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An *In Vitro* Study with an Ussing Chamber Showing That Unfractionated Heparin Crosses Rat Gastric Mucosa

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Abbreviations: PD, potential difference; R, resistance; Isc, short circuit current; anti-Xa activity, anti-factor Xa activity; anti-IIa activity, anti-factor IIa activity; APTT, activated partial thromboplastin time; UFH, unfractionated heparin; LMWH, low molecular weight heparin; MWCO, molecular weight cut off; GAG, glycosaminoglycan.

Abstract

Heparin, traditionally given parenterally, is used to treat and prevent thrombosis. Our previous results suggest that orally administered unfractionated heparin (UFH) is absorbed and has antithrombotic effects. However, there is little evidence indicating the site and mechanism of heparin absorption. Our aim was to determine if the stomach is an absorption site. Rat gastric mucosa was mounted in an Ussing chamber, and UFH was added to the mucosal buffer at pH 7.4. Potential difference (PD), resistance (R) and short circuit current (Isc) across the mucosa were determined comparing the mucosal to the serosal side. Mucosal and serosal buffers and tissue were analysed for chemical heparin and anticoagulant activity, anti- factor Xa (anti-Xa) and anti-factor IIa (anti-IIa) activity. The PD became more negative on UFH addition. Following a lag period, PD returned to the resting level. Changes in R followed those in PD, while Isc did not change. Heparin was found in the serosal and mucosal buffer and tissue. Heparin in the serosal buffer had anti-Xa and anti-IIa activity. Decreasing the pH of the mucosal buffer to 4.0, decreased the lag period for PD. Decreasing the concentration of UFH resulted in less pronounced changes in PD and less heparin in the serosal buffer. Changes in PD suggest that heparin moves across the mucosa. Presence of heparin in the serosal buffer and mucosal tissue, indicate that heparin crosses rat gastric mucosa. A stable Isc indicates passive diffusion contributes to heparin movement. The stomach could be a site for oral heparin absorption.

Introduction

The commercial antithrombotic drug heparin is traditionally given by intravenous and subcutaneous routes due to the belief that it is not absorbed from the gastrointestinal tract and is ineffective when given orally (Canadian Pharmaceutical Association, 2005). This is based on the assumption that the high negative charge and molecular weight of unfractionated heparin (UFH, average molecular mass of 10 000 to 15 000 daltons) prevent its absorption (Money and York, 2001) and that heparin is destroyed by stomach acids (Dal Pozzo et al., 1989). Little or no change in plasma activated partial thromboplastin times (APTT), an assay commonly used to measure heparin in plasma, following oral heparin administration has also supported the presumption that heparin is not absorbed (Leone-Bay et al., 1998).

Results from our laboratory and others, however, have challenged the historical belief that heparin is ineffective when given by the oral route. Administration of heparin *in vivo,* by parenteral routes, as well as *in vitro* experiments showed that UFH and low molecular weight heparins (LMWHs) bind avidly to endothelium (Hiebert and Jaques, 1976a; Hiebert and Jaques, 1976b; Glimelius et al., 1978; Barzu et al., 1985; Hiebert et al., 1993; Hiebert et al., 2001). Our observations also showed that orally administered heparin was found with endothelium despite low plasma levels (Hiebert et al., 1993). As well, oral UFH and LMWHs significantly reduced thrombotic incidence in both rat jugular vein and carotid

arterial models (Hiebert et al., 2001; Hiebert et al., 2000; Hiebert et al., 1996; Pinel et al., 2004). Furthermore, an increase in plasma anti-Xa activity and heparin in urine was observed in human subjects following oral administration (Hiebert et al., 2005). Therefore, these results suggest that heparin is absorbed following oral administration, and that the oral route may be a feasible alternative for administering heparin.

The site of oral heparin absorption and the mechanism responsible, however, are not known. Previous studies on heparin absorption from the gastrointestinal tract examined the small intestine (Sue et al., 1976), the anatomically favored and major site of absorption for most compounds. Sue et al.,1976 reported that when heparin was introduced into the gastrointestinal tract, increases in whole blood clotting time and plasma anti-Xa activity were observed. The anticoagulant effect was greater when heparin was placed in the stomach versus the small intestine. Furthermore, more chemical or [^{14}C] UFH was found in stomach tissue versus duodenum, jejunum, and ileum or colon tissue up to 24 hours following administration by stomach tube (Hiebert et al., 2000). These studies, therefore, suggest that the stomach may be a site for heparin absorption. Thus, the objective of the present study was to determine if orally administered heparin crosses rat gastric mucosa when mounted in an Ussing chamber. From these experiments, we conclude that heparin crosses rat gastric mucosa suggesting that the stomach may be a site for oral heparin absorption.

Methods

Chemicals

Bovine lung unfractionated heparin (156.2 Units/mg) was obtained from Scientific Protein Labs, Division of Viobin Corporation, Wisconsin, USA. Materials for gel electrophoresis; petroleum ether, glacial acetic acid, and acetone were obtained from VWR Canlab, Mississauga, ON, Canada; sodium barbital, hexadecyltrimethylammonium bromide, toluidine blue, and HCl were from Sigma-Aldrich, ON, Canada; and agarose was from Bio-Rad, Mississauga, ON, Canada. Materials for Kreb's buffer, $MgCl_2$-$6H_2O$,$CaCl_2$-$2H_2O$, NaCl, KCl, Na_2HPO_4, NaH_2PO_4-H_2O, $NaHCO_3$, mannitol, D-glucose, were from VWR Canlab. Molecular weight cut off (MWCO) 1000 dialysis tubing was purchased from Spectrum Laboratories Inc., RanchoDominguez, CA, USA. Materials for heparin extraction from mucosal tissues; protease from Streptomyces griseus was from Sigma-Aldrich; Tris, $CaCl_2$, isopropanol, and methanol were from VWR Canlab. The chromogenic assay kit (Accucolor™ Heparin®) for measurement of anti-Xa activity was obtained from Sigma-Aldrich. Reagents for measurement of anti-IIa activity, as outlined below, were from DiaPharma Group, Inc. West Chester, OH. QIAprep® Spin M13 Kit (50) columns for desalting the buffers were from Qiagen, ON, Canada. Chemicals for anaesthesia, chloral hydrate, sodium pentobarbital, magnesium sulfate, ethanol and propylene glycol were obtained from Sigma-Aldrich.

Gastric mucosa isolation from rats

Animals were obtained from Charles River Canada Company, St. Constant, Quebec, Canada and were handled in accordance with the Principles of Animal Care set by the Canadian Federation of Biological Societies. All animal procedures were approved by the Animal Care Committee of the University of Saskatchewan and performed according to the guiding principles of the Canadian Council on Animal Care. Male Wistar rats (n= 34, 250-300 g) were anesthetized by injection of Equithesin (chloral hydrate 4.2% w/v, sodium pentobarbital 0.98% w/v, magnesium sulfate 2.12% w/v, ethanol 10% v/v, propylene glycol 40% v/v, and sterile water to a volume of 100 ml: 1 ml/250 g rat) through the intraperitoneal route. The abdominal cavity was entered by a medio ventral incision. Retractors were placed on each side of the incision to hold the abdomen open. The stomach was removed by cutting its connections with the esophagus and duodenum, and was then placed in saline. The stomach was opened from the lesser curvature and then washed several times with saline until all stomach contents and blood were removed. The stomach was then stretched, mucosal side up, on the cork bottom of a plastic tray using pins to hold it in place. The tray was placed on ice to reduce the metabolic rate. The mucosa of the glandular portion of the stomach was carefully stripped from submucosa and serosa using a scalpel blade, leaving the mucosa intact for use in the Ussing chamber.

