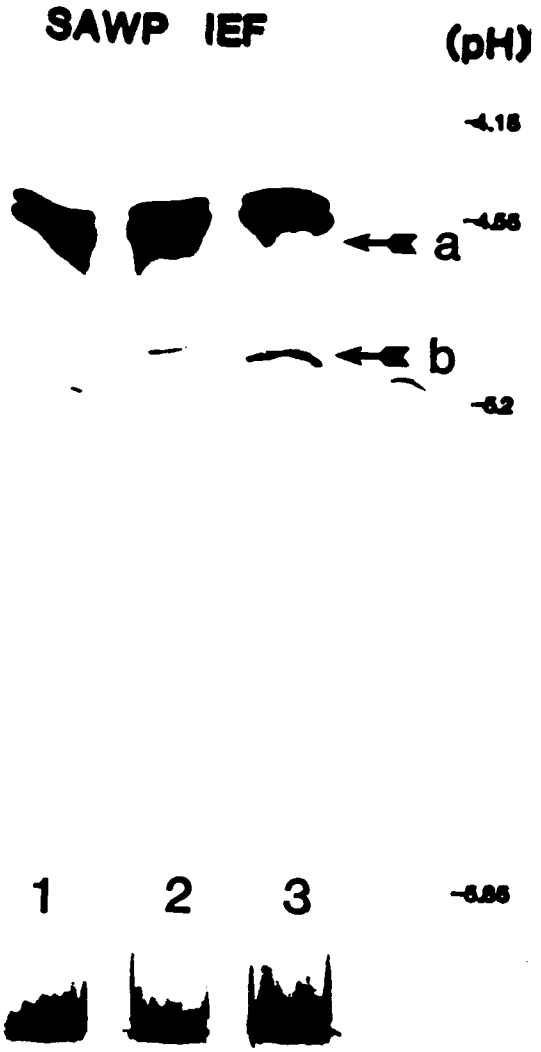

Figure 2: Isoelectric focusing of 1.0 mg of SAWP (all three lanes). (a) Indicates pH ≅ 4.5; (b) Indicates pH ≅ 5.0.

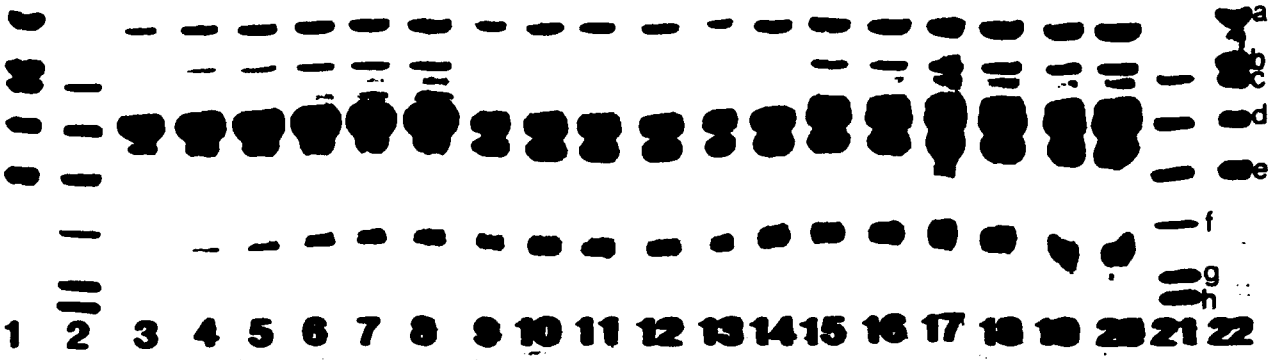

Figure 3: SDS-PAGE of the albumin fraction of SAWP(lanes 3-8), the amount of total protein varies from 15 to 30 mg. 8-18% Gradient Excel-Gel (Pharmacia), HWP (9-14), and SpAWP (lanes 15-20). Lanes 1 and 22 are the high molecular weight standards, and lanes 2 and 21 are the low molecular weight standards. (arrow indicates mol. wt. of albumin). Mol wt of protein standards is as follows: a-200, b-116, c-97, d-66, e-45, f-31, g-21, h-14 kD.

Vertical SDS-PAGE reveals much more striking differences between SAWP and HWP. The HWP separated with darker staining bands at 170, 140, 120, and 105 kD; moreover, at 72 kD a protein band that is definitely not present in the SAWP was revealed Fig 4). The SAWP presented a much darker band at 70 kD (Fig 4). Two dimensional analysis comparing the HWP and SAWP also shows a protein unique to HWP staining at approximately 72 kD (Fig. 5).

DISCUSSION

A comparison of Figures 1 and 2 reinforces the theory that the presence and concentrations of plasma proteins is changing with the season. While some of the HWP bands are obscure (Fig. 1), the presence of stain would suggest that the same proteins as in the SAWP gel (Fig. 2) might be present here in lower concentrations. As mentioned above, both of the IEF gels smeared around the pI of serum albumin which is 4.5 (Figs. 1 and 2). The smearing in the HWP gel (Fig 1) appears to be greater, and it may be that there are proteins whose presence is masked by the high albumin concentration.

SDS-PAGE analysis of the albumin-fractions (Fig. 3), as mentioned above, did give an indication of the seasonal variability in the concentrations of albumin-bound proteins. However, the inability to distinguish bands near 66kD (arrow) (the known molecular weight for serum albumin) made further evaluation difficult. SDS-PAGE with the discontinuous gel gradient strongly reinforces our theory of proteins whose presence/abscence regulate hibernation. Previously, Kondo and Kondo[11] identified three novel proteins in the blood of chipmunks and ground squirrels. They noted that these proteins disappear in the hibernating animals and then reappear when the animals become active; however, they did not identify the 72 kD peptide that we observed exclusively in the winter fractionation (Fig. 4).

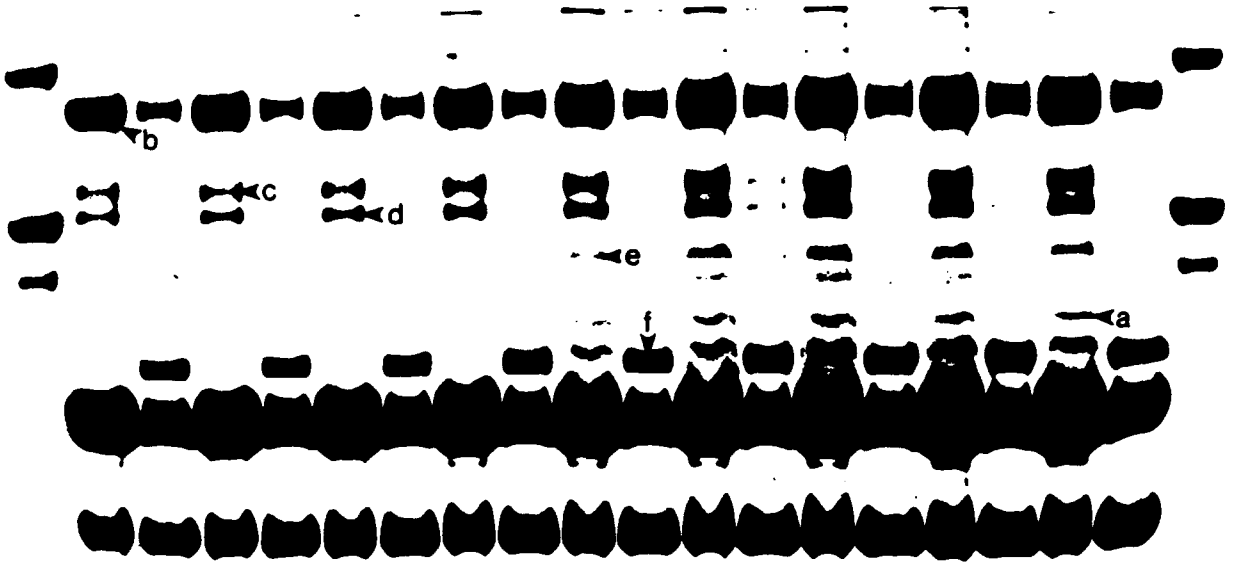

Figure 4. Separation of SpAWP and HWP in a discontinous vertical SDS-PAGE System. A 10% Homogenous gel (SDS-PAGE). Samples had β-Mercaptoethanol added and were heated to 95°C for 3 minutes prior to application to the gel. Vertical SDS-PAGE of HWP (even lanes 2-18) and SAWP (odd lanes 3-19). Lanes 1 and 20 represe... the molecular weight standards as indicated. The amount of total protein varies in 2 μg increments from 24 μg (lanes 2 and 3) to 40 μg (lanes 18 and 19). Note especially the 72 kD band unique to HWP (a). b. 170 kD band darker in the HWP; c. 140 kD band darker in the HWP; d. 120 kD band darker in the HWP; e. 105 kD band darker in the HWP; f. 70 kD band much darker in the SAWP.

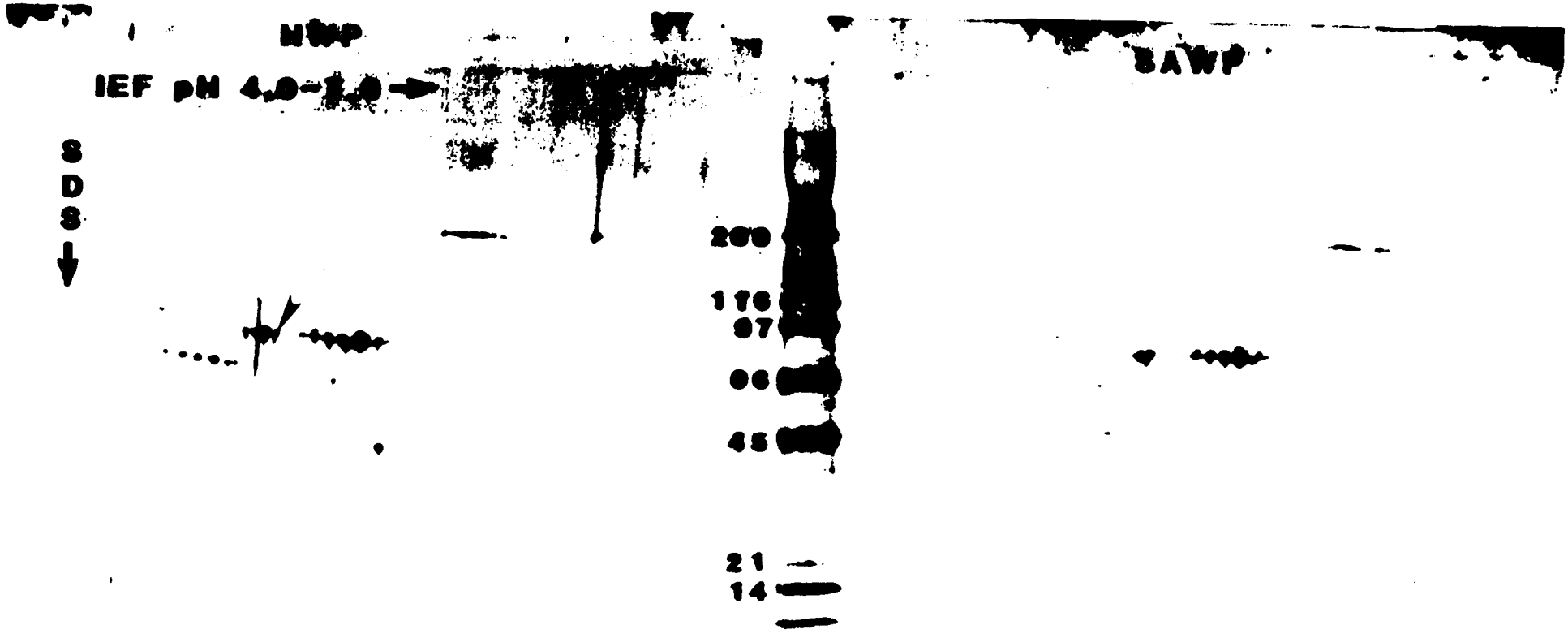

Figure 5: Two-dimensional analysis of HWP and SAWP. Arrow indicates peptide that is visible only in the HWP.

Differential gene expression is a common biological response to a variety of challenges. Srere *et al*[12] observed an elevation of α_2-macroglobulin in hibernating ground squirrels. Martin *et al*[13] has shown that in Richardson's and Columbian ground squirrels this is due to an increased synthesis of the corresponding mRNA in the animal's livers. Similarly our studies have shown in increase in a 170kD protein, at the approximate molecular weight of α_2-macroglobulin, in hibernating woodchucks. It is possible that the other quantitative changes in plasma proteins between the summer-active and winter hibernating animals is also due to differential gene expression.

Vertical SDS-PAGE (Fig. 4) revealed clear differences between the plasma from winter-hibernating and summer-active woodchucks. The association of hibernation induction or metabolic inhibition with one or more of the peptide bands has not yet been established. However, the 72 kD band in the HWP, which is absent in the SAWP, is now the main focus of research efforts in our laboratory. Furthermore, the differences in the protein bands in the SAWP and HWP separations are in harmony with the hypothesis that a peptide(s) that is unique or increased in the winter is responsible for hibernation. Additional support for this hypothesis comes from the presence of a novel spot at 72 kD in the HWP when it is separated by two-dimensional SDS-PAGE (Fig. 5). Further analysis through peptide sequencing, tissue culture assays and opiate receptor assays may reveal if this is the separated HIT-active component.

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II. DEVELOPMENT OF A RAPID *IN VITRO* BIOASSAY TO FACILITATE PURIFICATION AND CHARACTERIZATION OF THE HIT MOLECULE AND RELATED FACTORS

We have recently developed a rapid *in vitro* assay system used for both purifying and characterizing HIT-like molecules present in the plasma of hibernating woodchucks. This system, which replaces the seasonally restrictive ground squirrel bioassay, measures inhibition of DNA and protein synthesis (metabolic inhibition at the cellular level). Three cell lines are used for this *in vitro* bioassay system: 1) TRMP - a dog kidney epithelial cell line;[1] 2) CREF - a rat embryo fibroblast cell line;[2] and 3) SB3 - a human kidney tumor (Wilm's) cell line.

Although we have previously reported that an albumin fraction from hibernating woodchuck plasma could induce hibernation in summer-active ground squirrels[3], we decided to develop the *in vitro* assay system with unfractionated plasma. This approach allowed us to define a number of the activities of this plasma. Some of these activities may be due to molecules capable of lowering or stimulating cellular metabolism but not capable of inducing hibernation in summer-active animals. Therefore, this new approach may allow us to identify and ultimately purify relevant molecules that would not have been detected with the *in vivo* bioassay.

To monitor the inhibition of DNA synthesis, we used two separate assays. In the first, the cells were serum starved for 24 hours and then restimulated with 5% fetal bovine serum (FBS) in the presence of various concentrations of plasma obtained from winter-hibernating or summer-active woodchucks.