Measurements of heparin movement across gastric mucosa using an Ussing chamber.

An EVC 4000 voltage/current clamp (NaviCyte, Harvard Apparatus, Inc.) was used for transport studies across the gastric mucosa and included three components: (1) vertical diffusion Ussing chambers, in which the mucosa acted as a partition between the two hemi-chambers thus creating two separate compartments: the mucosal compartment and the serosal compartment; (2) electrodes for sensing the voltage and for passing current; (3) instrumentation which measured both voltage and current. Immediately following separation from submucosa and serosa, the mucosa was mounted on the pins of one hemi-chamber, and the matching hemi-chamber was attached to seal the diffusion apparatus. As a control, an additional portion of the mucosa was frozen for later glycosaminoglycan (GAG) extraction.

The assembled chamber was placed in a block heater connected to a circulating water bath, which maintained a temperature of 37ºC. The reservoirs on each side of the mucosa were filled with warmed (37ºC) oxygenated Kreb's Ringer bicarbonate buffer ($MgCl_2$-$6H_2O$, 1.1 mM; $CaCl_2$-$2H_2O$, 2.15 mM; NaCl, 113.96 mM; KCl, 5.03 mM; Na_2 HPO_4, 1.65 mM; NaH_2PO_4-H_2O, 0.30 mM; $NaHCO_3$, 25 mM) of pH either 7.4 or 4.0. As an energy source, D-glucose (40 mM) was added to the serosal-side (serosal) buffer. Mannitol (40 mM) was added to the mucosal-side (mucosal) buffer to provide an equivalent osmotic load between mucosal and serosal buffers. Buffers in the reservoirs were circulated by gas lift (95% O_2 /

5% CO_2), controlled by valves (Precision Instrument Design, Los Altos, CA). Buffers (1.5 ml / hemi-chamber) were added to each side of the chamber simultaneously to prevent hydrostatic pressure effects. The exposed mucosal surface area was 2.5 cm^2.

Harvard/ Navicyte Micro-Reference voltage measuring electrodes (2.5 mm x 5.0 cm) were placed on either side of the mucosa as close as possible to reduce the magnitude of the series resistance of the solution. Electrodes used for passing current were placed in the rear of the chambers, as far as possible from the mucosa to ensure uniform current density across the mucosa. Voltage and current electrodes were connected to the amplifier. After eliminating asymmetries in the voltage measuring electrodes, the following basic electrical properties across the mucosa were measured at specific intervals: potential difference (PD) or voltage difference across the mucosa (ΔV) in mV, resistance (R) in mΩ, and short circuit current (Isc) in mA (a measure of the net active ion transport across the mucosa).

Using the pulse generator, a current of 15 mA was passed across the mucosa and ΔV was recorded. The R was then calculated using Ohm's law:

$$R = \Delta V_t I^{-1} \quad (1)$$

where ΔV_t is voltage difference across the mucosa at a specific time and I is the passed current across the mucosa of 15 mAmps.

Finally, the transmucosal current was clamped to zero (i.e. there was no net transmucosal current flow) under the current clamp mode and ΔV_t was measured. Since R of the tissue is known, the short circuit current (Isc) can then be calculated:

$$Isc = \Delta V_t R_t^{-1} \quad (2)$$

The tissue was stabilized in buffer for 40 min with electrical measurements taken every five min. Heparin was then added to the mucosal buffer by adding 0.1 ml of the heparin stock solution to obtain a final concentration of 10 or 0.7 mg/ml. Electrical measurements were continued every two min for an additional 84 min. Mucosal and serosal buffers as well as mucosal tissues were then collected and frozen at -4ºC for later extraction and analysis.

Assessment of mucosal injury

Trypan blue was used to determine any existing minor damage in the isolated mucosa in some experiments. Gastric mucosal tissue was mounted in the Ussing chamber and UFH was added to the mucosal buffer at pH 4.0. The experiment was performed for 124 min as described above. Then, 100 µl of trypan blue stock solution (0.3 mg/ml) was added to the mucosal buffer. After 15 min, mucosal and serosal buffers were collected and absorbance was read at 590 nm. Serosal buffer without trypan blue was used as a control. The trypan blue concentration in samples was determined by comparing the absorbance to a reference curve prepared with known amounts of the dye.

As well the mucosal tissue was punctured with a 25G needle to cause intentional injury. The electrical parameters, PD, R and Isc were determined with addition of heparin.

Heparin extraction from buffers

Mucosal and serosal buffers were dialyzed in distilled water for 48 h using molecular weight cut off (MWCO) 1000 dialysis tubing. The dialyzed buffers were then dried and analyzed for chemical heparin as well as heparin bioactivity by agarose gel electrophoresis and anti-Xa and anti-IIa activity respectively.

Heparin extraction from mucosa

GAGs were extracted from treated and untreated mucosal tissue by a modified published method (Jaques, 1977). Mucosa was minced, defatted with acetone and isopropanol/petroleum ether (1:1), and digested by protease in 0.1M Tris buffer with 0.1M $CaCl_2$ at pH 8.0 at 37°C. Digests were purified by precipitating with 1%NaCl in acetone and methanol. The precipitates were then dried.

Identification and chemical measurement of extracted heparin

Agarose gel electrophoresis was used to identify and measure heparin in extracts. Dried powders were dissolved in suitable volumes of water and applied to agarose gel slides, along with the administered heparin as a reference. Gels were fixed in 0.1% hexadecyltrimethylammonium bromide and air-dried. Slides were stained with 0.04% toluidine blue in 80% acetone, and background color

was removed using 1% acetic acid. Heparin was identified by electrophoretic migration as compared to reference material and amounts were determined by densitometry.

Measurement of anti-factor Xa (anti-Xa) and anti-factor IIa (anti-IIa) activity in buffers

Mucosal and serosal buffer extracts were desalted using QIAprep® Spin M13 Kit columns. Columns were washed twice with distilled water by centrifuging at 5000 rpm for 5 min. Dried powders from mucosal and serosal buffer extracts were dissolved in suitable volumes of distilled water and were then added to the washed columns. After 15 min, columns were washed twice with distilled water and centrifuged at 5000 rpm for 5 min. The membrane of the spin column was then washed using a pipette with 200 µl of distilled water. The washes were collected and dried obtaining a desalted buffer extract. Mucosal buffer extracts were dissolved in 1 and 10 ml of distilled water and serosal buffer extracts were dissolved in 100 µl of distilled water for the measurement of anti-Xa and anti-IIa activity.

A chromogenic assay was used to identify and measure heparin anti-Xa activity in the desalted buffer extracts. Human antithrombin III (200 µl) was added to a plastic test tube. Next, 25 µl of heparin standard or buffer extracts was added, mixed, and incubated at 37°C for 2 min. Bovine factor Xa (200 µl) was then added with mixing and incubated at 37°C for 1 min. Factor Xa substrate (200 µl)

was added to the same tube and incubated for exactly 5 min. Finally, 200 µl acetic acid was added with mixing to terminate the reaction. The solution was read at absorbance of 405 nm. Similarly a chromogenic assay was used to measure anti-IIa activity in the desalted buffer extracts. Human antithrombin III (25 µl) was added to a plastic test tube along with 25 µl of heparin standard or buffer extracts and 200 µl of buffer (Tris 50 mmol/l, NaCl 175 mmol/l, Na_2EDTA-$2H_2O$ 7.5 mmol/l, pH 8.4) with mixing and incubation at 37°C for 3-4 min. Human thrombin 100 µl was then added for 30 sec to 200 µl of the above mixture followed by addition of the chromogenic substrate S-2238 (200 µl) for 1 min. Finally, 300 µl acetic acid was added with mixing to terminate the reaction. The absorbance of the sample was read at 405 nm. Heparin concentrations in samples for both assays were determined by comparing the absorbance to a reference curve prepared with known amounts of heparin.