Figure 6 depicts results with the TRMP cells and Figure 7 with the CREF cells, demonstrating the complete to partial inhibition of the FBS-stimulated DNA synthesis observed with the winter plasma (WWP). For the CREF cells, even at a concentration as low as 0.5% winter-hibernating plasma, significant inhibition of FBS-stimulated DNA synthesis was observed. For both cell lines, equivalent

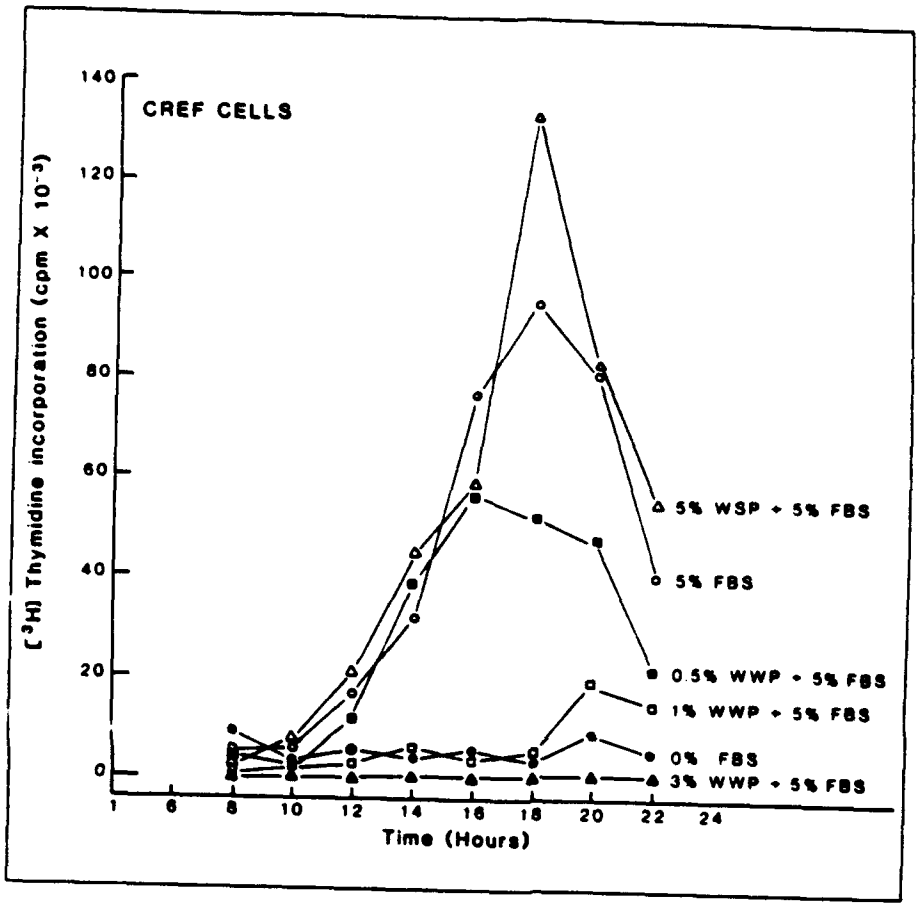

Figure 6. Complete to partial inhibition of FBS-stimulated DNA synthesis in CREF cells exposed to WWP. Serum-starved CREF cells stimulated by 5% FBS also showed increased incorporation of ^{3}H-thymidine beginning at 10-12 h with a peak at 16-18 h. When supplemented with winter plasma at 5% or 3% (as shown), the ^{3}H-thymidine incorporation was totally inhibited. Partial inhibition was observed at concentrations as low as 0.5% of winter plasma. Summer plasma at 5% with 5% FBS enhanced ^{3}H-thymidine uptake above that observed for 5% FBS alone. This experiment demonstrated that the CREF cells were extremely sensitive to inhibition by winter woodchuck plasma.

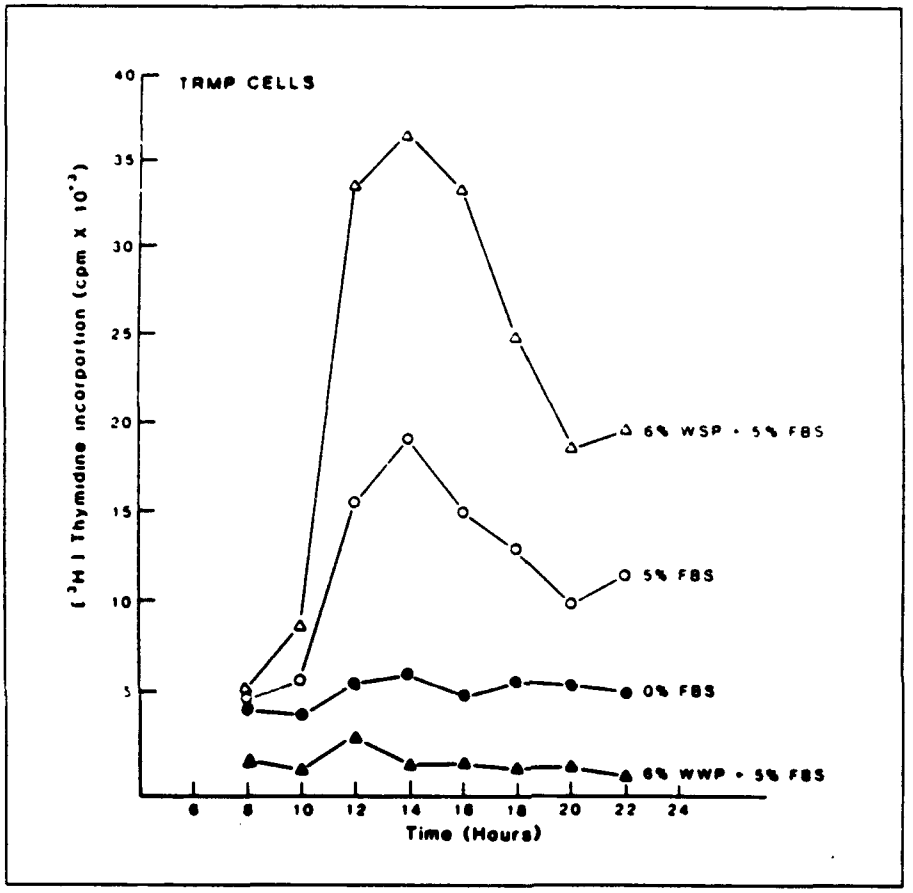

Figure 7. Complete to partial inhibition of FBS-stimulated DNA synthesis in TRMP cells exposed to winter woodchuck plasma (WWP). Plasma from winter-hibernating woodchucks inhibited fetal bovine serum (FBS) stimulation of DNA synthesis in serum-starved TRMP cells. Serum-starved TRMP cells stimulated with 5% FBS show increased incorporation of ^{3}H-thymidine beginning at 10-12 hr with a maximum of 16-18 hr. When supplemented with 6% winter plasma (as shown), the incorporation of ^{3}H-thymidine was inhibited, falling to a level below that seen for cells maintained in 0% FBS. However, summer plasma at the same concentration (6%) enhanced incorporation of ^{3}H-thymidine above that seen for 5% FBS alone. Similar results were obtained for winter and summer plasma at 7.5% and 5%, demonstrating the presence of an inhibitory factor in the winter woodchuck plasma.

concentrations of the summer-active plasma (WSP) enhanced the FBS stimulation of DNA synthesis. For the second assay, continuously growing cells were exposed to various concentrations of winter plasma, and DNA synthesis was measured every hour for six hours. Figure 8 demonstrates that with the CREF cells DNA synthesis slowed and eventually halted in these cells in a concentration-dependent manner. Although not shown in this figure, the summer-active plasma did not inhibit DNA synthesis. To monitor inhibition of protein synthesis, we also used continuously growing cells. The winter plasma was found to inhibit protein synthesis, but equivalent amounts of summer plasma did not. Figure 9 demonstrates these results for the TRMP cells.

The above results with serum-starved cells stimulated with FBS and summer plasma suggested that the summer plasma may also play a role in the stimulation of DNA synthesis (Figs. 6 and 7) To test this hypothesis, we stimulated serum-starved TRMP cells with concentrations of summer plasma ranging from 0.5%-5% in the absence of FBS and found significant stimulation (not shown).

This demonstrated that the woodchuck summer-active plasma contained potent mitogenic activity. The detection of such activity in the summer plasma was quite surprising, because plasma from most species, relative to serum, is mitogen poor.(4) Identical experiments with winter-hibernating plasma demonstrated that, at concentrations as low as 2%, DNA synthesis did not occur in the TRMP cells. However, when the plasma concentration was dropped to 0.5%, the winter plasma displayed significant mitogenic activity. Figure 10 demonstrates that both summer-active and winter-hibernating plasma could stimulate detectable amounts of DNA synthesis in the TRMP cells at concentrations as low as 0.01%, whereas FBS could not stimulate DNA synthesis at concentrations below 0.25%.

With the establishment of this *in vitro* system, we have begun work to determine whether this system will function as a bioassay for purifying both the inhibitory (HIT-active) and mitogenic activities present in the woodchuck plasmas. In these experiments, both summer-active and winter-hibernating plasmas are passed over an Affi-Gel Blue affinity column, and three fractions are collected: the pass-through,

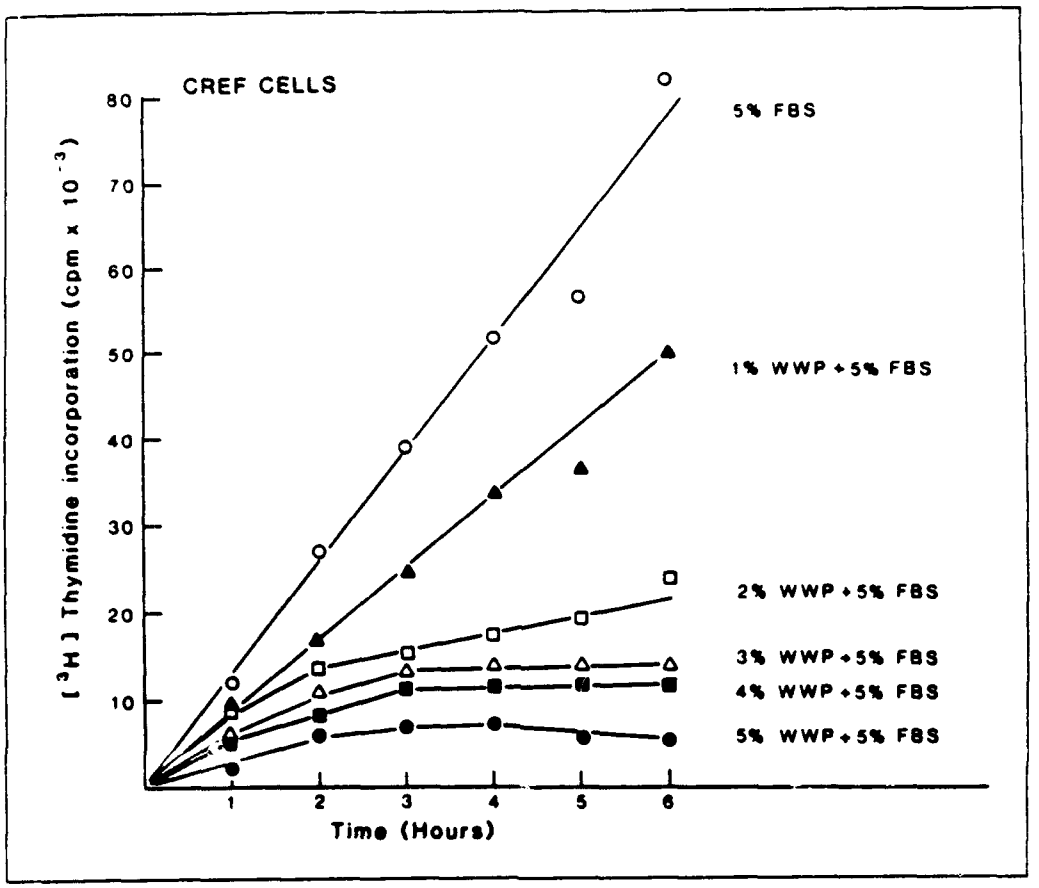

Figure 8. DNA synthesis measured every 6 hours in continuously growing CREF cells exposed to various concentrations of WWP. Plasma from winter-hibernating woodchucks inhibits DNA synthesis in continuously growing CREF cells. CREF cells growing in DMEM supplemented with 5% FBS were treated with varying concentrations of winter plasma. Significant inhibition of ^{3}H-thymidine incorporation was observed. The level of inhibition was directly proportional to the concentration of winter plasma added, and significant inhibition was observed at concentrations as low as 1%.

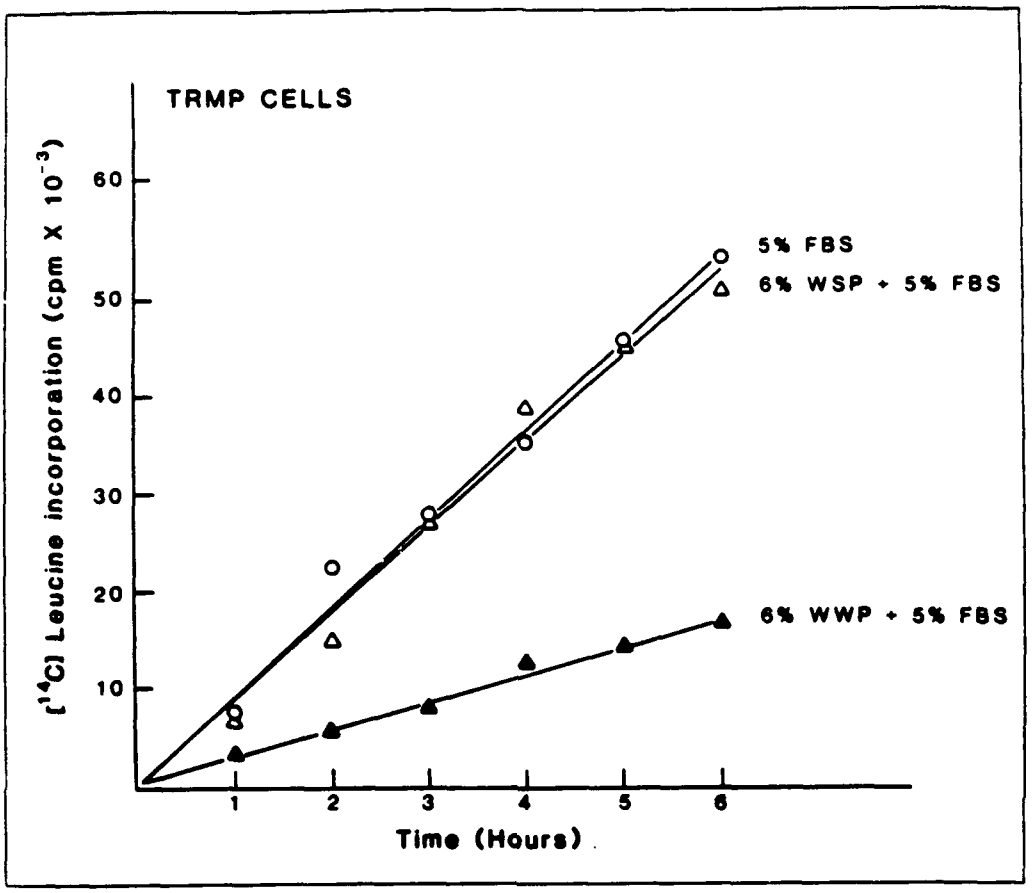

Figure 9. Inhibition of protein synthesis in TRMP cells following addition of 6% WWP to culture media and absence of protein synthesis Inhibition following addition of 6% WWP. Plasma from winter-hibernating woodchucks inhibits protein synthesis in continuously growing TRMP cells. TRMP cells growing in DMEM supplemented with 5% FBS were treated with 6% each of summer and winter woodchuck plasma in the presence of ^{14}C-leucine. Decreased incorporation of ^{14}C-leucine was observed with the 6% winter plasma relative to that seen with both 6% summer plasma plus 5% FBS or with 5% FBS alone.