Data analysis and statistical procedures

All data are expressed as mean ± standard error of the mean (SEM). A one-tailed unpaired t-test was used to determine significant differences in the lag period before PD began to return to the resting level in different environments. A two-tailed t-test was used to measure differences in heparin concentrations in serosal buffers, experimental mucosa, and control tissue under different conditions, as well as to measure differences in PD increase upon heparin addition to the mucosal side of the Ussing chamber. A paired two-tailed t-test was used to measure differences in trypan blue concentrations between

mucosal, and experimental and control serosal buffers. Values were considered significant at $P<0.05$.

Results

Electrical parameters of rat gastric mucosa following addition of UFH to the mucosal side at pH 7.4

To observe heparin movement across rat gastric mucosa under neutral conditions, as a control compared to the usual acidic environment of the stomach, the gastric mucosa was first placed in the Ussing chamber with buffers at pH 7.4. The electrical properties of the membrane were recorded every 5 min. Results are shown in Fig.1. When UFH (10 mg/ml) was added to the buffer on the mucosal side of the mounted gastric mucosa after 40 min of stabilization, the PD became more negative, and the charge difference between the mucosal and serosal side of the membrane increased compared to PD prior to UFH addition (Fig 1A). After a lag period of 18.8 ± 4.7 min, the PD began to decrease. The PD achieved the previous resting level 39.3 ± 17.6 min later, after which the PD continued to change with time and became positive compared to the resting level. R of the mucosa followed a pattern similar to that seen for the PD (Fig.1B). The R decreased upon addition of UFH and then began to increase reaching the resting level 23 ± 4.2 min later, following a lag period of 14.6 ± 4.3 min. Isc did not change after heparin addition (Fig.1C). No change was observed in PD, R, or Isc, if buffer was added to the mucosal side instead of UFH (Fig.1a, b, and c).

The presence of chemical heparin in mucosal and serosal buffers and experimental tissue was measured. In addition to the mucosal buffer, heparin was found in the serosal buffer (65.0 ± 20.6 µg/ml) as well as the mucosal tissue (51.9 ± 21.5 µg/g) when UFH was added to buffer at pH 7.4 (Table 1 and Fig. 2). Heparin was not found in mucosal tissue obtained from the same stomach and not placed in the Ussing chamber. Based on recovery from the mucosal tissue the rate of movement of heparin across the mucosal tissue was calculated to be 0.47 ± 0.10 µg/ cm^2/ min.

Electrical parameters of rat gastric mucosa following addition of UFH to the mucosal side at pH 4.0

Since the stomach pH is acidic *in vivo*, electrical parameters of rat gastric mucosa were measured in our *in* vitro model with the mucosal buffer at pH 4.0, the average pH in rat stomach (Eastman and Miller, 1935). When UFH was added to the mucosal buffer at pH 4.0, PD increased as in the neutral condition (pH 7.4). After a lag period, the PD began to decrease to the previous resting level as shown in Fig. 3A. There was a trend toward a significant decrease in the lag period of 9.2 ± 2.8 min in the acidic environment compared to 18.8 ± 4.7 min in the neutral environment ($P < 0.06$, one-tailed t-test). PD reached the previous resting level 40.5 ± 7.9 min after the lag period in the acidic environment compared to 39.3 ± 17.6 min in the neutral environment. Isc changed little in the acidic environment following the addition of UFH (Fig. 3B). Heparin recovery was 111.3 ± 40.5 µg/ml from the serosal buffer when heparin was added to mucosal

buffer at pH 4.0 compared to 65.0 ± 20.6 μg/ml in the neutral condition (P< 0.3, two-tailed t-test) (Table 1). Furthermore, 26.9 ± 6.6 μg/g heparin was recovered from the mucosal tissue when UFH was added to the mucosal buffer at pH 4.0 compared to 51.9 ± 21.5 μg/g in the neutral condition (P=0.3, two-tailed t-test) (Table 1). The rate of movement of UFH across the mucosal tissue at pH 4 was calculated to be 0.80 ± 0.19 μg/ cm^2/ min.

Biological activity of heparin

To determine if the heparin recovered in the serosal buffer had anticoagulant activity, anti-Xa activity and anti-IIa activity were measured in the desalted buffer extracts at pH 4. Anti-Xa and anti-IIa activity were found in both the mucosal and serosal buffer extracts. Heparin found in the serosal buffer extracts based on anti-Xa activity was 2.5 ± 0.3 μg/ml compared to 63.2 ± 1.5 μg/ml for the mucosal buffer, and based on anti-IIa activity was 2.6 ± 0.3 and 29.8 ± 3.1 μg/ml for the serosal and mucosal buffer respectively. The anti-Xa/IIa ratio was 2.08± 0.31 and 0.54 ± 0.11 for the mucosal and serosal buffer respectively.

Changes in electrical parameters of rat gastric mucosa following addition of UFH at different concentrations at pH 4.0

To determine the effect of heparin concentration on its movement through the gastric mucosa, heparin concentration was decreased and electrical properties of the tissue were measured. Decreasing the concentration of UFH from 10 to 0.7

mg/ml resulted in a change in PD that was less negative on addition of heparin to the mucosal buffer. The PD changed by –2.4 ± 0.5 mV when 10 mg/ml was added compared to -0.9 ± 0.1 mV when 0.7 mg/ml heparin was added to the mucosal buffer ($P< 0.02$, two-tailed t-test, Fig. 4A). The lag period was 25 ± 1.2 min before the PD began to decrease to the previous resting level. Isc did not change when 0.7 mg/ml UFH was added to the mucosal buffer (Fig. 4B). Heparin recovered from the serosal buffer (18.3 ± 9.4 µg/ml), when 0.7 mg/ml of heparin was added to the mucosal buffer, was significantly less compared to 111.3 ± 40.5 µg/ml when 10 mg/ml heparin was added ($P< 0.05$, two-tailed t-test) (Table 1). Rate of movement of heparin across the mucosal tissue was calculated to be 0.13 ± 0.09 µg/ cm^2/ min.

Identification of mucosal damage

Trypan blue was used to detect any existing subtle damage in the isolated tissue sample tested. Mucosal and serosal buffers were collected 15 min after adding 100 µl of trypan blue stock solution (0.3 mg/ml) to the mucosal buffer following experiments lasting 124 min. The concentration of trypan blue in the mucosal and serosal buffers was determined and compared to control serosal buffer without trypan blue. The concentration of trypan blue in the mucosal buffer was significantly different than that of the serosal buffer (Table 2), $P< 0.0001$, two-tailed t-test. There was no difference in the concentration of trypan blue in the serosal buffer compared to the control buffer (Table 2) $P = 0.20$, two-tailed t-test.