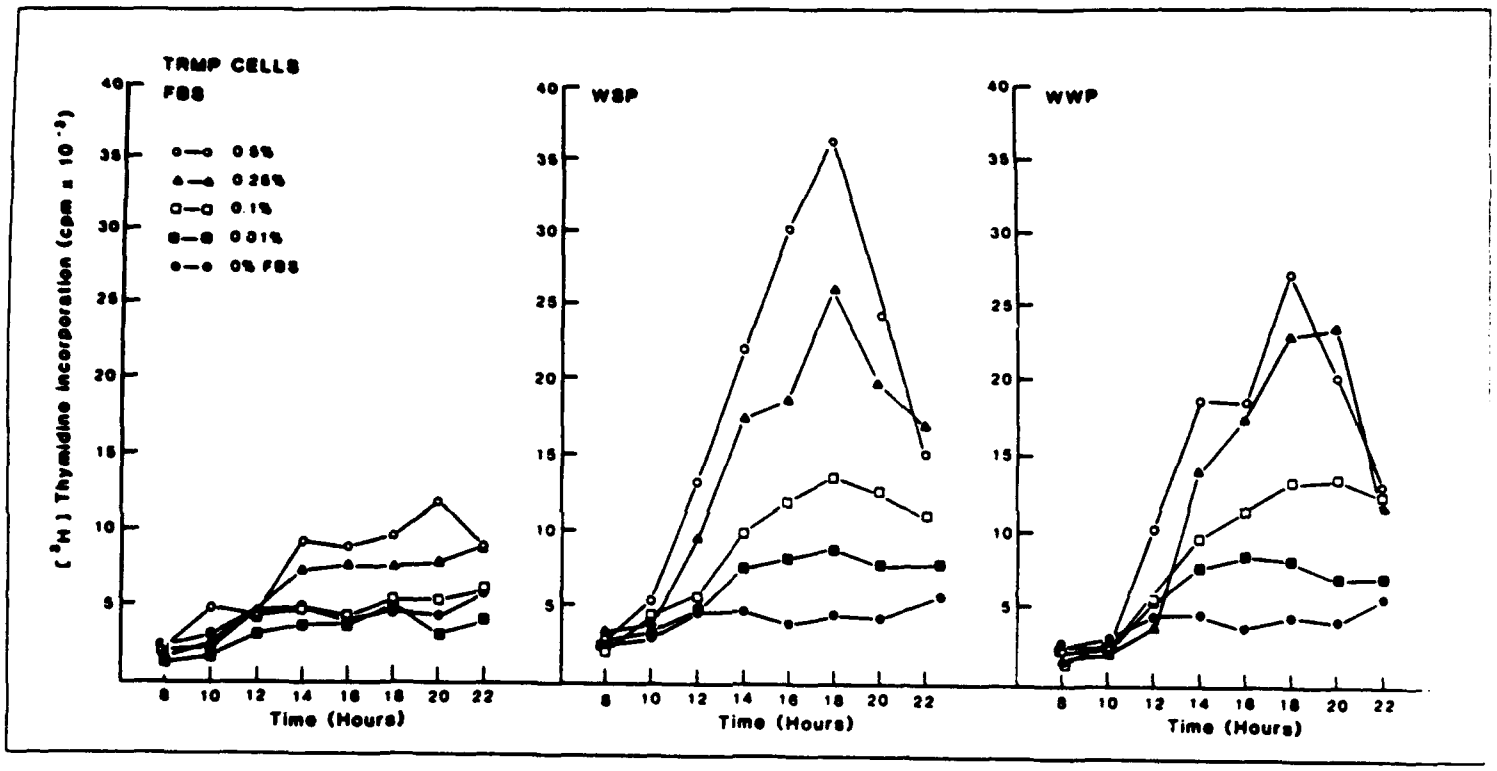

Figure 10. Stimulation of DNA synthesis in TRMP cells exposed to WWP and WWP at concentrations as low as 0.01%. Low concentrations of plasma from both winter-hibernating and summer-active woodchucks stimulated DNA synthesis in serum-starved TRMP cells. Serum-starved TRMP cells treated with summer or winter woodchuck plasma at concentrations ranging from 0.5%-0-01% were stimulated to incorporate ^{3}H-thymidine. FBS at this low concentration demonstrated only marginal stimulation of ^{3}H-thymidine. This would indicate the presence of a mitogen in both summer and winter woodchuck plasma. However, the mitogen in the winter plasma was revealed upon dilution.

proteins removed from the column with 0.2M sodium phosphate, and proteins eluted with 1.4 M NaCl. The last fraction is dialyzed after collection. Table 1 demonstrates that the bulk of the inhibitory activity from the winter-hibernating plasma is eluted with high salt. This fraction is enriched for albumin.[3] Table 2 demonstrates that the bulk of the mitogenic activity from both the summer-active and winter-hibernating plasma is also eluted in this peak, although significant amounts are also found in the other fractions when the TRMP cells are used as a target. We have not yet tested these fractions for inhibition of protein synthesis or their effect on calcium release. Nonetheless, it should be clear that our *in vitro* assay system will prove useful for purifying these biologically active molecules and characterizing their effect at the cellular levels.

The Effect of Delta Opioids DADLE and DPDPE on Cell Growth

We have previously demonstrated that only the *delta* opioid DADLE could mimic the activity of the HIT molecule in inducing hibernation in summer-active ground squirrels, the traditional bioassay[5]. Moreover, utilizing the multiorgan autoperfusion system developed by Chien, *et. al.*[6], we were able to demonstrate that infusions of DADLE at 1 mg/kg had a dramatic effect on extending effective preservation time of the multiorgan autoperfusion system from baseline levels of 14.8 hours to almost 45 hours. This was comparable to the effect seen with infusions of HIT containing plasma into the multiorgan autoperfusion system[7]. We were therefore interested in determining if DADLE would also mimic the metabolic inhibitory activity of the HIT-containing plasma in the recently utilized tissue culture assay. In addition, we also used DPDPE ([D-Pen ^{2,5}] - Enkephalin), a new and potent enkephalin from (Peninsula Labs, Belmont, CA) which, unlike DADLE, is selective exclusively for the *delta* opioid receptor with little or no affinity for μ or κ receptors. Moreover, DPDPE, is a synthetic pentapeptide, designed to sterospecifically resist enzymatic digestion by enkephalinases commonly found in serum.

Table 1. Inhibition of DNA synthesis by Affigel-blue column fractions[a]

% Inhibition[b]

Frac.[a]	TRMP	Cells	CREf	Cells	SB3	Cells
wpf[c]	spf[d]	wpf	spf	wpf	spf	
0	100	0	100	7	100	0
1	4	0	39	27	95	16
2	48	18	0	52	0	0
3	100	30	100	35	100	5

a: Serum-starved cells were stimulated with 5% FBS in the presence of 7.5% plasma or concentrated fractions from the Affigel-Blue column.
b: DNA synthesis was measured by determining 3H-thymidine incorporation during the interval of 12-18 hrs after FBS stimulation. The % inhibition was determined by comparing the amount of thymidine incorporation in the presence of the different column fractions with the amount observed with 5% FBS alone.
c: wpf = protein fractions obtained from the winter-hibernating plasma.
d: spf = protein fractions obtained from the summer-active plasma.
e: The tested fractions are as follows: 0 = unfractionated plasma, 1 = protein that passed through the column, 2 = protein that was weakly bound to the column and removed with the 0.02M phosphate buffer, 3 = protein eluted with 1.4M NaCl (albumin fraction).

Table 2. Stimulation of DNA synthesis by Affigel-blue column fractions[a]

% Stimulation[b]

Frac.[a]	TRMP	Cells	CREF	Cells
	wpfc	spf[d]	wpf	spf
0	133	108	127	112
1	105	30	17	1
2	78	0	17	1
3	94	83	95	75

a: Serum-starved cells were stimulated with 0.5% plasma or concentrated plasma protein fractions
b: DNA synthesis was measured by determining 3H-thymidine incorporation at 12-18 hours after stimulation of the serum-starved cells. The % stimulation was determined relative to stimulation with 5% FBS.
c: wpf = protein fractions obtained from winter-hibernating plasma
d: spf = protein fractions obtained from summer-active plasma
e: Tested fractions are the same as in Table *1*

MATERIALS AND METHODS

TRMP - a dog kidney epithelial cell line (Turker et. al.[1]) is used. The cells are routinely grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). To measure DNA synthesis $5X10^4$ cells are plated per 18mm well (Corning, 24 well plates) and allowed to attach in the presence of 10% FBS for 24 hr.

A.) Effect of Opiate Peptides DADLE or DPDPE on DNA-Synthesizing Activity of Logarithmically Growing Cells

The cells are placed in growth medium (control) or growth medium with various concentrations of DPDPE or DADLE and ^{3}H-thymidine. The time of incubation is 4 hours. The cells are then washed with ice-cold phosphate buffered saline (PBS), fixed with 50% ethanol (1 hour), washed twice with ice-cold PBS, 4 times with ice-cold 5% TCA, twice with 100% ethanol and dissolved in 1% SDS with 0.5M KOH. Aliquots of this lysate are then counted with liquid scintillation to determine the relative amount of DNA incorporated.

B.) Effect of Opiate Peptides DADLE or DPDPE on DNA-Synthesizing Activity of Serum Starved Cells Stimulated with FBS.

The cells are placed in serum-free medium for 24 hours and then in medium containing 0.25% FBS (control) and various concentrations of DPDPE or DADLE. At 12.5 hours after incubation the cells are exposed to ^{3}H-thymidine for 2 hours and then the relative amount of DNA incorporated are determined as described above.

RESULTS

Effect of Opiate Peptides on DNA-Synthesizing Activity of Logarithmically Growing Cells

The experiments with actively growing cells involved plating 5 x 10^4 cells in 18mm wells in the presence of 2.5% FBS. 24 hours later the cells were exposed to the concentrations of DPDPE and DADLE that are shown in Table 3 in the presence of ^{3}H-thymidine. ^{3}H-thymidine incorporation is used as a relative index for the rate of DNA replication. After four hours the cells were processed as described in the Materials and Methods section.

Table 3: **Effect of Opiate Peptides DPDPE and DADLE on DNA-Synthesizing Activity of TRMP Cells In Culture**

Logarithmic culture in the presence of DPDPE or DADLE (4 hr)

Testing substance	Concentration of peptide (ug/ml)	^{3}H-thymidine incorporation (cpm)	^{3}H-thymidine incorporation (% to control)
DPDPE	100	1969.5 ± 221.9	69.9 ± 3.0
	10	2199.0 ± 244.3	78.4 ± 2.0
	1	2206.8 ± 213.1	78.7 ± 2.0
DADLE	100	2540.0 ± 311.4	90.5 ± 4.5
	10	2473.2 ± 159.9	88.1 ± 0.6
	1	2758.0 ± 163.6	98.3 ± 1.2
control	—	2805.5 ± 199.9	100

Effect of Opiate Peptides on DNA-Synthesizing Activity of Serum Starved Cells Stimulated With FBS

The experiment with the serum starved cells is performed by plating 5 x 10^4 cells per well as above. Eight hours later the medium with FBS is removed and replaced with medium without serum. The cells are thus "starved" for serum for 24 hours, which forces the cells out of the cell cycle into G_0 (i.e. quiescent cells). The cells are then re-exposed to medium with 2.5% FBS in the presence of DADLE or DPDPE. When cells are re-exposed to FBS they re-enter the cell cycle resulting in a wave of DNA synthesis (ie. S phase) beginning approximately 10 hours later and lasting for approximately 8 hours.

Table 4. The Culture After Starvation (24 hr) in the Presence of DPDPE or DADLE (14.5 hr)

Testing substance	Concentration of peptide (ug/ml)	^{3}H-thymidine incorporation (cpm)	^{3}H-thymidine incorporation (% to control)
DPDPE	100	4136.0 ± 298.2	102.9 ± 7.5
	10	4030.6 ± 263.4	100.2 ± 8.0
	1	3878.8 ± 228.2	96.0 ± 8.5
DADLE	100	1978.0 ± 422.7	49.2 ± 4.0
	10	1770.0 ± 173.0	44.0 ± 2.5
	1	1998.4 ± 181.1	49.7 ± 3.0
control	—	4020.8 ± 578.8	100

DISCUSSION

The results in Table 3 demonstrated that DPDPE, but not DADLE, could reduce DNA replication by 20-30% in the actively growing cells. Since cells in S phase (i.e. the phase during which DNA replication occurs) will usually continue to progress through this phase until it is completed, the results suggest that the DPDPE is blocking cells from entering S phase, but not inhibiting DNA replication if the cells are in S phase at the time they were exposed to DPDPE. This possibility could be confirmed by pretreating the cells with 100 μg/ml DPDPE for 10 hours (which is enough time for all cells in S phase to exit) and then exposing the cells to ^{3}H-thymidine for 4 hours (still in the presence of DPDPE. If the hypothesis is correct, the cells should exhibit very little ^{3}H-thymidine incorporation when compared with untreated control cells.

The results in Table 4 demonstrated that DADLE could inhibit DNA replication in the FBS stimulated cells by approximately 50% as compared with untreated controls, but that DPDPE had no effect. Since

no difference was seen when comparing 100 ug/ml DADLE with 1 ug/ml, it will be important to repeat this experiment with decreasing concentrations of DADLE to show a concentration region in which inhibition is dose dependent.

One possible explanation for these results is that DADLE and DPDPE block different opiate receptors (DADLE has some affinity for mu receptors as well as delta receptors) and that the serum starved cells use one set of receptors to re-enter the cell cycle and a second set of receptors to enter DNA synthesis. It is a possibility, that these growth inhibiting effects of opiate peptides such as DADLE and DPDPE may be caused by the ability of both delta and mu receptors to mediate the inhibition of adenylate cyclase (Burns, et al.[8]).

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INTRODUCTION

III. Extending Organ Survival Time Utilizing An Opiate-Like Hibernation Induction Trigger (HIT)

We have previously demonstrated that an albumin fraction derived from plasma of deeply hibernating woodchucks contains a potent metabolic inhibitor which we have referred to as the hibernation induction trigger (HIT). By infusing primates IV or ICV with this HIT-active fraction, we have induced profound behavioral and physiological depression mimicking natural hibernation including the appearance of an anesthetized state, hypothermia, bradycardia, decreased renal function and long-term feeding inhibition. The aforementioned behavioral and physiological depression can be reversed or retarded by infusion of the opiate antagonists naloxone and naltrexone[1-4]. Such evidence indicates that the HIT molecule may initiate its potent metabolic inhibitory effects through specific peripheral and central opioid receptors. Moreover, these studies indicate the great clinical potential of this opiate-like molecule(s) in nonhibernating recipients.

A new autoperfusion multiorgan preparation has been developed in our laboratories in which the dogs are anesthetized and artificially ventilated. The heart, lungs, liver, pancreas, duodenum and both kidneys are removed *en bloc* while being perfused by the heart and oxygenated by the lungs. A respirator is used for ventilation. Fresh blood, electrolytes, antibiotics and other nutrients are infused by intravenous dripping. No anticoagulants or inotropic drugs are necessary. The heart continues to pump blood to all the organs and the lungs oxygenate the blood. The liver keeps its normal biological functions. Electrolyte and water balance is maintained and metabolic waste is removed by the kidneys. This preparation preserves the natural anatomic, as well as physiologic connections of the organs without foreign material and requires little intervention. In the original study, liver congestion reduced survival time to an average of 14.8 hours[5] However, when plasma from deeply hibernation woodchucks was infused into the multiorgan autoperfusion system an average survival time of 44 hours was obtained under non-sterile conditions[6] as seen in Figure 11. Comparable multiorgan survival

times (averaging 46 hours were achieved when the autoperfusion system was infused with the delta opioid, DADLE,[7] (Figure 12) which mimics the natural biologic activity in hibernators of the winter hibernating plasma[8]. The aforementioned studies have clearly demonstrated the profound beneficial effects of HIT and infusions of delta opiate on extending tissue viability in a multiorgan autoperfusion system in which no central nervous system control exists.

In our most recent studies, we utilized the canine multiorgan autoperfusion system and infusion of HIT containing plasma to study lung preservation and to provide left lungs for transplantation after more than 24 hours of preservation. These studies indicated the transplanted left lung from the multiorgan autoperfusion block was able to support the anesthetized dog after the opposite pulmonary artery was occluded. These studies are detailed as follows:

MATERIALS AND METHODS

Animals Used

Six pairs of adult mongrel dogs weighing 15 to 20 kg each were used in the study. The body weights of recipients and donors were matched within $\pm$3 kg. For organ wet/dry weight ratio comparisons, control data were obtained from tissue samples of the heart, lungs, liver, pancreas, duodenum, and kidney from 15 normal dogs.

The organs were preserved for at least 24 hours. Left lungs were transplanted into the left chest of recipient dogs. After transplantation, the dogs were observed for 24 hours while receiving anesthesia and mechanical ventilation. Organ functions were monitored, and the animals were sacrificed at the end of observation period.

All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH Publication No. 85-23, revised 1985).

Pretreatment

All donor dogs were given neomycin 2 gm orally once a day for three days before surgery to reduce gut flora. All dogs were fasted for 10 hours prior to surgery. Donor dogs also received 10 ml of HIT-containing plasma intravenously 2 hours prior to the operation.

Sample Procurement

Woodchucks *(Mormota monax)* weighing 3-5 kg serve as the source of winter hibernating plasma. Blood was drawn by heart ventricular puncture while these animals were in deep winter hibernation as evidenced by having a core temperature of 4.0-6.0°C, a heart rate of 1 or 2 bpm, and one or two respirations per minute.

Surgical Technique

The technique used to harvest the organs was similar to that previously reported[6 and 9]. A brief review of this technique follows.

Dogs were anesthetized with sodium pentobarbital 30 mg/kg, i.v., intubated, and mechanically ventilated. With the animal in the supine position, the abdomen was opened. The liver, pancreas, duodenum, and both kidneys were dissected free, along with the abdominal aorta and the inferior vena cava. The chest was then opened, and the heart, and lungs along with the aorta and the superior and inferior vena cava (IVC), were dissected free. The trachea was transected and an endotracheal tube was inserted for ventilation. Several catheters were placed for pressure measurement, fluid transfusion, and sample collection: one was placed in the left ventricle (LV) through the right carotid artery for LV pressure measurement and dp/dt monitoring; one was placed in the abdominal aorta for arterial pressure measurement and blood gas sampling; one was placed in the IVC for venous pressure measurement and blood sampling; one was placed in the portal vein for portal venous pressure measurement and fluid infusion; one was placed in the common bile duct for collecting the bile; one was placed in the duodenum for collecting duodenal and pancreatic fluid; and one was placed in each ureter for urine collection. A transonic flow probe (Transonic Systems Inc. Ithaca NY) was placed around the descending aorta to monitor blood flow.