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Changes in electrical parameters of rat gastric mucosa following addition of UFH to damaged mucosa

To determine the electrical parameters when the mucosa was intentionally injured, the stomach mucosal preparation was damaged by puncture with a 25 G needle. When UFH (10 mg/ml) was added to the mucosal side of the damaged mucosa, the PD became negative but to a lesser degree compared to the intact mucosa. The PD reached -0.9 ± 0.3 mV when the mucosa was damaged compared to -1.75 ± 0.4 mV when it was intact. The PD began to decrease to its previous resting level immediately without a lag period. At 47 ± 4.7 min after drug addition, the PD reached a plateau without continuing to rise (Fig. 5A). The Isc decreased immediately after heparin addition but reached a plateau approximately 20 min after heparin addition (Fig. 5B). Heparin was recovered from serosal and mucosal buffers as well as tissues (Table 1). The amount of heparin recovered from the serosal buffer with the mucosa damaged was 162.5 ± 8.3 μg/ml. This was comparable to 111.3 ± 40.5 μg/ml recovered from the serosal buffer of the intact mucosa ($P < 0.3$, two-tailed t-test). Rate of heparin movement across the mucosal tissue with intentional damage was calculated to be 1.16 ± 0.04 $\mu g/cm^2$/ min.

Discussion

As a potent and safe drug, heparin has been widely used by parenteral administration for the prevention and treatment of thrombo-embolic disease. Although heparin is not considered effective when administered orally, results from our laboratory and others have shown evidence of oral heparin absorption (Hiebert et al., 1993; Hiebert et al., 2001; Hiebert et al., 2000; Hiebert et al., 1996; Hiebert et al., 2005; Engelberg, 1995). Heparin was recovered from aortic and vena caval endothelium following oral administration of unfractionated heparin to rats. If concentrations of heparin with endothelium in these vessels were applied to endothelium in the whole body, it was calculated that considerable amounts of the total heparin administered could be found with the endothelium (Jaques et al., 1991; Hiebert et al., 1993). Oral heparin was demonstrated to have antithrombotic activity in rat jugular vein (Hiebert et al., 2000; Hiebert et al., 2001), venous stasis (Costantini et al., 2000) and carotid arterial thrombosis models (Pinel et al., 2004), further indicating that heparins are absorbed following oral administration. Evidence of heparin absorption was also seen in humans (Engelberg, 1995; Hiebert et al., 2005). Little is known about the site of heparin absorption. Tissue distribution studies showed that orally administered heparin was found in gut and non-gut tissues. Considerable heparin was recovered from the stomach tissue, suggesting that the stomach may be a site of absorption (Hiebert et al., 2000), thus our present study was to determine if heparin crosses rat gastric mucosa. The glandular portion of the rat stomach,

from which the mucosa was obtained, is similar to that of the human and has been used as a model for human disease (Ichikawa et al., 2000).

Heparin was found in the serosal buffer as well as mucosal tissue after heparin addition to the mucosal buffer of the Ussing chamber. Furthermore, the heparin recovered from the mucosal buffer had anti-Xa and anti-IIa activity. These findings clearly demonstrate that heparin crosses rat gastric mucosa and retains its anticoagulant activity. The anti-Xa/IIa ratio did not increase also indicating that heparin is not broken down when it crosses rat gastric mucosa, since longer heparin chains are needed for anti-IIa activity than anti-Xa activity (Boneu, 2000). The rate of transport was 70% of that when the gastric mucosa was intentionally damaged (0.80 $\pm$ 0.19 versus 1.16 $\pm$ 0.04 μg/ cm^2/ min) indicating that heparin easily crosses the mucosa. The Ussing chamber model differs from the in vivo situation by a number of factors including a small surface area and lack of an active circulation. If heparin was applied to the whole stomach, not only a surface 2.5 cm^2 in size, with an active circulation it is expected that a considerable amount of heparin could cross the stomach mucosa. Time could also be a factor. Our previous studies showed that heparin remains in the stomach in high concentrations at least 4 h after oral administration and could still be detected in the stomach after 24 h (Hiebert et al., 2000) a much longer time period than the 84 min used in this experiment.

Changes in PD across rat gastric mucosa also support heparin transport. When the mucosal side is compared to the serosal side, a change in PD is seen the moment heparin is added to the mucosal buffer, such that the mucosal buffer becomes more negative compared to the serosal buffer, and is likely due to the anionic charge on heparin. The PD remains negative and with time returns to the previous resting level and beyond suggesting that heparin binds to the tissue and moves across the mucosa to the serosal side as supported by chemical measurement of heparin and anti-Xa and anti-IIa activity in the serosal buffer. These results are not an artifact of mucosal damage, since trypan blue dye added to the mucosal buffer was not found in the serosal buffer (Table 2), confirming the absence of any minor increases in permeability in the mucosal tissue. Furthermore, intentional damage of the mucosa caused a smaller change in PD, on addition of heparin, from that observed using undamaged mucosa, likely due to heparin moving freely from the mucosal to the serosal side.

Changes in the PD observed following heparin administration were also dose-dependent (Fig. 4). The PD became more negative and the lag period was shorter when 10 mg/ml compared to 0.7 mg/ml of heparin was added to the mucosal side. It is possible that the greater the heparin concentration, the easier its penetration through the mucosal tissue. This is supported by a decreased amount of heparin recovered from the serosal buffers when less heparin is added to the mucosal side.

The idea that heparin is absorbed in the stomach is in agreement other findings. Sue et al. observed a much greater anticoagulant effect when heparin was placed in the stomach compared to the small intestine (Sue et al., 1976). Our recent *in vivo* studies in rats indicated that more heparin was found with endothelium when heparin was placed in the stomach compared to the small intestine (Hiebert et al., 2007).

Ionization of heparin may also affect its movement across the gastric mucosa. Placing heparin into an acidic mucosal buffer at pH 4, the average pH in rat stomach on a normal diet (Eastman and Miller, 1935), reduced the lag period before the PD begins to return to baseline, compared to 7.4 (Fig. 3) . The reduced lag period suggests a more rapid drug movement at lower pH levels. This result is supported by the recovery of more chemical heparin from the serosal buffer when experiments are conducted at pH 4.0 compared to pH 7.4 (Table1) . The carboxylate groups of heparin have a pKa between 2 and 4 (Casu and Gennaro, 1975). Since an acidic environment may reduce ionization of weak acidic groups as seen in heparin, these results imply that ionization of heparin can be an important factor in determining the rate of absorption of heparin from the gastrointestinal tract. Our results agree with previous studies. Others have reported improvement in heparin absorption from the small intestine when the environment is made more acidic. When heparin was dissolved in diluted acids and added to the duodenum, whole blood clotting time was significantly increased compared to when heparin was dissolved in water (Sue et al., 1976).

Instillation of buffers with heparin into dog duodenal loops, indicated that systemic anticoagulant effects were obtained at pH 4.0 but not pH 8.0 (Loomis, 1959).

Although this study does not deal with the mechanism responsible for heparin movement across gastric mucosa, observations of electrical changes support the idea that passive diffusion is at least partly responsible for heparin transport. The Isc did not change in the 84 min period after heparin addition to the mucosal buffer, suggesting passive diffusion since a change in Isc is indicative of active transport (Cooke and Dawson, 1978). Furthermore, R of the tissue, an indicator of the tissue permeability, decreases and becomes more negative when heparin is added to the mucosal side of the chamber. The R progressively increases with time and may reflect the movement of heparin across the mucosa. It is likely that as heparin crosses the mucosa, the concentration on the mucosal side progressively decreases, thus decreasing the concentration gradient across the mucosa. This change in R suggests passive diffusion. However, we cannot rule out active transport as part of heparin movement through the gastric mucosa since R and PD continue to go beyond the previous resting level. Active transport processes may be activated when the concentration of heparin decreases helping its movement across the mucosa. Further studies are required to evaluate the exact contribution of passive and active transport in the movement of heparin across the stomach mucosa.