As shown in Figure 13, the entire system, including the heart, lungs, liver, pancreas, duodenum, both kidneys, and their connecting blood vessels, was removed from the body and placed in a temperature controlled fluid filled bath containing lactated Ringers, heparin 10 mg/L, and neomycin 100 mg/L.

Interventions

The bath solution was prewarmed and maintained around 32°C. Mechanical ventilation was maintained by a Harvard ventilator. Tidal volume of 500-700 ml, rate 10 to 20 rpm, and PEEP of 4-8 cm H_2O were maintained. A gas mixture of 50% O_2 1 3% CO_2 + 47% N_2 was delivered via the ventilator. A 5% dextrose solution containing the following drugs was infused through the portal vein at 10 to 20 ml/hour: calcium chloride (1 g/L), insulin (50 units/L), mannitol (12.5 g/L), methylprednisolone (500 mg/L), penicillin (1,000,000 units/L), and Flagyl (500 mg/L). A fat emulsion (Soyacal, 2 ml) and methylprednisolone (30 mg) were injected through the portal vein every 2 hours. Blood transfusions were given as needed to maintain aortic systolic pressure between 75 and 100 mmHg and CVP between 0 and 10 mmHg. Plasma was given instead of whole blood if the hematocrit was higher than 45%. Four milliliters of plasma from hibernating woodchucks was infused through the portal vein every 4 hours during the preservation period.

Monitoring

Aortic pressure, central venous pressure, and portal venous pressure were monitored and recorded on a Gould multichannel recorder (Gould Inc. Centerville, OH) throughout the preservation period. Blood gas measurements were performed every hour using an IL Blood Gas-Electrolyte Analyzer (Instrumentation Laboratory, Lexington, MA), or continuously monitored using PB-3300 Blood Gas Monitor (Puriton-Bennett, Anahein, CA). Respiratory pressure, tidal volume, and PEEP for the lungs were recorded every hour, as were observable changes in the lungs, such as color, atelectasis, air leaking, and edema. Blood samples were also taken every 4 hours for hematological analysis and biochemical analysis including liver, kidney, and pancreatic function tests as well as heart isoenzyme determinations. Temperature, urine and bile production, and duodenal and pancreatic secretions were monitored and recorded every hour.

Determination of Tissue Wet/Dry Weight Ratio.

At the termination of the experiment, tissue specimens were taken from each organ for tissue wet/dry ratio determination. Tissue samples were blotted to remove excess fluid, and wet weight was measured. The dry weight was determined after the samples had been dried in an oven at 85°C for 72 hours.

Statistical Analysis.

All laboratory test results obtained before the experimental procedure (blood gases, hematocrit, blood chemistries, hematology, lactic acid, and enzymes for heart, liver, pancreas, and kidney functions) were used as normal controls. These controls were compared to the results obtained during the preservation period. Hemodynamic values and urine output were measured immediately after harvesting and during the preservation period and these results were compared. Tissue wet/dry weight ratios for all the organs were compared with those obtained from normal dogs.

For comparisons within a group, ANOVA and Student-Newman-Keuls tests were used to compare the data measured during the preservation period with those obtained preoperatively. If a comparison was needed between the study group and the control group at a certain point, an unpaired Student t-test was used. All data are expressed as mean$\pm$standard error of the mean, with statistical significance assigned at $p<0.05$.

RESULTS

The amount of blood or plasma used during preservation ranged from 900 to 2400 ml, with an hourly blood or plasma transfusion rate of 33 to 100 ml. Total urine output from the organ block ranged from 610 to 1860 ml. The average hourly urine output was 48$\pm$9 ml. Total bile output ranged from 45 to 120 ml and averaged 3.1$\pm$0.5 ml/hour. Total pancreatic and duodenal output ranged from 30 to 250 ml, with an average hourly output of 4.5$\pm$1.1 ml.

Lung Function During Preservation.

Tidal volume was maintained from 500-700 ml for the dogs used in this study. When a gas mixture of 50% O_2, 3% CO_2, 47% N_2 was used, arterial oxygen tension ranged from 201±42 to 326±14 mmHg, carbon dioxide tension ranged from 21±2 to 33±3 mmHg, and arterial pH values ranged from 7.33±0.02 to 7.45±0.03. These parameters did not change appreciably during the preservation period (Figure 14). The maximum inspiratory pressure ranged from 13±2 to 22±3 mmHg. Calculated airway resistance ranged from 0.012±0.003 to 0.019±0.004 mmHg/ml. A slight increase in airway resistance occurred at 24 hours (Figure 15). Pulmonary systolic pressure ranged from 22 to 29 mmHg during the preservation period and had a slight increase at the end of preservation.

Lung color change was related to how well the lungs were protected during harvesting. In one experiment, portions of the lung surface were damaged because of longer exposure to room air during harvesting. Atelectasis seemed to occur in these damaged areas at approximately 20 hours. In other experiments, in which the lungs were well protected during harvesting, the lungs tissue appeared normal after 24 hours of preservation. They were light in weight, pink in color, pliable to the touch with no gross atelectasis, blotching, bleeding or air leakage. The lung tissue wet/dry weight ratio after more than 24 hours of preservation was 4.94±0.17. This was not significantly different from the lungs of the control dog (4.91±0.10). In some experiments, lung tissue samples were taken during the preservation period for electron microscopy studies.

Function Of Other Organs During Preservation.

Aortic pressures and heart rate were stable during the preservation period. No inotropic drug administration was required. Aortic systolic pressure ranged from 86±6 mmHg to 98±14 mmHg and was easily adjusted by blood or plasma transfusions. Aortic diastolic pressures ranged from 45±4 to 62±9 mmHg. Left ventricular end diastolic pressures ranged from 2.2±0.6 to 3.6±1.3 mmHg. The heart rate ranged from 67±3 to 91±9 beats per minute. Central venous pressure ranged from 1.2±0.8 to 3.2±1.1 mmHg. Mean left ventricular maximum dp/dt was 1240±171 mmHg/second at the beginning of

preservation, and 1350±160 mmHg/second after 24 hours. Mean left ventricular dp/dt/p was 13.8±2.9 (second^{-1}) at the beginning of preservation and 13.9±0.9 (second^{-1}) at 24 hours. Blood flow in the descending aorta ranged from 256±37 ml/min to 365±41 ml/min. Calculated systemic resistance ranged from 10232±511 to 15039±1967 (dynes. sec. cmS) and showed no significant change during the preservation period (Figure 16). The average serum lactic acid level was 1.25±0.22 mMol/L before surgery. It increased to 3.48±0.71 mMol/L after surgery ($p<0.05$), decreased gradually to 1.59±0.37 at 8 hours, and then increased gradually to 3.61±0.74 at 24 hours ($P<0.05$, Figure 17).

Liver function remained good during the entire preservation period. ALP decreased and GGT remained stable during the preservation period. Both AST and ALT levels increased slightly. However, only in one experiment was the increase of AST significant. In the other experiments, AST remained very low during the preservation period (Figure 18). AST and ALT are the most sensitive enzyme tests for detecting liver parenchymal cell damage while ALP and GGT are highly sensitive tests for detecting biliary tract obstruction or damage. Serum LDH was 54±10 U/L before the operation, increased immediately to 203±56 U/L at 4 hours ($p<0.025$), and then decreased to 120±14 UL at 8 hours and remained stable thereafter. The level of LDH_1 isoenzyme, used for diagnosis of acute myocardial infarction and hemolysis, increased somewhat during the preservation period but remained within normal limits. The level of LDH_3, an indicator of pulmonary emboli, decreased during preservation. The level of LDH_5, an indicator of hepatic congestion and liver injury, remained stable during preservation (Figure 19). The serum amylase level ranged from 533±95 to 807±130 U/L and remained very stable in each organ block.

Blood urea nitrogen (BUN) averaged 16.8±1.7 mg/dL before the surgery. It decreased to 5.0±1.1 mg/dL at 8 hours ($p<0.001$) and remained at this low level throughout the preservation period. Serum creatinine levels decreased from 1.06±0.05 mg/dL before surgery to 0.12±0.02 mg/dL at 8 hours ($p<0.00001$) and remained low throughout the preservation period (Figure 10).

The level of serum electrolytes, including potassium, sodium, chloride, and calcium, remained stable during the preservation period. Blood glucose levels were usually higher due to continuous infusion of

glucose. Red blood cell concentrations were kept stable during the preservation period by blood or plasma transfusions. Bleeding was minimal even though the dissection was extensive. The WBC count was 12.4±1.6 (10^3/uL). It decreased to 3.7±0.7 (10^3/uL) at 8 hours and continuously decreased to 0.88±0.12 (10^3/uL) at 24 hours ($p<0.001$). Blood platelet levels decreased from 355±22 (10^3/uL) before the surgery to their lowest level of 154±29 (10^3/uL) at 24 hours ($p<0.0025$, Figure 21).

Serum cortisol concentrations ranged from 8 to 60 μg/dL, and serum insulin concentrations ranged from 6 to 400 μU/ml during the preservation period. The higher concentration of insulin in some organ blocks was a result of the presence of insulin in the infusion solutions. The serum thyroxine levels were 0.9 to 1.7μ/dL during the preservation period. This level was lower than normal because the preservation block did not include the thyroid gland.

At the end of the experiments, tissue wet/dry ratios of the preserved organs were measured and compared with the ratios of tissue samples taken from normal dogs. Wet/dry ratios of the heart, lung, liver, and pancreas were similar to the ratios of controls. However, wet/dry ratios of the kidney and duodenum were higher than those of normal dog organs (Figure 22).

TRANSPLANTATION OF THE LUNGS AFTER PRESERVATION

Operation of transplantation

The times of preservation were 24 hours (3 dogs), 25 hours (1 dog), 30 hours (1 dog), and 33 hours (1 dog), averaging 26.7±1.4 hours. The transplantation technique was a modification of the one reported by Veith and Richards[10]. The dog was anesthetized with sodium pentobarbital 30 mg/kg, intubated, and artificially ventilated. The chest was opened through the 5th intercostal space. The left pulmonary artery was dissected free from its origin to the first branch. The pericardium was opened over the main pulmonary artery. The right main pulmonary artery was dissected free, and a 10 mm IVM OC hydraulic vascular occluder (In Vivo Metric, Healdsburg, CA) was sutured around it for later occlusion. The pericardial incision was extended inferiorly, exposing the anterior aspect of the left atrium and the left

inferior pulmonary vein. The posterior mainstem bronchus was separated from the left atrium. Two atraumatic clamps were placed across the left pulmonary artery, and the left pulmonary artery was divided near its first branch.

The left mainstem bronchus was occluded with an angled atraumatic clamp and divided proximal to the origin of the upper lobe. The left atrium was further mobilized by dividing the fat and visceral pericardium along the superior border of the left atrium. An angled atraumatic clamp was placed across the left atrium as far medially as possible without occluding the right inferior pulmonary vein. The left pulmonary veins were then transected at their junction with the left atrium, and the intervening tissue was incised over a clamp. The left lung was removed.

In the preservation block, heparin sodium 5 mg was infused into the venous line. Dissection of the left lung was performed to expose the left pulmonary artery, the left mainstem bronchus, and the left atrium, as in the recipient. A suture was used to encircle the left pulmonary artery. The pericardium over the main pulmonary artery was opened. A transfusion cannula was inserted into the left pulmonary artery through the main pulmonary artery, and fast infusion of cooled Collins solution was administered to cool the left lung. The suture around the left pulmonary artery was tied over the infusing cannula as close to the main pulmonary artery as possible. The left atrium was opened for fluid drainage. After 1000 ml of preservation solution had been infused and the lung tissue was cold, the left lung was removed with the pulmonary artery, the left mainstem bronchus, and the left atrium. The removed left lung was wrapped in an ice-cooled wet towel and placed in the recipient for anastomosis.

Both ends of the left atrium were anastomosed using two everting mattress sutures of 4-0 prolene. Air was flushed out with saline before the sutures were tied off. Next, the bronchial anastomosis was performed. Two 3-0 prolene sutures were used for end-to-end anastomosisr. After the bronchial anastomosis was completed, the bronchial clamp was removed, allowing expansion and ventilation of the transplanted left lung.

The donor and recipient pulmonary arteries were anastomosed with continuous 4-0 prolene sutures. Saline solution was used to fill the artery and expel air before the last stitch. The clamps were released, and any leaks were repaired. The chest was closed in layers, and the hydraulic occluder was brought out through the incision.

Treatment of the recipient animals after the transplantation

The right pulmonary artery was occluded after transplantation. In 3 recipients, the occlusion was performed immediately after the transplantation. In the other 3 dogs, the occlusion was performed 1-6 hours after the transplantation due to lung damage sustained during the transplantation procedure. The dogs were maintained on anesthetic and artificial ventilation after the operation. A gas mixture of 50% O_2, 3% CO_2, and 47% CO_2 was used. A Gould pressure transducer was connected to the inspiration tubing for continuous measurement of inspiratory pressures for airway resistance calculations. An arterial line was placed in the femoral artery for arterial pressure monitoring and blood gas sampling. A venous line was placed in the femoral vein for fluid infusion and blood sampling. Arterial blood samples were taken every hour for blood gas measurement, and venous blood samples were taken every four hours for hematological, blood chemistry, and enzyme measurements. An intravenous drip of 5% glucose plus penicillin (1,000,000 units/L) and Flagyl (500 mg/L) was given slowly. The animal was sacrificed after 24 hours of observation, and lung tissues were examined for pathologic changes after transplantation.

HEMODYNAMIC AND FUNCTIONAL STUDIES AFTER TRANSPLANTATION

Blood pressures: Arterial blood pressures remained stable after the transplantation without the need for blood transfusion or inotropic drug administration. Aortic systolic pressure ranged from 121±2 to 141±5 mmHg, aortic diastolic pressure ranged from 73±7 to 91±4 mmHg, and pulse pressure ranged from 43±2 to 54±7mmHg. Arterial blood pressure was slightly lower during the observation period because some blood loss occurred in the chest. Heart rate ranged from 90±6 to 129±13 bpm (Figure 23). No arhythmia occurred after the transplantation.

Lung function: Tidal volume ranged from 600 to 700 ml after transplantation. Maximum airway pressure ranged from 19±1 to 24±2 mmHg, and calculated airway resistance ranged from 0.029±0.002 to 0.036±0.003 mmHg/ml during the observation period. These values did not change appreciably after transplantation (Figure 24). Arterial oxygen tension was maintained from 205±39 to 360±57 mmHg, arterial carbon dioxide tension was maintained from 23±2 to 34±2 mmHg, and arterial pH was maintained from 7.33±0.04 to 7.51±0.02 (Figure 25). Some damage was induced in two transplanted lungs, because of longer operations. The damage spread after transplantation; some areas of atelectasis were found, and frequent lung expansions were required. In one experiment, the recipient had a high fever and high value of white blood cell count before transplantation. Oxygen tensions were low after transplantation, and opposite pulmonary artery occlusion was delayed. This dog died 8 hours earlier than expected 24-hour observation. At autopsy, heart worms were discovered in the distal pulmonary arteries. Mean lung tissue wet/dry ratio obtained 24 hours after transplantation was 5.23±0.23, which was higher than that of normal dogs (4.91±0.10).

Function of other organs

In all liver-related enzyme studies, AST increased gradually after transplantation. However, ALT increased only slightly after 16 hours. ALP and GGT remained stable after transplantation (Figure 26). Total bilirubin increased from 0.1 to 0.2 mg/dL at 24 hours. Total LDH level increased two-fold at 24

hours; however, the highest enzyme value was still within the normal range. The LDH_1 isoenzyme level increased during the observation period but remained within the normal limits. Both LDH_3 and LDH_5 levels remained normal or slightly below normal during the observation period. Creatinine phosphokinase (CK) levels increased dramatically after transplantation as a result of surgery. CK-MB also rose after transplantation ($p<0.01$, Figure 27). Serum amylase level remained stable after transplantation, as did serum lactate levels. The latter test is a good indication of the overall metabolic state of the recipient animal. Serum potassium, sodium, chloride, and calcium levels all were within normal limits during the observation period. Serum protein and albumin levels were slightly lower after the transplantation because no blood transfusions were given. Red blood cell levels were stable after the transplantation. White blood cell levels increased during the observation period; this increase probably resulted from bacterial contamination or from a reaction to surgery. The platelet levels were stable after transplantation.

All recipient dogs maintained normal urine output after transplantation. Tests indicated good renal function, including stable or lower blood urea nitrogen and creatinine.

DISCUSSION

Our preliminary studies utilizing HIT and DADLE have achieved the longest average survival time in autoperfusion history, and one of the longest survival times in the history of organ preservations. Our experiments in lung transplantation after more than 24 hours of preservation following HIT infusions were among the very few reported experiments in which the lungs could be preserved for such a long time and still maintain good function after transplantation.