In conclusion, recovery of heparin in the serosal buffer and the mucosal tissue as well as anti-Xa and anti-IIa activity in the serosal buffer, support the idea of heparin movement across the rat gastric mucosa. Decrease in PD with time following an increase in PD upon UFH addition, also suggests heparin transport. Less pronounced changes in PD following decreasing concentrations of heparin added to the mucosal buffer, suggests heparin transport is concentration dependent. Furthermore, a decrease in the lag phase before the PD begins to return to the baseline with lower mucosal pH in the presence of heparin suggests that suppressing ionization may increase drug movement. Finally, indirect results from changes in R and Isc after heparin addition suggest that passive diffusion may play some role in the heparin movement across the mucosa. These results support the idea that heparin may be absorbed from the stomach following oral administration. Further studies are required to understand how important gastric absorption of heparin may be in comparison to the small intestine. Moreover, considerable work is still required to evaluate mechanisms of heparin absorption from the stomach mucosa including the contribution of passive and active transport.

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References

Barzu T, Molho P, Tobelem G, Petitou M and Caen J (1985) Binding and endocytosis of heparin by human endothelial cells in culture. *Biochim Biophys Acta* **845**:196-203.

Boneu B (2000) Low molecular weight heparins: Are they superior to unfractionated heparins to prevent and to treat deep vein thrombosis? *Thromb Res* **100:**113-120.

Canadian Pharmaceutical Association. (2005) *Compendium of Pharmaceuticals and Specialities*. Ottawa, Canada.

Casu B and Gennaro U (1975) A conductimetric method for the determination of sulphate and carboxyl groups in heparin and other mucopolysaccharides. *Carbohydr Res* **39**:168–176.

Cooke HJ and Dawson DC (1978) Transport characteristics of isolated newborn rabbit ileum*. Am J Physiol Endocrinol Metab* **234**:E257-E261.

Costantini V, Deveglia R, Stabile A and Nenci GG (2000) Absorption and antithrombotic activity of unfractioned heparin after intraduodenal administration in rats. *Blood Coagul Fibrinolysis* **11**:7-13.

Dal Pozzo A, Acquasaliente M, Geron MR and Andriuoli G (1989) New heparin complexes active by intestinal absorption: I-multiple ion pairs with basic organic compounds. *Thromb Res* **56**:119-124.

Eastman IM and Miller EG (1935) Gastrointestinal pH in rats as determined by the glass electrode. *J Biol Chem* **110**:255-262.

Engelgerg H (1995) Orally ingested heparin is absorbed in humans. *Clin Appl Thrombosis/Hemostasis* **1**:283-285.

Glimelius B, Busch C and Hook M (1978) Binding of heparin on the surface of cultured human endothelial cells. *Thromb Res* **12**:773-782.

Hiebert LM and Jaques LB (1976a) The observation of heparin on endothelium after injection. *Thromb Res* **8**:195-204.

Hiebert LM and Jaques LB (1976b) Heparin uptake on endothelium. *Artery* **2**: 26-37.

Hiebert LM, Wice SM and Jaques B (1996) Antithrombotic activity of oral unfractionated heparin. *J Cardiovasc Pharmacol* **28**:26-29.

Hiebert LM, Wice SM and Ping T (2005) Increased plasma anti-Xa activity and recovery of heparin from urine suggest absorption of orally administered unfractionated heparin in human subjects. *J Lab Clin Med* **145**:151-155.

Hiebert LM, Wice SM, Mcduffie NM and Jaques LB (1993) The heparin target organ--the endothelium: Studies in a rat model. *Q J Med* **86:**341-348.

Hiebert LM, Wice SM, Ping T, Herr D and Laux V (2001) Antithrombotic efficacy in a rat model of the low molecular weight heparin, reviparin sodium, administered by the oral route. *Thromb Haemost* **85**:114-118.

Hiebert LM, Wice SM, Ping T, Hileman RE, Capila I and Linhardt RJ (2000) Tissue distribution and antithrombotic activity of unlabeled or ^{14}C-labeled porcine intestinal mucosal heparin following administration to rats by the oral route. *Can J Physiol Pharmacol* **78**:307-320.

Hiebert LM, Wice SM and Abdelhameed T (2007) Evidence for the absorption of heparin by rat stomach. *Biomed & Pharmacother* **61**:68-74.

Ichikawa T, Ishihara K, Kusakabe T, Hiruma H, Kawakami T and Hotta K (2000) CGRP modulates mucin synthesis in surface mucus cells of rat gastric oxyntic mucosa. *Am J Physiol Gastrointest Liver Physiol* **79**:G82-G89.

Jaques LB (1977) Determination of heparin and related sulfated mucopolysaccharides. *Methods Biochem Anal* **24**:203-312.

Jaques LB, Hiebert LM and Wice SM (1991) Evidence from endothelium of gastric absorption of heparin and of dextran sulfates 8000. *J Lab Clin Med* **117**: 122-130.

Leone-Bay A, Paton DR, Variano B, Leipold H, Rivera T, Miura-Fiura-Fraboni J, Baughman RA and Santiago N (1998) Acylated non-α-amino acids as novel agents for the oral delivery of heparin sodium, USP. *J Control Release* **50**:41-49.

Loomis TA (1959) Absorption of heparin from the intestine. *Proc Soc Exp Biol Med* **101**:447-449.

Money SR andYork JW (2001) Development of oral heparin therapy for prophylaxis and treatment of deep venous thrombosis. *Cardiovasc Surg* **9**:211-218.

Pinel C, Wice SM and Hiebert LM (2004) Orally administered heparins prevent arterial thrombosis in a rat model. *Thromb Haemost* **91**: 919-926.

Sue TK, Jaques LB and Yuen E (1976) Effects of acidity, cations and alcoholic fractionation on absorption of heparin from gastrointestinal tract. *Can J Physiol Pharmacol* **54**:613-617.

Footnotes

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Legends for Figures

Figure 1. Changes in electrical parameters across rat gastric mucosa on heparin (10 mg/ml) or Kreb's buffer addition to the mucosal buffer. The potential difference (PD) became more negative when the mucosal side was compared to the serosal side immediately after addition of heparin to the mucosal buffer. PD returned to the resting level with time (A). Changes in resistance (R) followed the pattern of changes in PD (B). Short Circuit Current (Isc) did not change after heparin addition (C). Results are shown as mean ± SEM of 8 experiments. No change was observed in PD (a), R (b), or Isc (c) if buffer alone was added to the mucosal buffer. Results are shown as mean ± SEM of 3 experiments.

Figure 2. An electrophoretic gel showing heparin recovered from buffer and tissue following unfractionated heparin addition to the mucosal buffer. Using agarose gel electrophoresis, heparin was found in both the mucosal and serosal buffer as well as in experimental tissue 84 min after heparin addition to the mucosal buffer. Standard and recovered heparin samples were dissolved in measured amounts of distilled water and 2 µl was applied to each lane at the top of the gel, level with the negative sign. Lane 1, Standard unfractionated bovine lung heparin 2 mg/ml. Lane 2, Mucosal buffer extract dissolved in 1000 µl of water. Lane 3, Mucosal buffer extract dissolved in 10000 µl of water. Lane 4, Serosal buffer extract dissolved in 50 µl of water. Lane 5, Standard unfractionated bovine lung heparin 2 mg/ml. Lane 6, Control mucosal tissue

extract where heparin was not added to the mucosal buffer, dissolved in 50 µl of water. Lane 7, Experimental mucosal extract dissolved in 50 µl of water. All material extracted from buffers is similar in migration and color to the original heparin. A blue band, moving slower than standard heparin, and likely chondroitin sulfate, is seen in the control mucosa (Lane 6). This band is seen in the experimental mucosa along with heparin (Lane 7).

Figure 3. Effects of changing the pH of the mucosal buffer on the electrical parameters across rat gastric mucosa on addition of unfractionated heparin (10 mg / ml) to the mucosal buffer. Unfractionated heparin was added to the mucosal buffer at pH 4.0. Changes in potential difference (PD) and short circuit current (Isc) are compared to those when heparin is added at pH 7.4. The lag period before the PD began to rise was less at acidic pH versus neutral pH (A). Isc did not change after heparin addition (B). Results are shown as mean ± SEM of 6 experiments.

Figure 4. Effects of changing the concentration of unfractionated heparin in the mucosal buffer on the electrical properties across rat gastric mucosa. Decreasing the concentration of unfractionated heparin in the mucosal buffer results in less pronounced changes in potential difference (PD) (A). Short circuit current (Isc) did not change after heparin addition (B). Results are shown as mean ± SEM of 5 experiments.

Figure 5. Effects of injury on electrical parameters across rat gastric mucosal tissue following addition of unfractionated heparin to the mucosal side. Damaging the mucosa resulted in less pronounced changes in potential difference (PD) (A) and variable changes in short circuit current (Isc) (B). Results are shown as mean ± SEM of 4 experiments.

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Table 1. Heparin recovered from buffers and tissues following addition to mucosal buffer when rat gastric mucosa is placed in an Ussing chamber for 124 min

Treatment	Mucosal Buffer (µg)	Serosal Buffer (µg)	Control Mucosa (µg)	Experimental Mucosa (µg)
UFH (pH 7.4) 10 mg / ml, (n=6)	3917.7 ± 271.3	65.0 ± 20.6	0.0 ± 0.0	51.9 ± 21.5
UFH (pH 4) 10 mg / ml, (n=5)	2400.0 ± 597.0	111.3 ± 40.5	0.0 ± 0.0	26.9 ± 6.6
UFH (pH 4) 0.7 mg / ml, (n=5)	545.0 ± 237.2	18.3 ± 9.4	0.0 ± 0.0	15.0 ± 6.6
UFH (pH 4) 10 mg / ml, damaged mucosa, (n=4)	2500.0 ± 333.3	162.5 ± 8.3	0.0 ± 0.0	17.5 ± 2.0

Table 2. Trypan blue recovered from buffers after addition to the mucosal buffer when rat stomach mucosa is placed in an Ussing chamber for 124 min

Buffers	Trypan blue (μg/ml)
Mucosal buffer	$40.00 \pm 4.65 \times E^{-8}$
***Experimental serosal buffer**	1.43 ± 0.16
****Control serosal buffer**	$1.35 \pm 7.07 \times E^{-3}$

***** Serosal buffer obtained when trypan blue is added to the mucosal buffer.

** Serosal buffer obtained when trypan blue is not added to the mucosal buffer.

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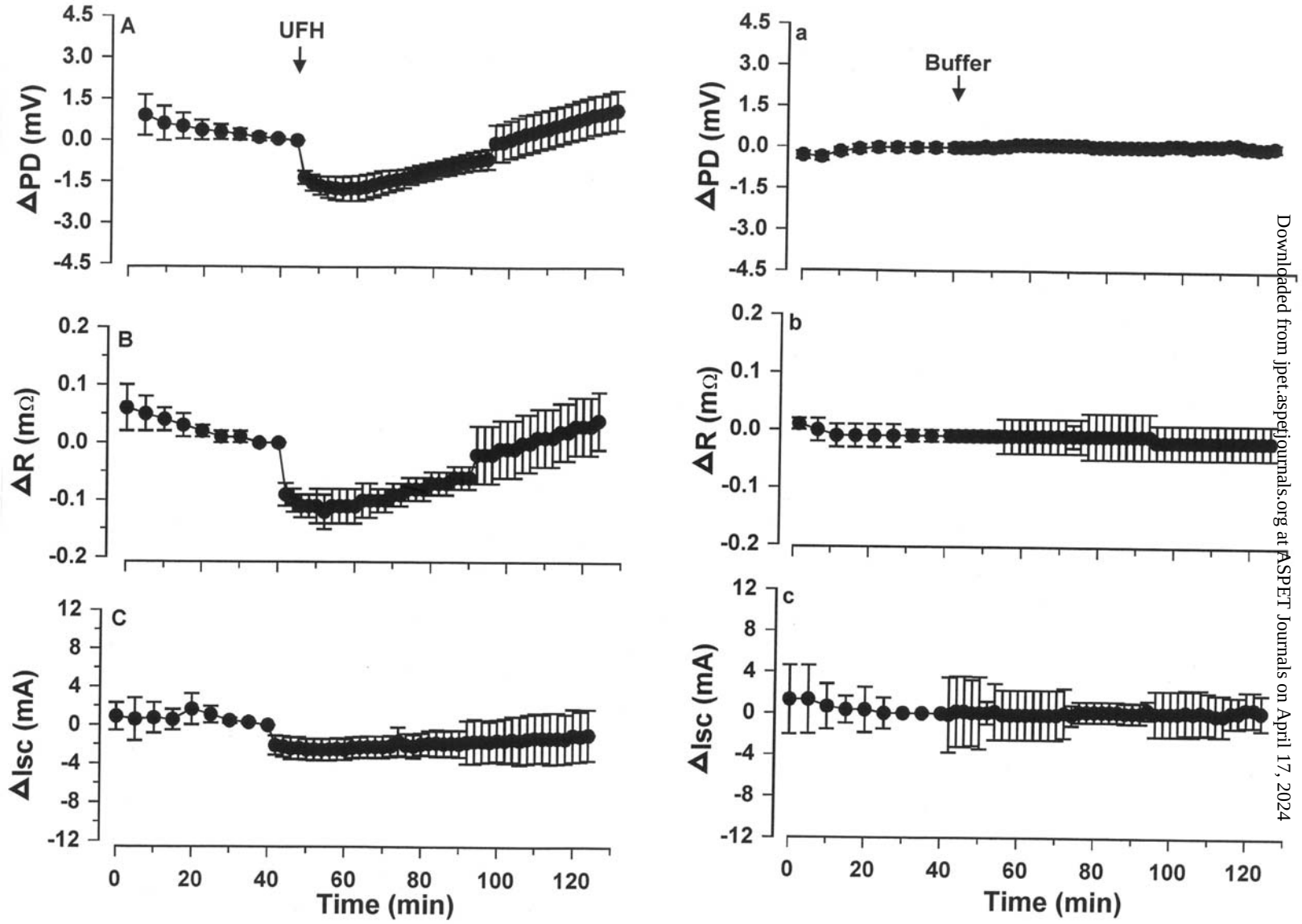