From our preliminary studies, the multiorgan autoperfusion system appears to have the following advantages:

1. Each specialized organ is functionally integrated with all others by an integrated metabolic regulatory system. The efficient exchange of energy and substrates between organs is crucial for inducing the steady state favorable to maintaining life. The liver plays a central role in this biologic homeostasis and is being increasingly recognized as a critical organ in the pathogenesis of multiple organ failure. The inclusion of 6 major organs in one system enhances survival of individual organs.
2. There is no ischemic time from harvesting to preservation so that the damage to the organs is reduced to a minimum.
3. A natural circulation is preserved in the system so that no foreign material is needed, and blood trauma is reduced substantially.
4. A relatively complete metabolic cycle is present. Functional disturbance to the organs is kept minimal.
5. The blood volume is large enough so that no mechanical assistance is necessary.
6. The set-up is relatively simple and the procedure is highly reproducible. The whole harvesting procedure takes less than 2 hours to complete.
7. The organ's functions can be easily studied while they are preserved, allowing the appropriate viability tests to be conducted prior to transplantation.

8. Since all the organs are preserved *en bloc* outside the body, it is also an excellent model for physiologic, pharmacologic or pathologic studies.

There are several reasons which make lung tissue preservation much more difficult than for other organs: 1) The unique, delicate architecture of the lung poses a special problem in preservation. Methods effective for the short-term storage of kidneys, livers, and hearts do not necessarily work for lungs. 2) Total functional dependence is placed on the preserved lung after it is transplanted. This makes evaluation of functional adequacy more critical during preservation.

From our preliminary studies, the multiorgan autoperfusion system has the following disadvantages:

1) Organ retrieval requires a well trained operating team. Exposing the lung for a long period of time reduces its preservation time.
2) The organ block is relatively bulky, and transportation from site to site is more difficult than for a single organ.
3) Unlike hypothermic storage, in which the organ can be ignored after it is placed in a refrigerator, the autoperfused block needs continuous observation, and monitoring and adjustments by trained clinical scientists.

Our studies have shown that HIT and the delta opioid DADLE can markedly extend tissue survival time in organ preservation. If further studies of the HIT molecule(s) and DADLE confirm their protective effect, possibly as a potent metabolic inhibitor in tissue preservation, they could have a great potential in clinical patient care, such as in trauma treatment, multiorgan failure, and organ transplantation.

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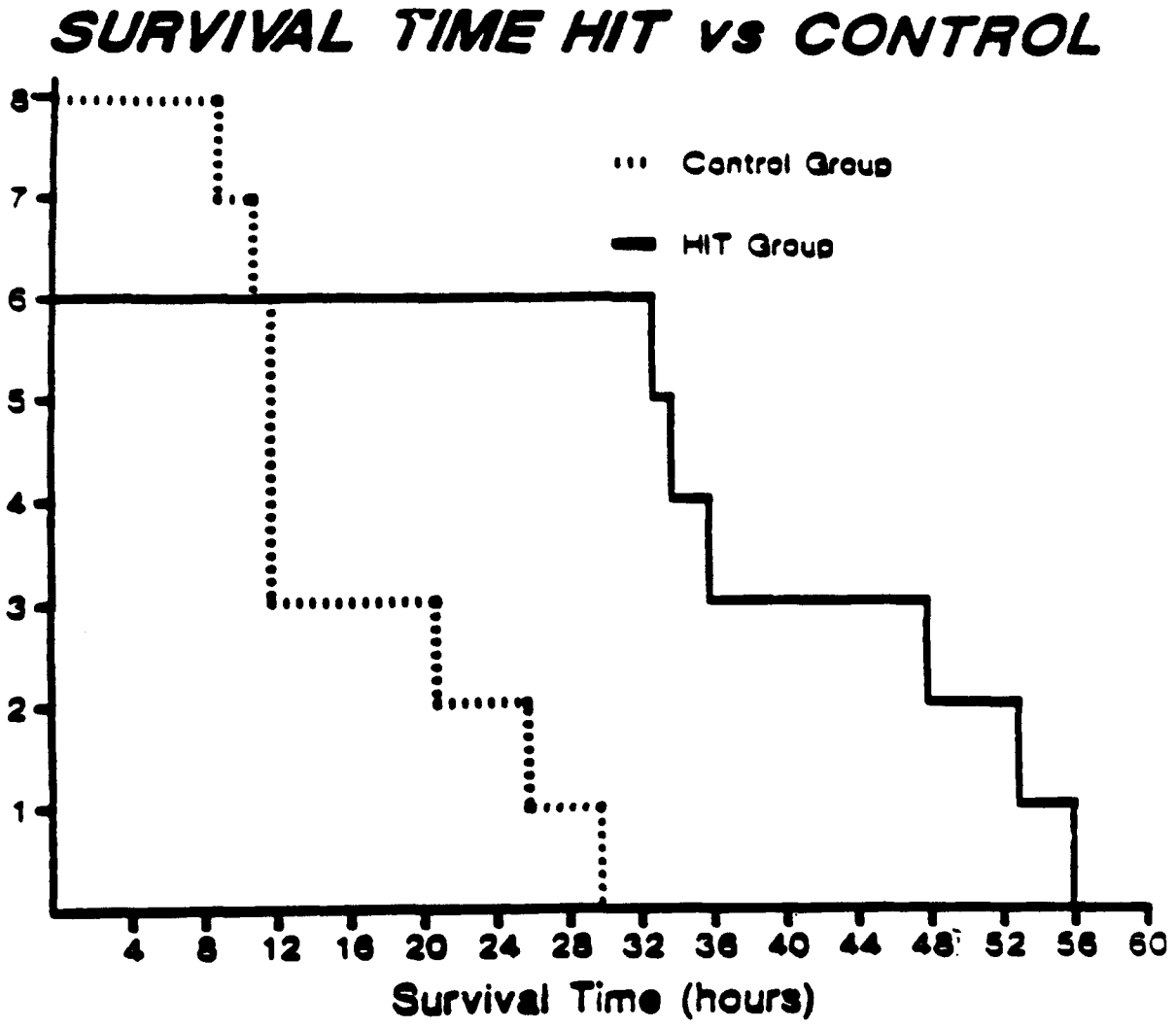

Figure 11. Survival of individual multiorgan preparations in the control group (n=8) versus the HIT treated group (n=6)

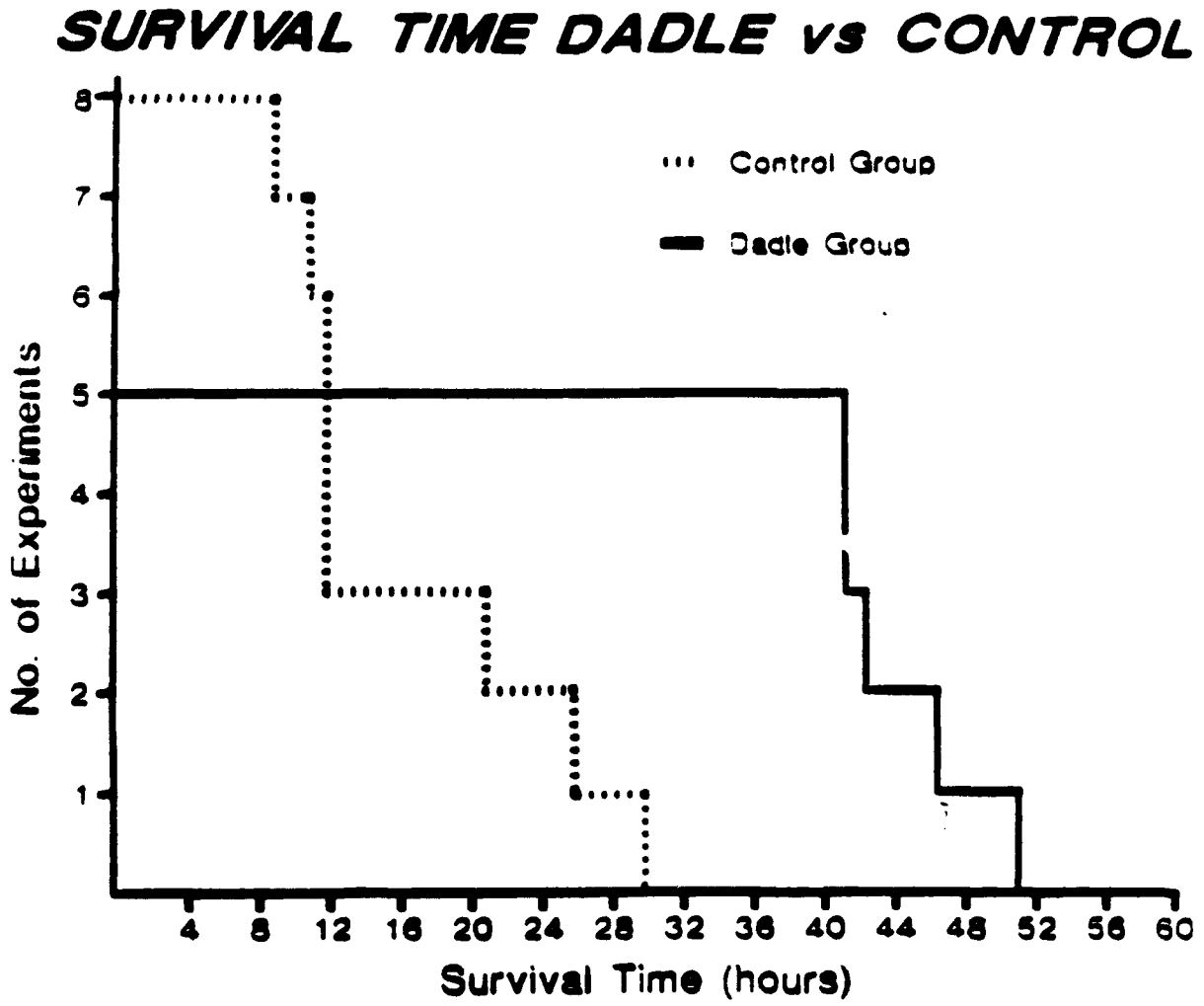

Figure 12. Survival of individual multiorgan preparations in the control group (n=8) versus the DADLE - treated group (n=5).

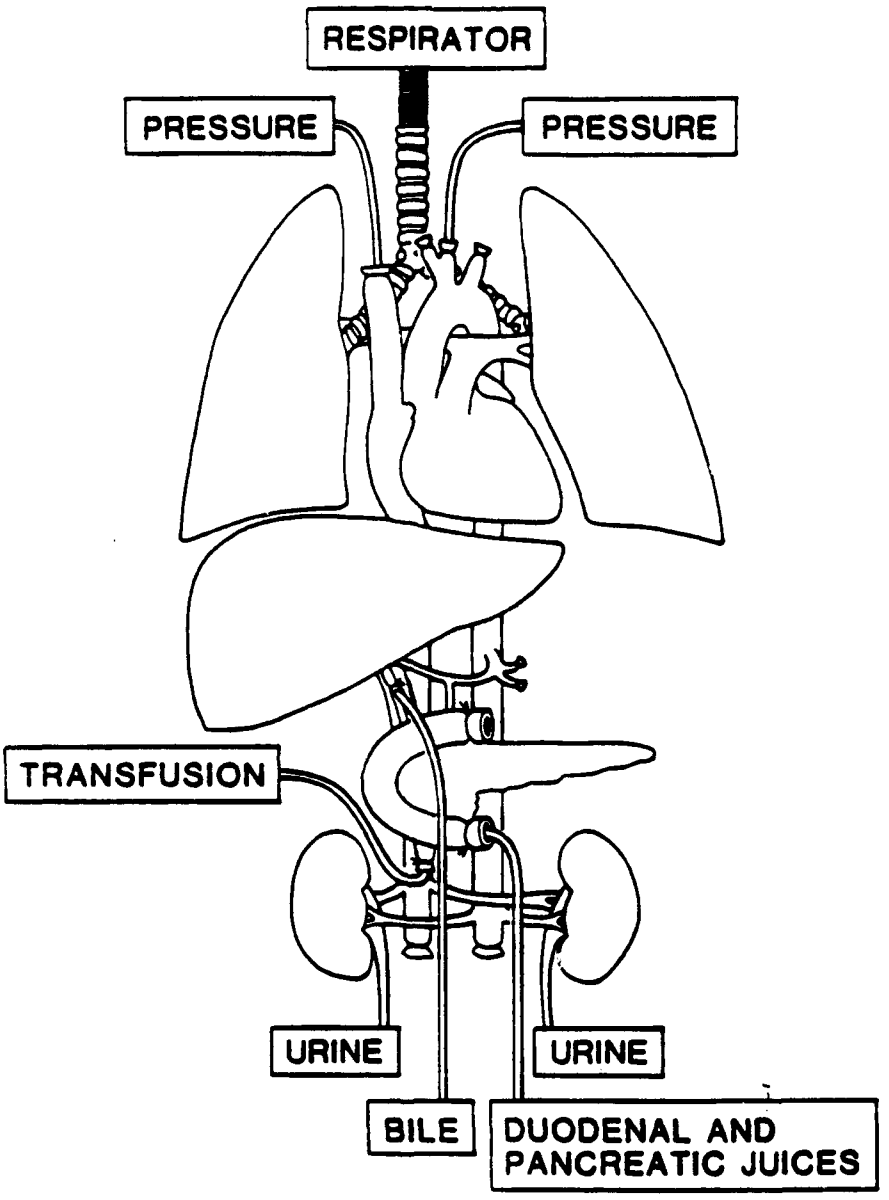

Figure 13. The preservation block used in this study. The heart, lungs, liver, pancreas, duodenum, and kidneys were preserved together.

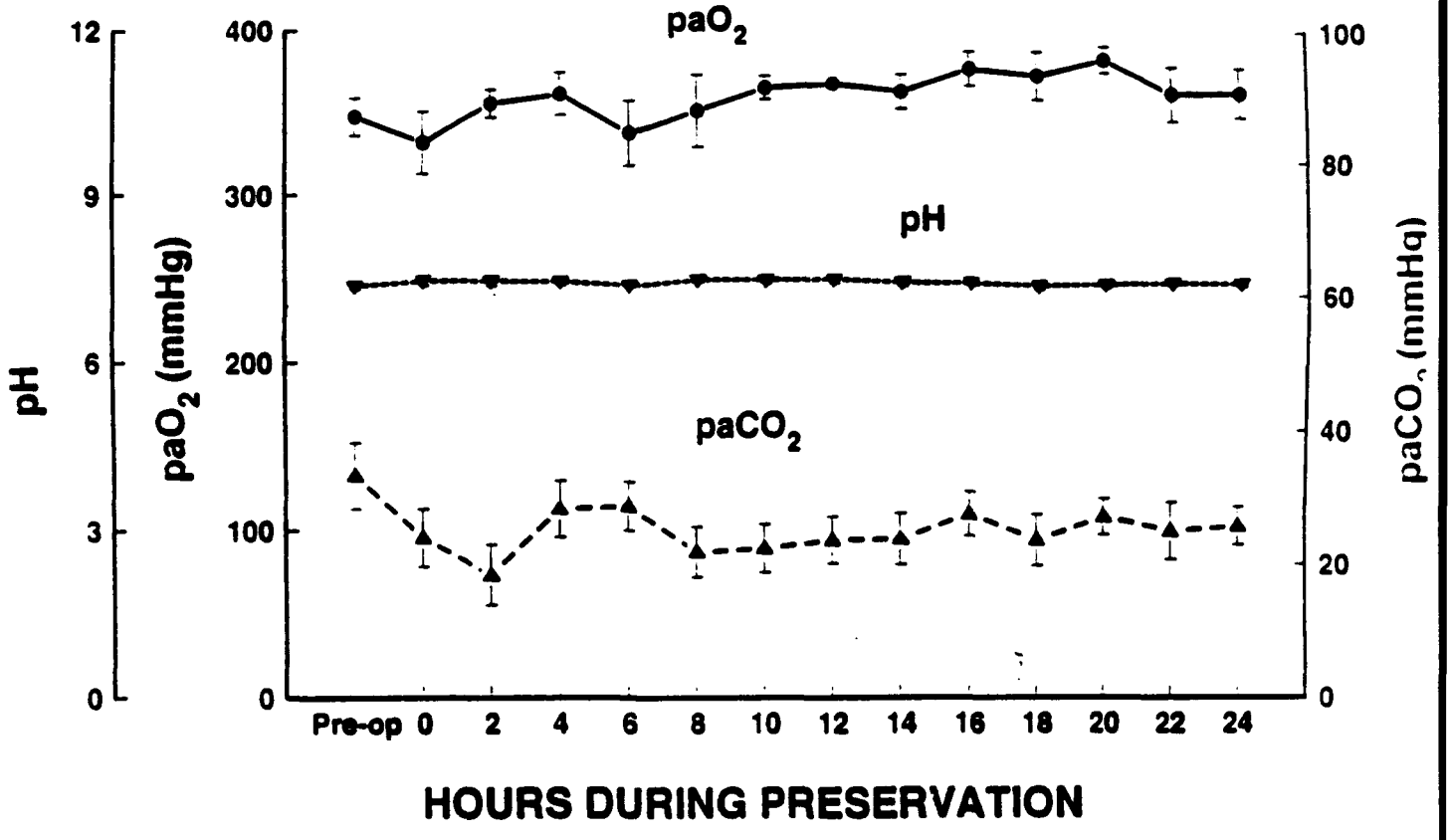

Figure 14. Change of arterial oxygen tension (paO2), arterial carbon dioxide tension ($paCO_2$), and pH during the preservation period.