Figure 1

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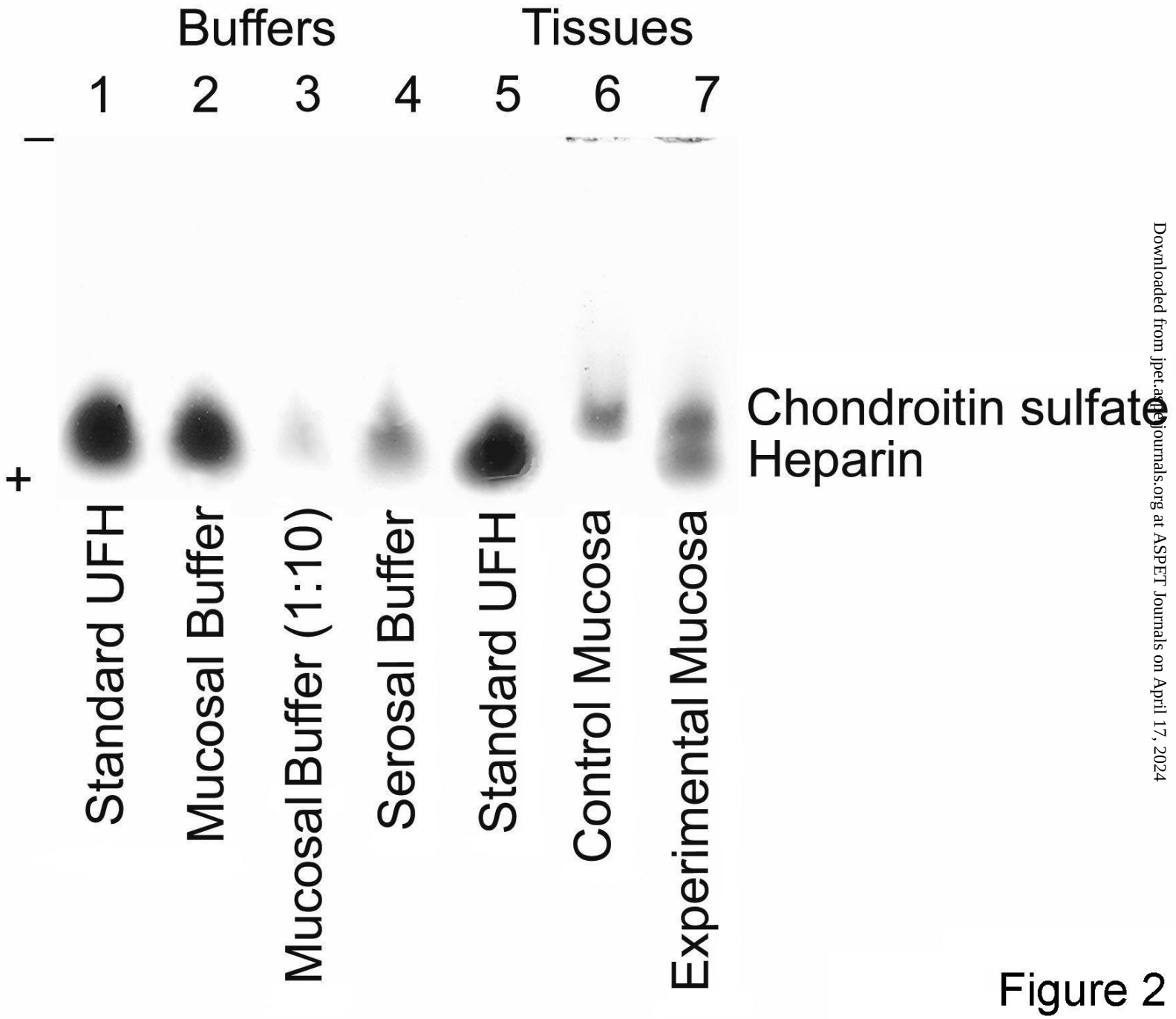

Figure 2

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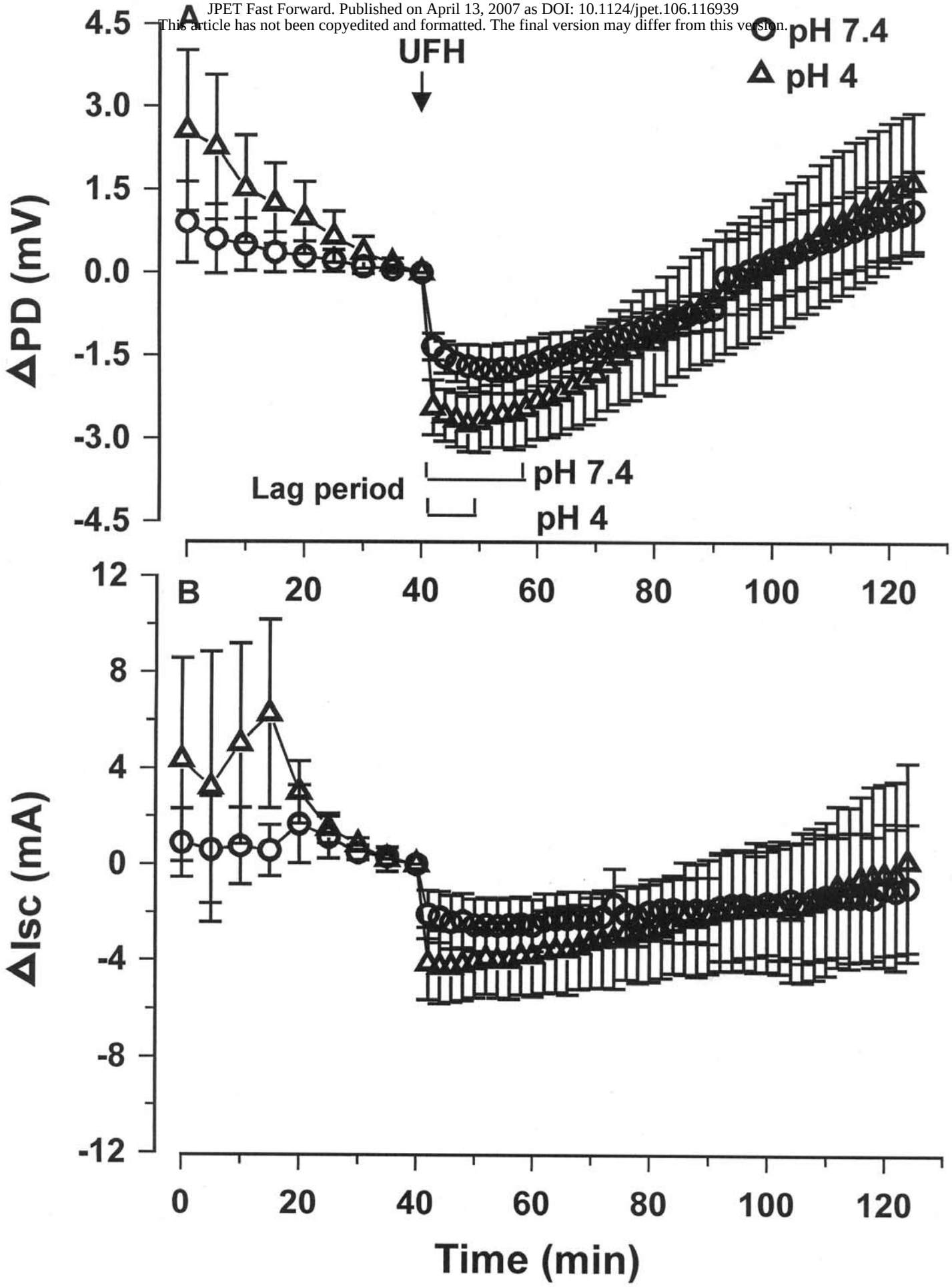