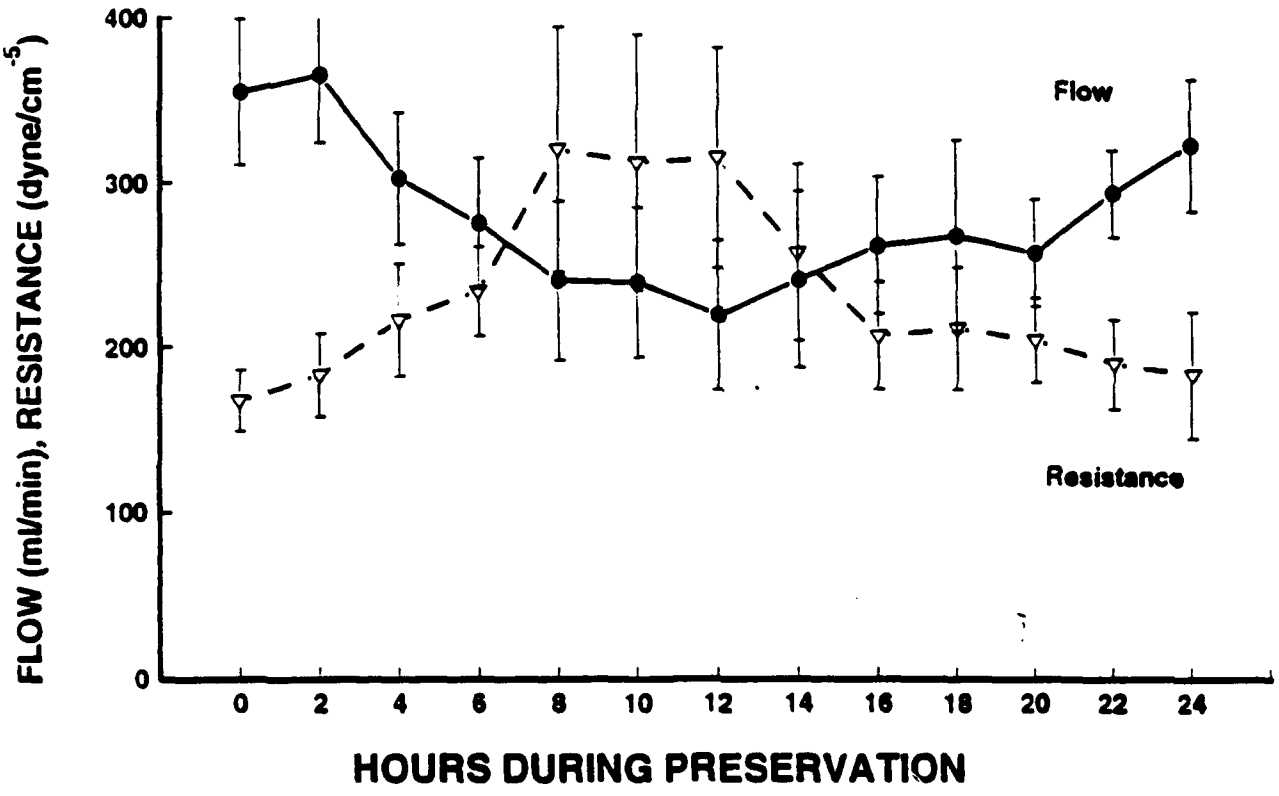

Figure 15. Maximum airway pressure and resistance during the preservation period.

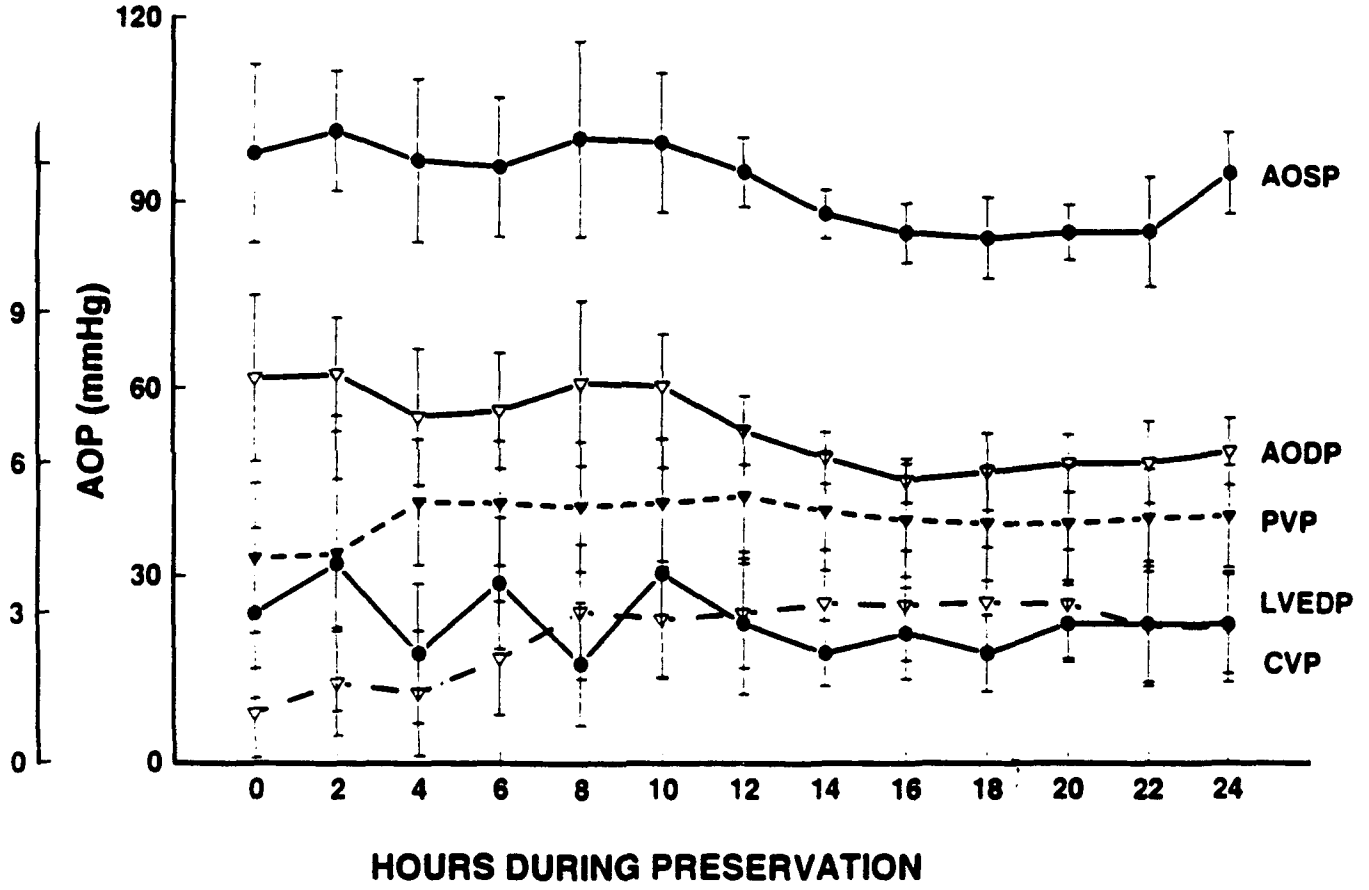

Figure 16. Aortic systolic pressure (AOSP), diastolic pressure (AODP), left ventricular end-diastolic pressure (LVEDP), central venous pressure (CVP) and portal venous pressure (PVP) during the preservation period.

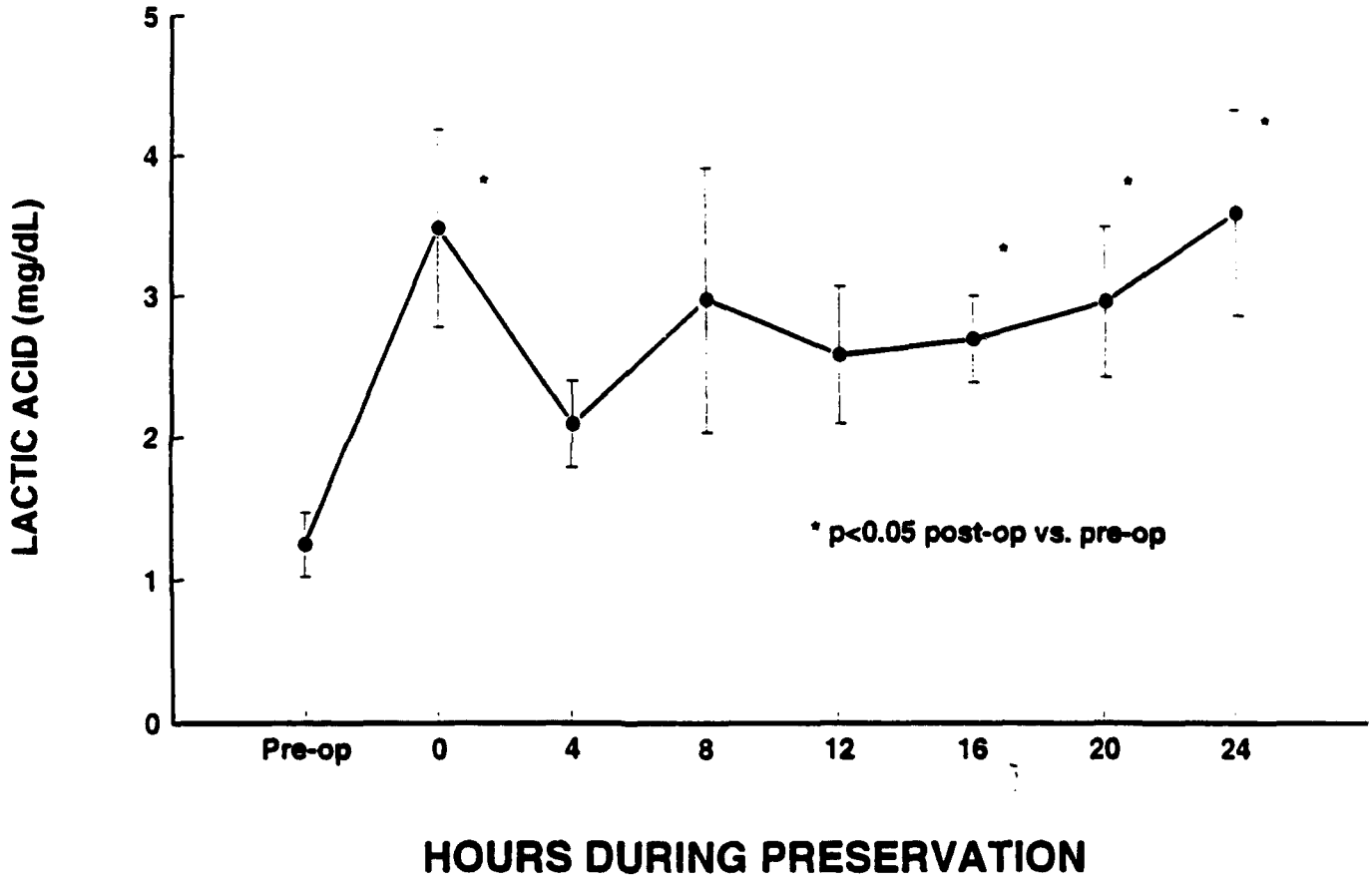

Figure 17. Change of serum lactic acid during the preservation period.

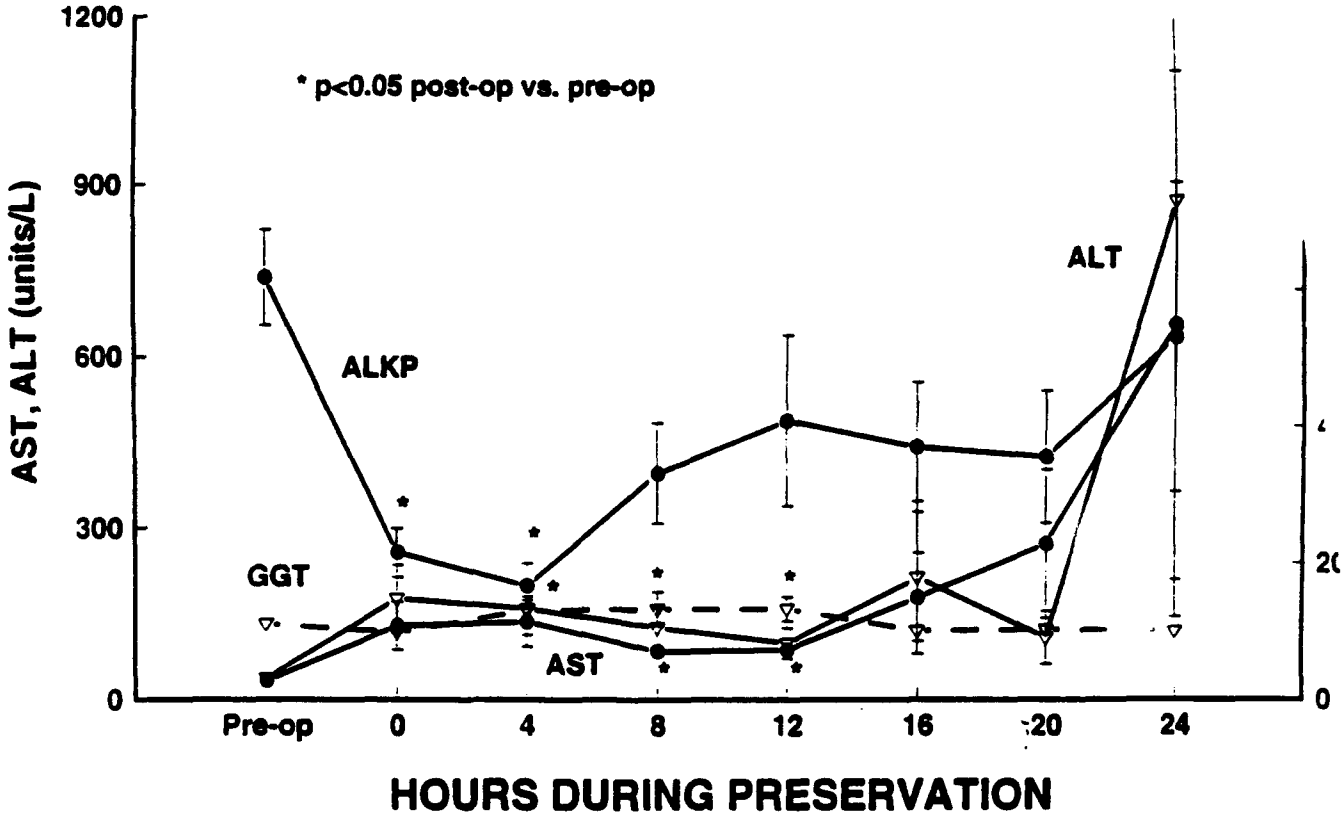

Figure 18. The change of liver-related enzymes during the preservation period. AST (aspartate amino-transferase), ALT (alanine amino-transferase), ALP (alkaline phosphatase), GGT (gamma glutamyl transpeptidase).

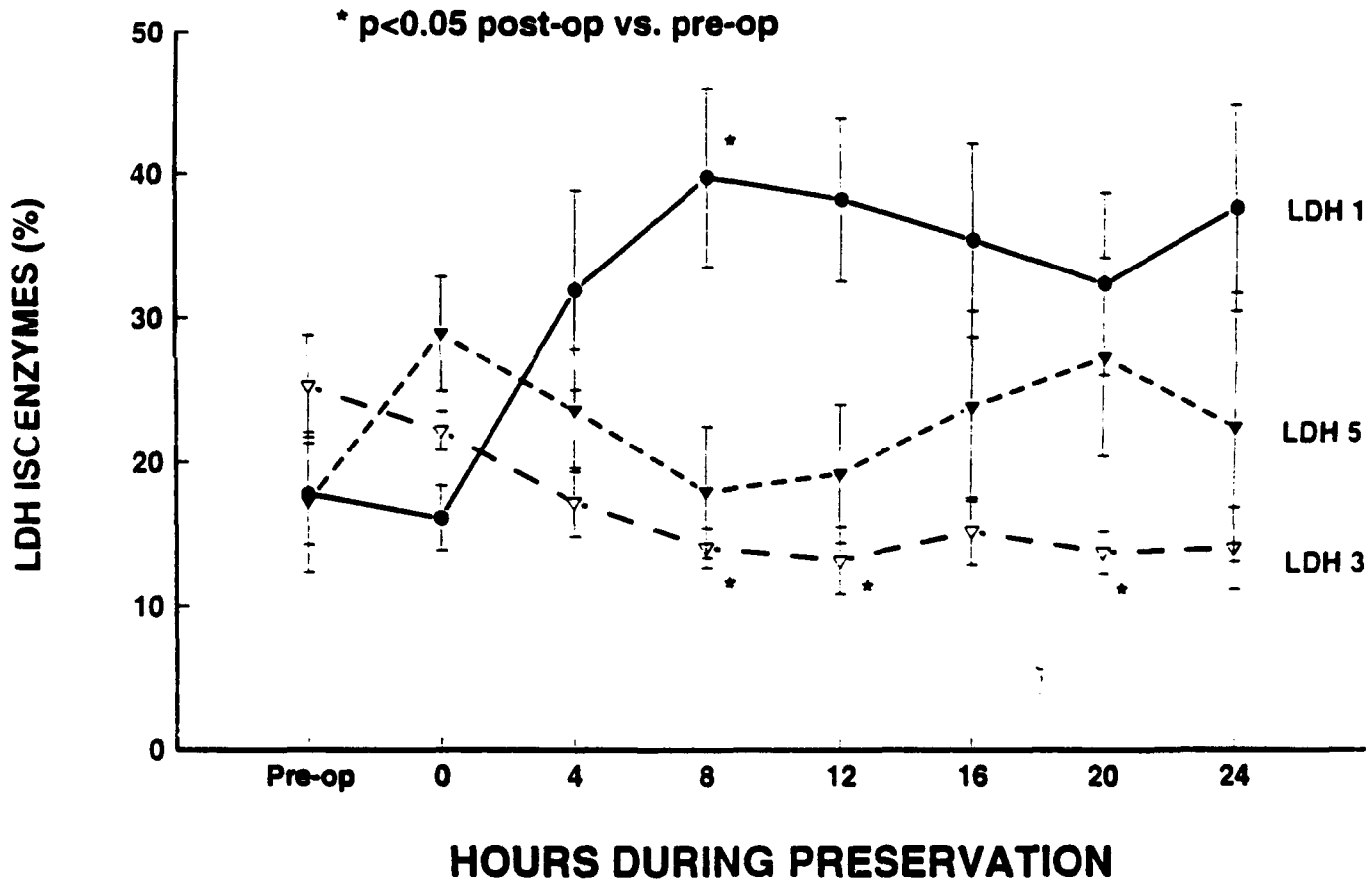

Figure 19. LDH isoenzyme 1, 3, and 5 profile during the preservation period.

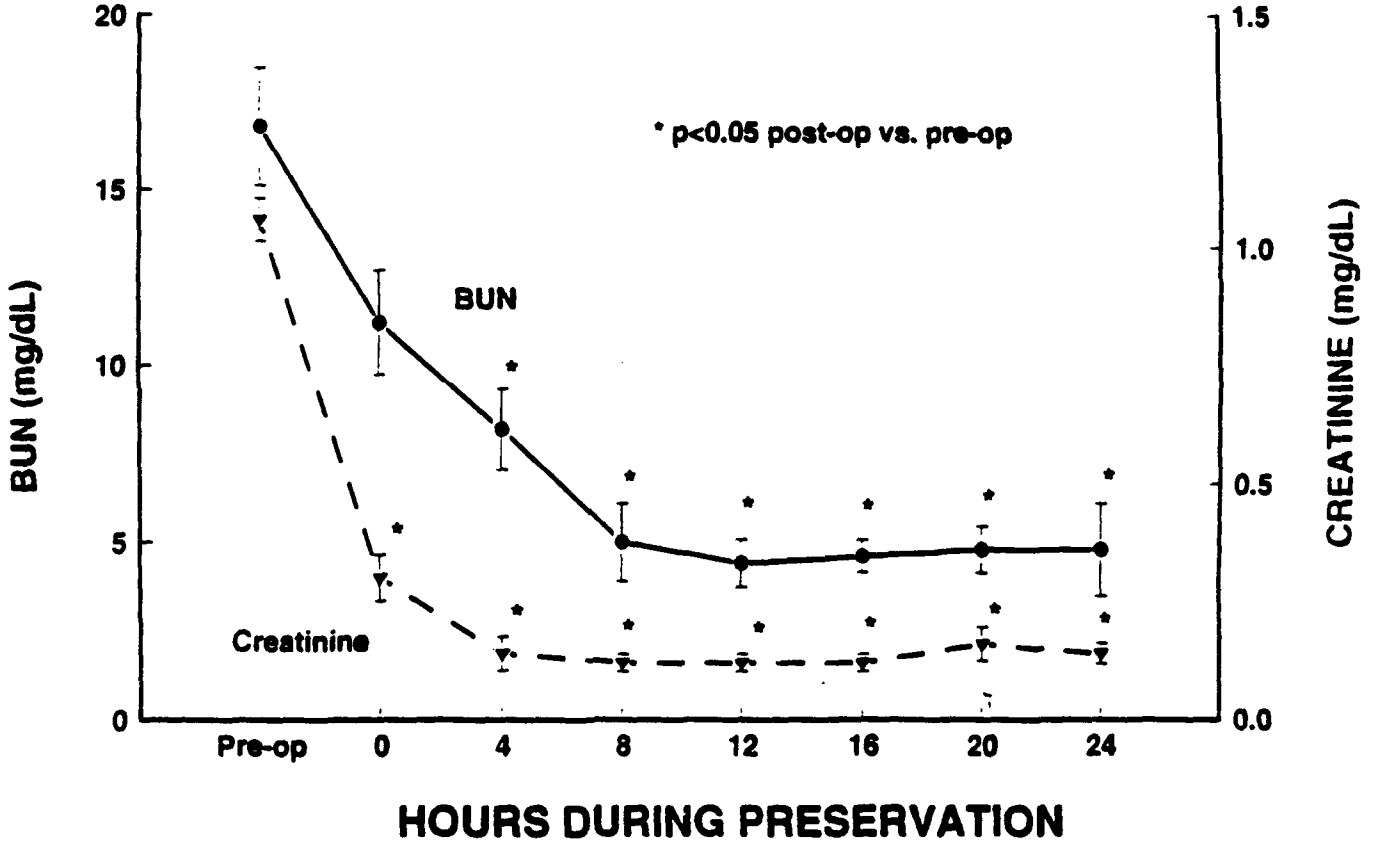

Figure 20. Blood urea nitrogen (BUN) and creatinine levels during the preservation period.

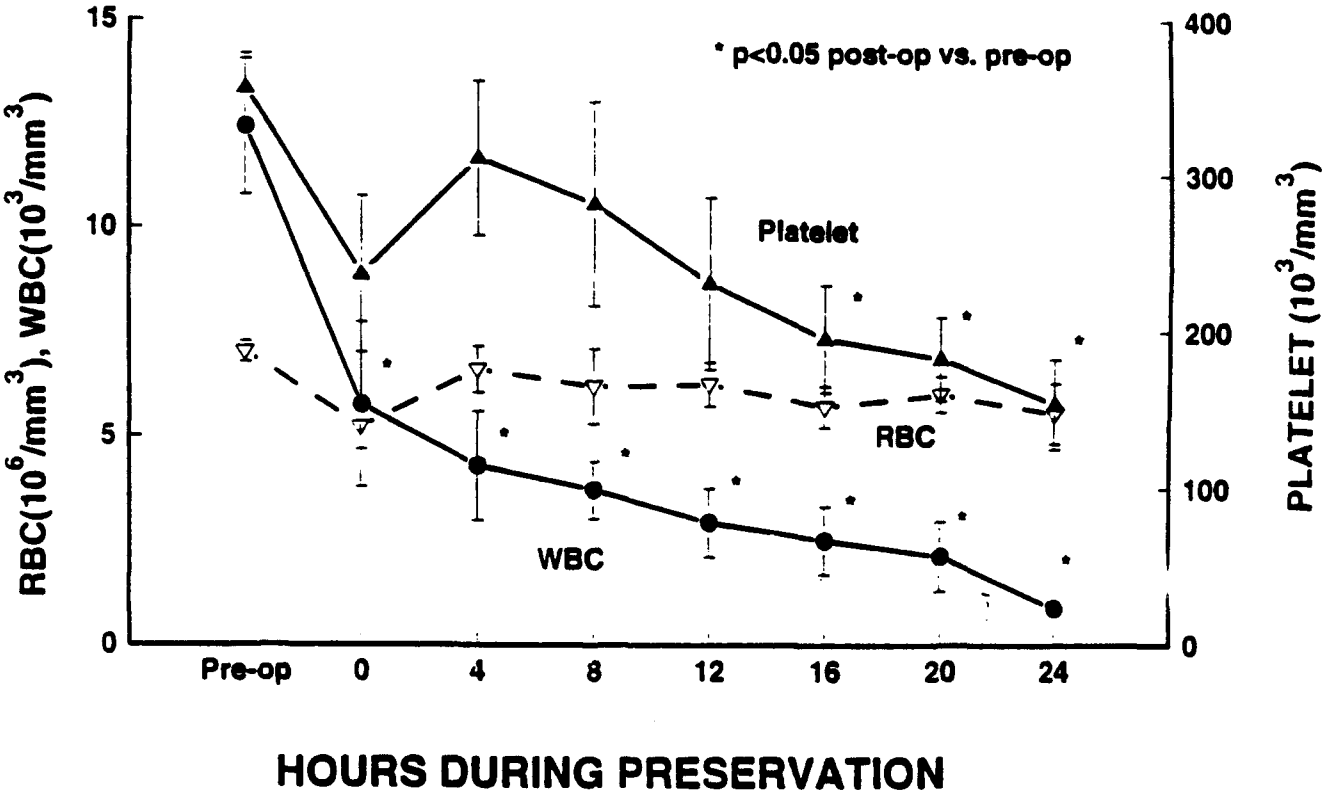

Figure 21. Change of blood cell counts during the preservation period. Red blood cell (RBC) levels remained stable, but white blood cell (WBC) and platelet levels decreased.

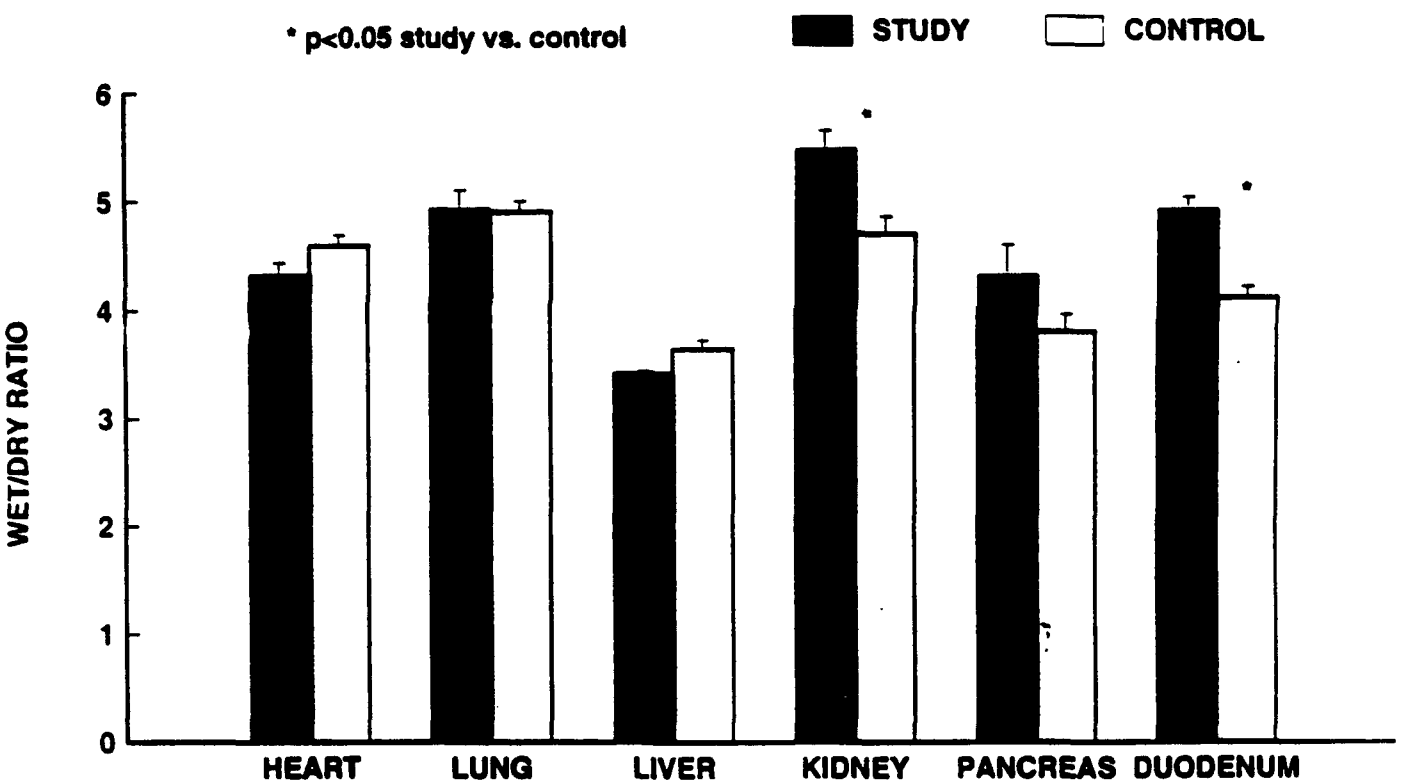

Figure 22. Tissue wet/dry ratios after preservation.

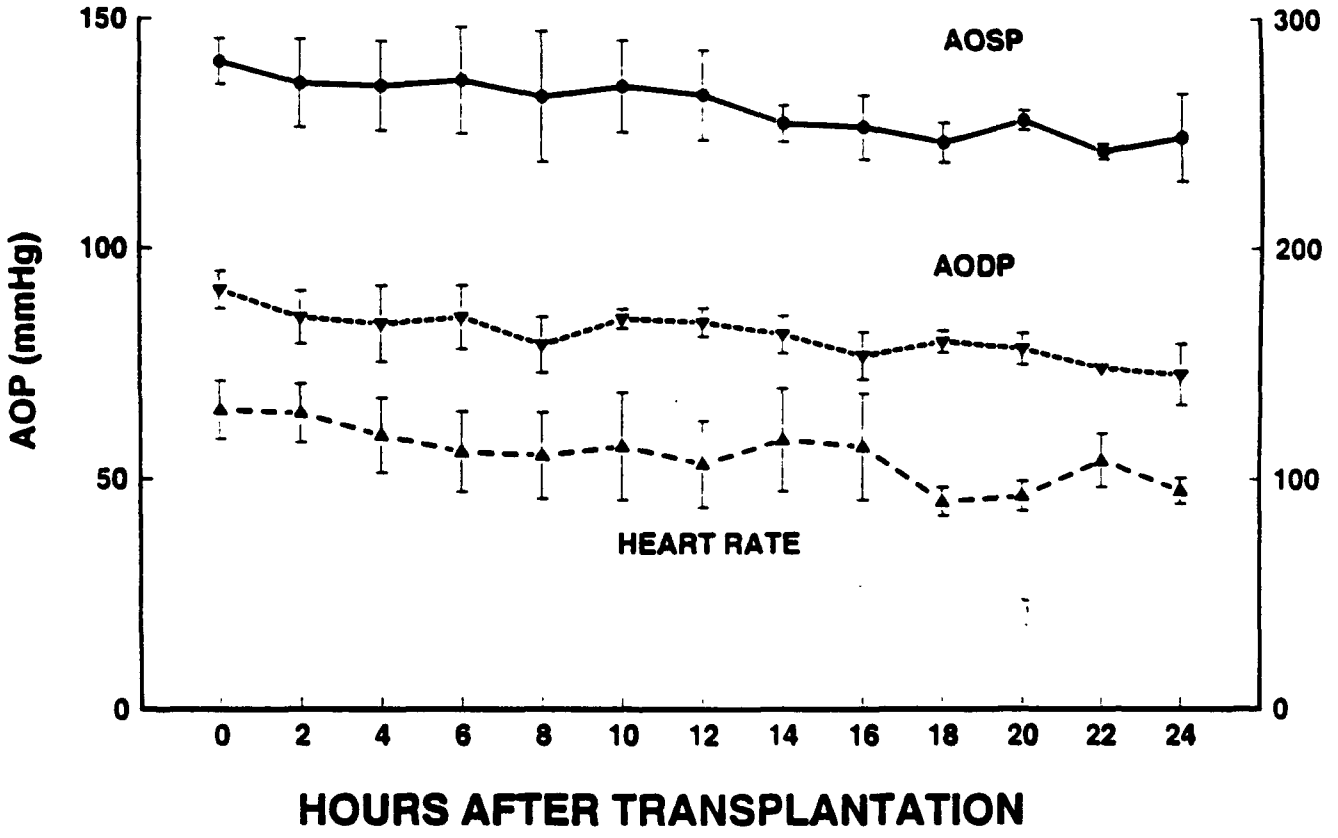

Figure 23. Blood pressures and heart rate after transplantation. AOSP (aortic systolic pressure), AODP (aortic diastolic pressure).