Figure 3

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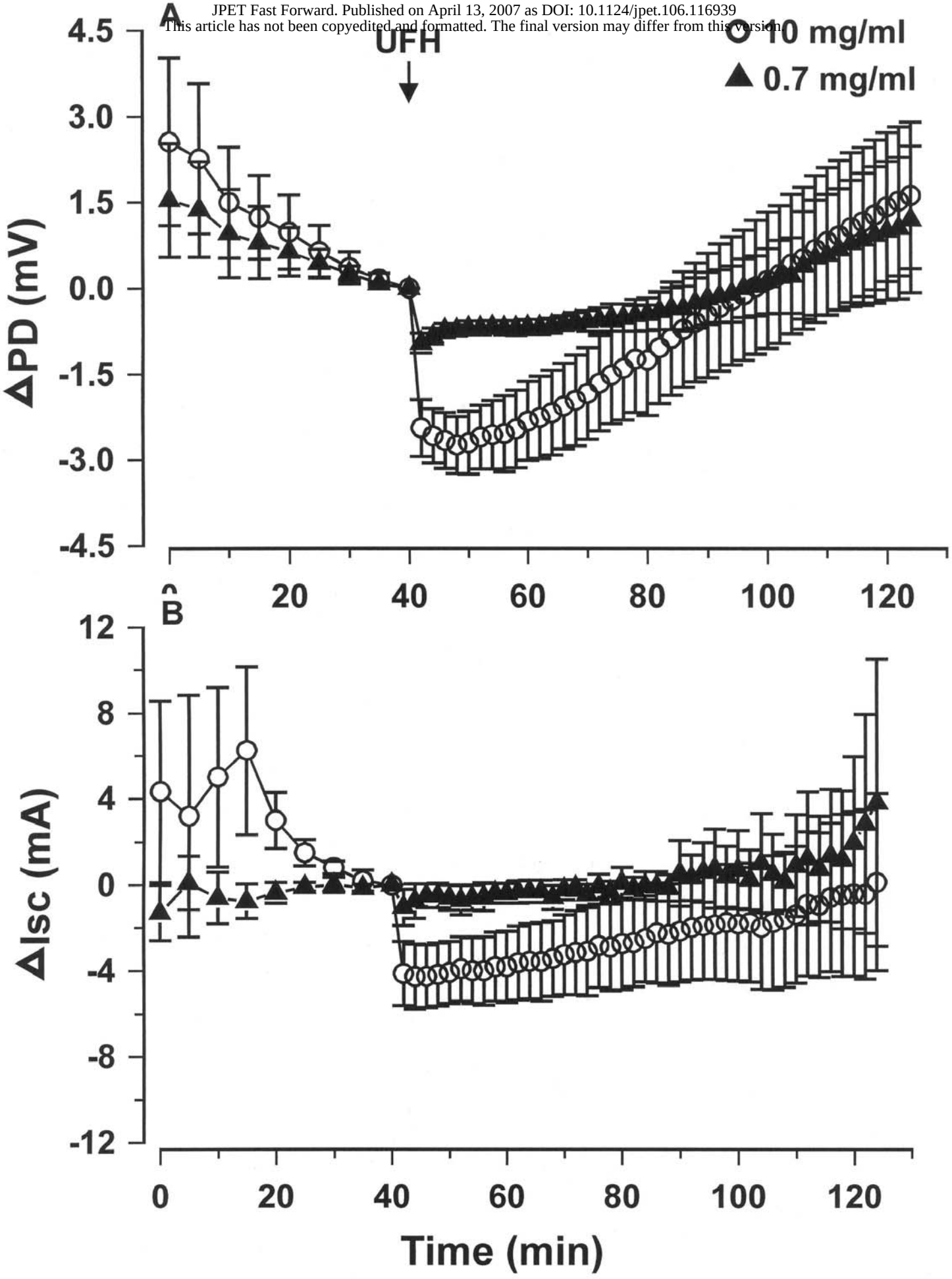

Figure 4

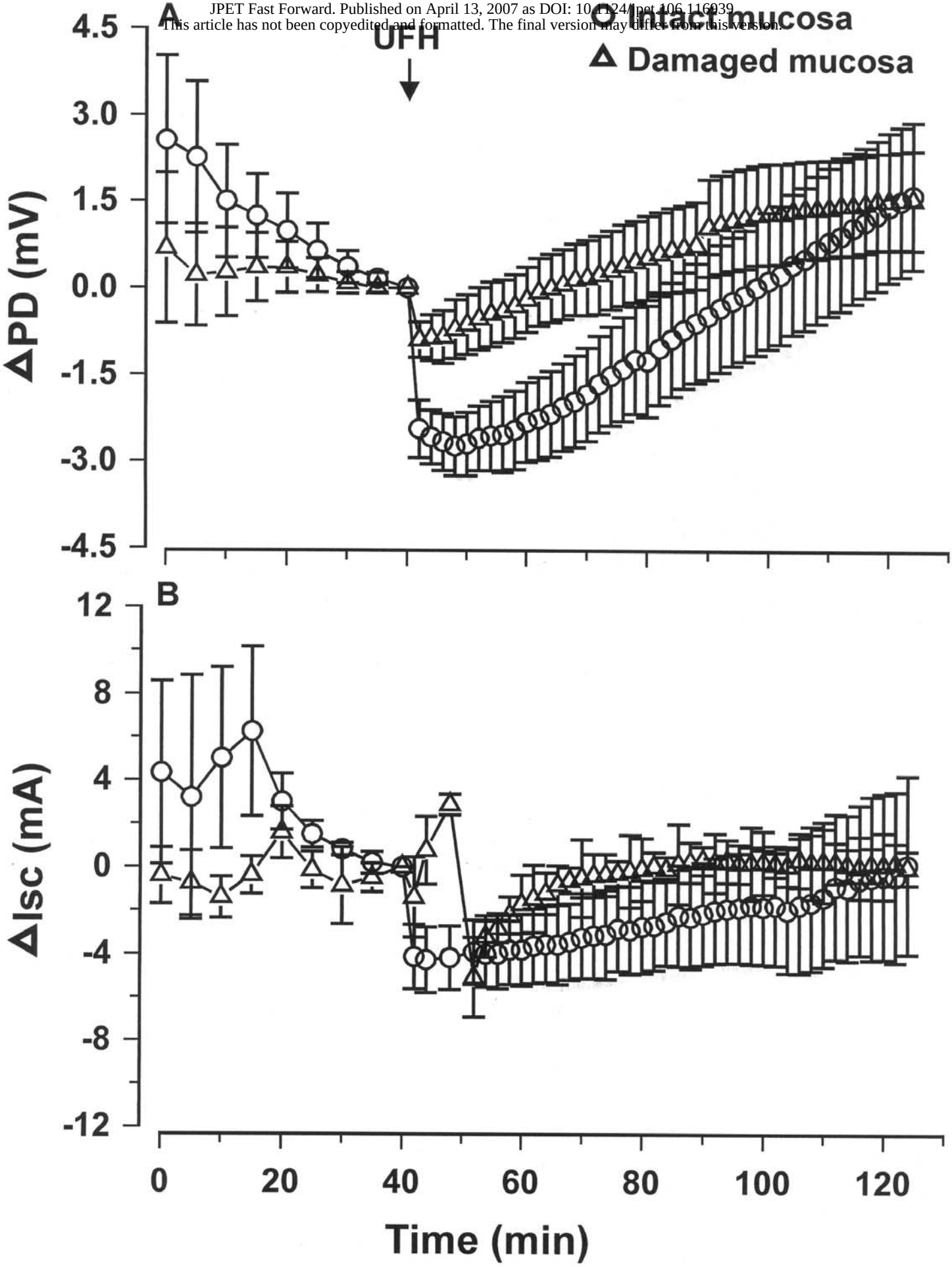

Figure 5