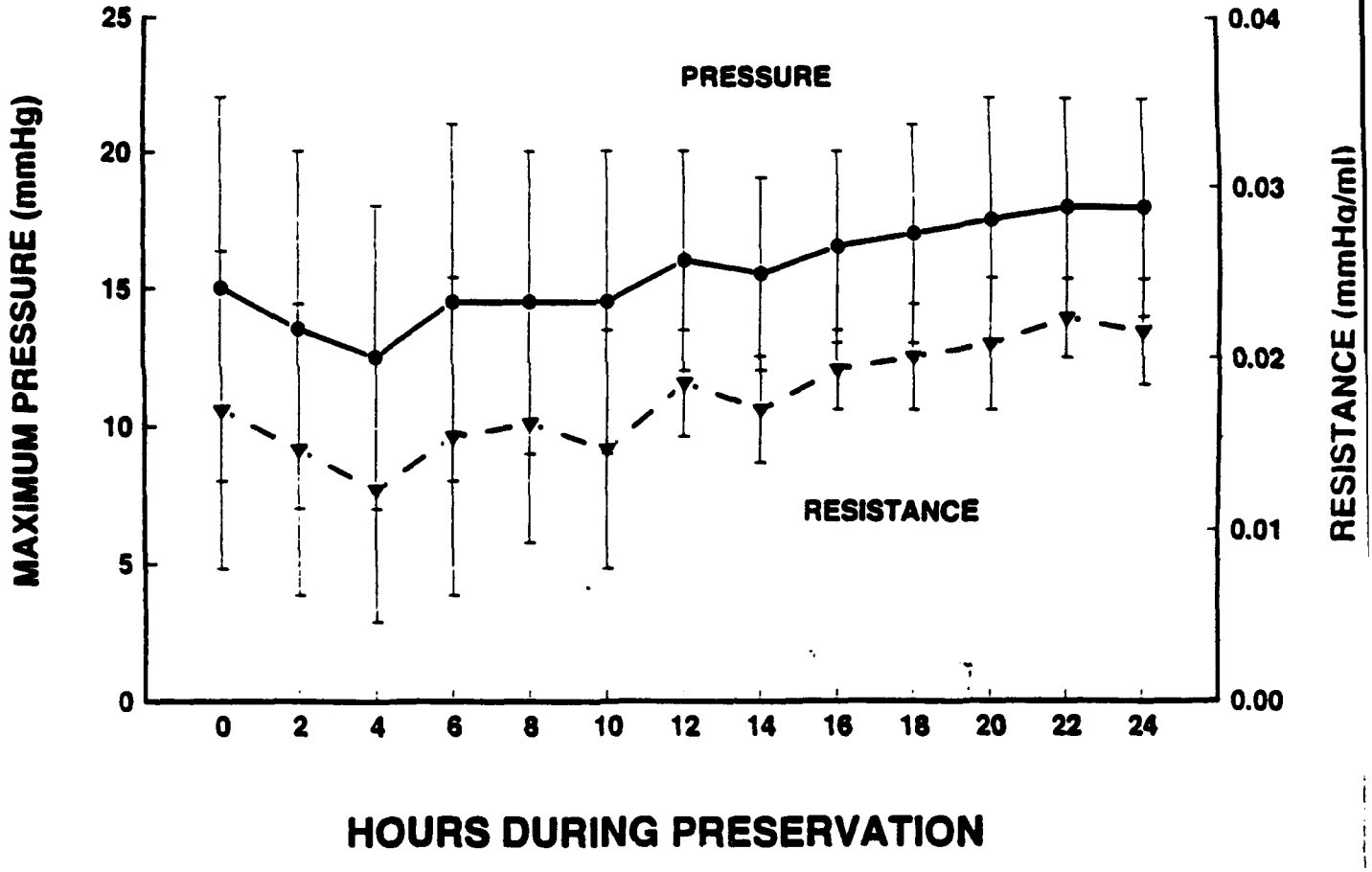

Figure 24. Maximum airway pressure and airway resistance after transplantation.

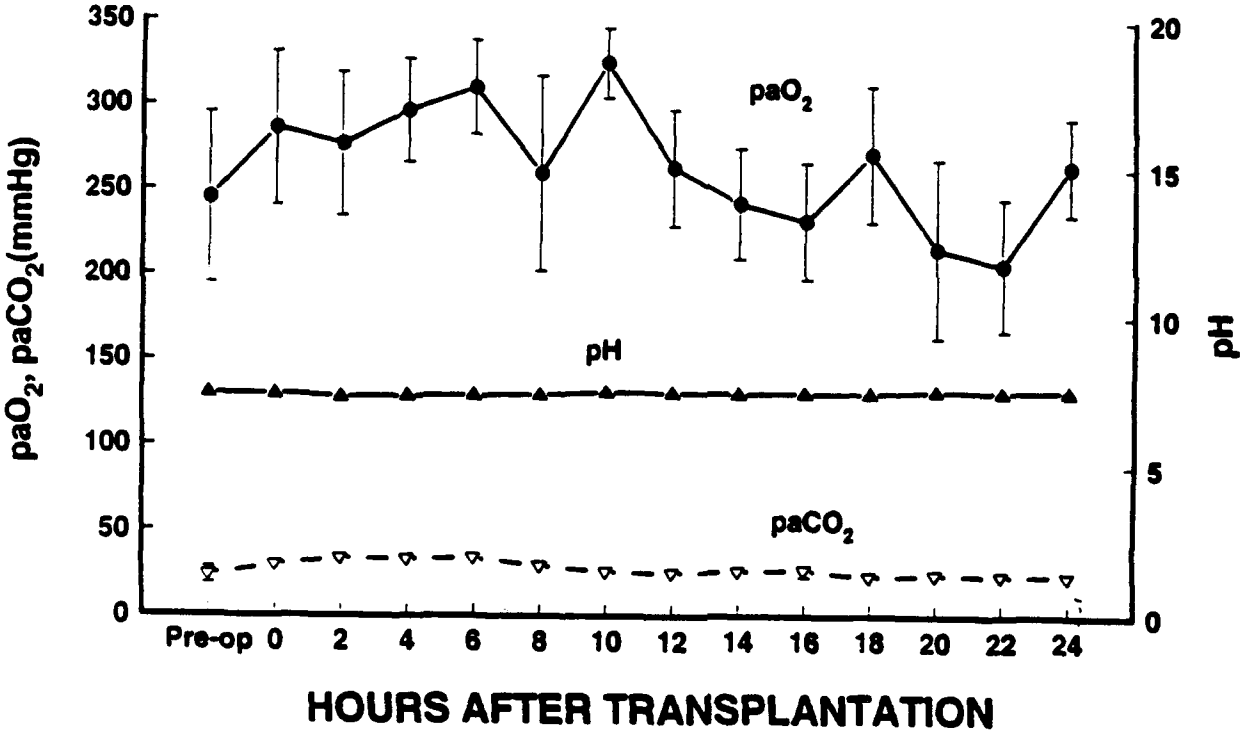

Figure 25. Arterial oxygen tension (paO2), carbon dioxide tension (paCO2), and pH after transplantation.

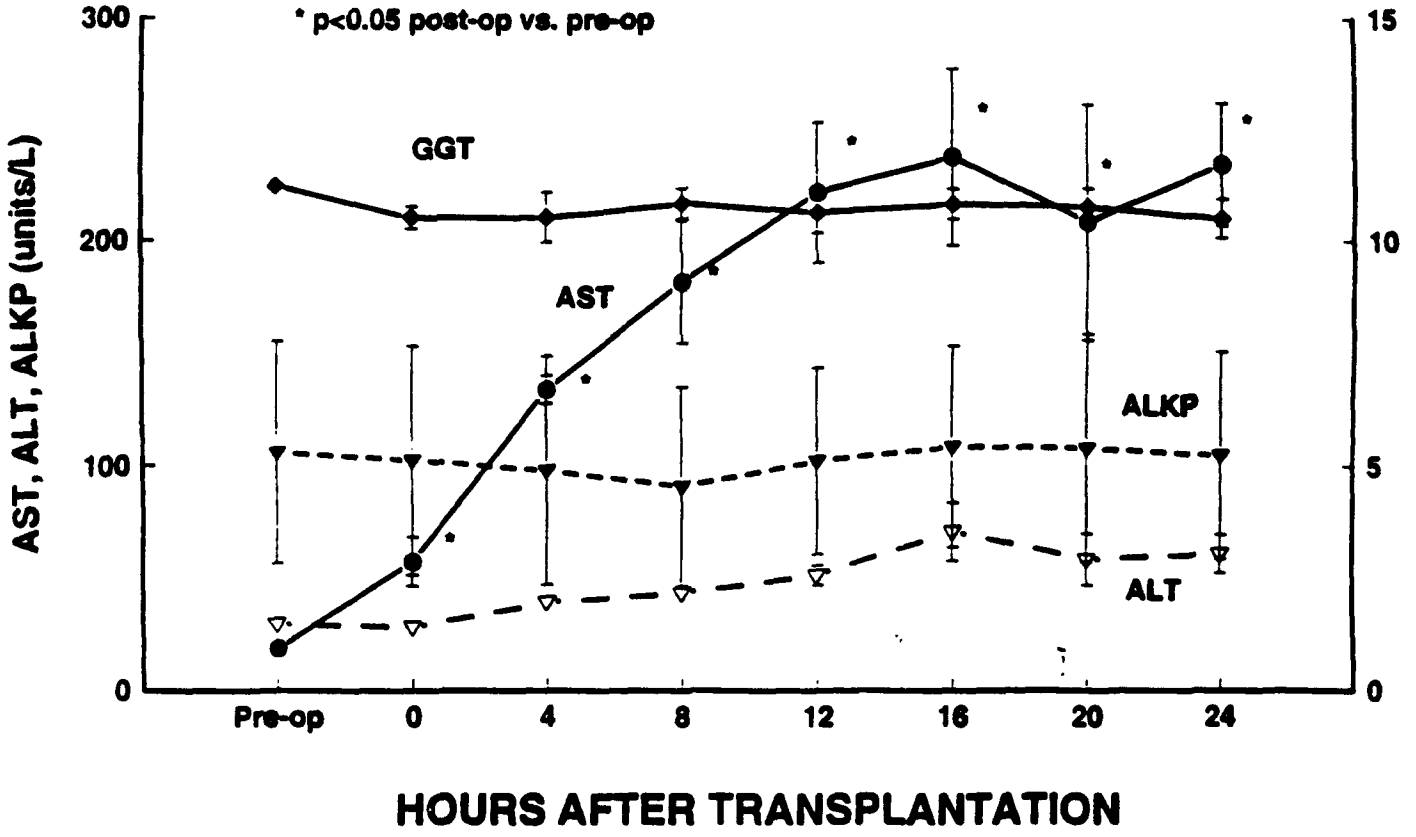

Figure 26. Change of liver-related enzymes after transplantation. AST (aspartate amino-transferase), ALT (alanine amino-transferase), ALP (alkaline phosphatase), GGT (gamma glutamyl transpeptidase).

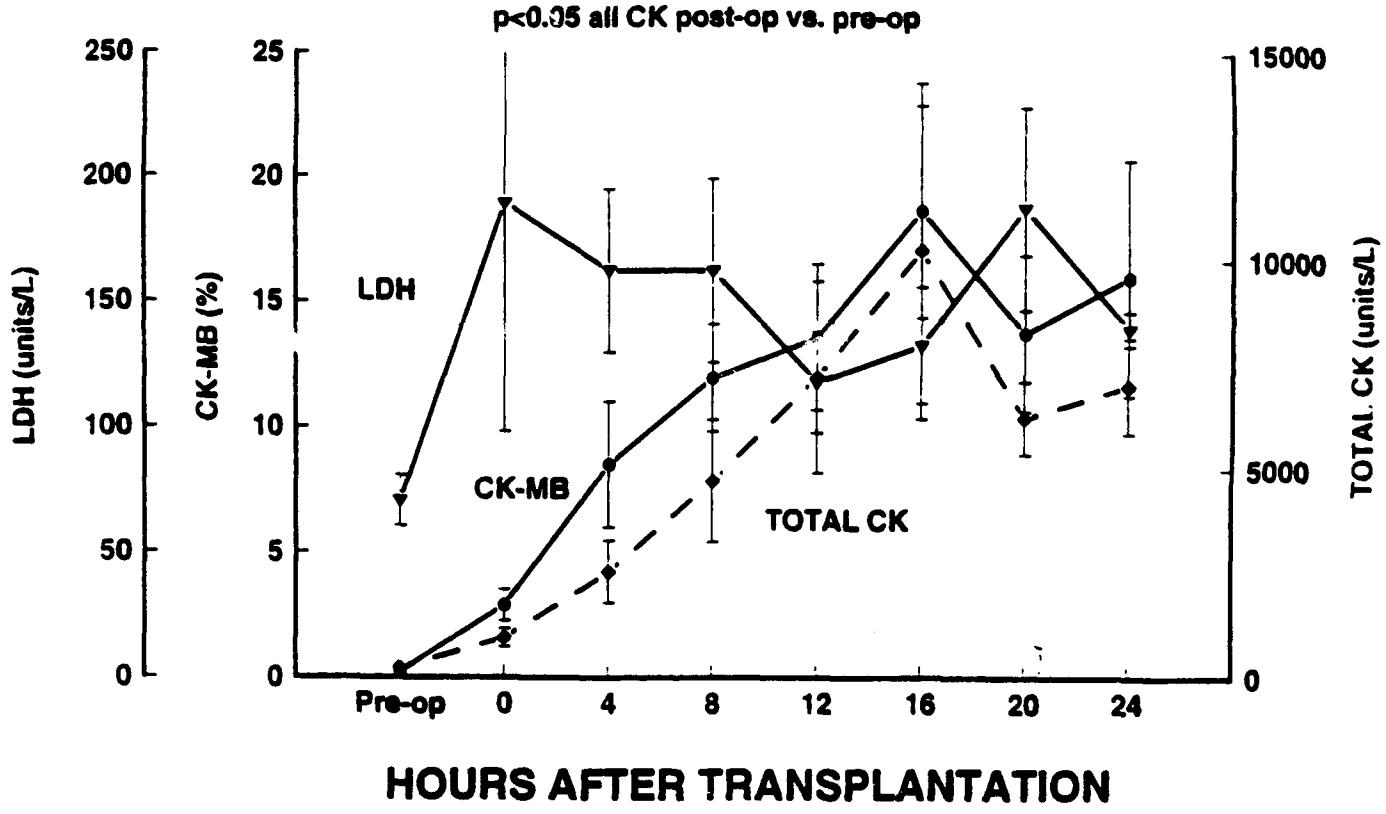

Figure 27. Change of LDH (lactic dehydrogenase), CK (creatine phosphokinase), and CK-MB levels after transplantation.

INDIVIDUALS RECEIVING CONTRACT SUPPORT

NAME	ROLE IN PROGRAM	% EFFORT
Sufan Chien, MD (change to Steven Bowling, MD)	Co-In	25%
Mitchell Turker, Ph.D.	Co-In	10%
Inna Kruman	Postdoctoral Fellow	100%
Noel Horton	Ph.D. Candidate Toxicology	100%