# INHIBITION OF THE ACTIVE LYMPH PUMP IN RAT MESENTERIC LYMPHATICS BY HYDROGEN PEROXIDE

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## ABSTRACT

The lymphatic system plays an important role in the regulation of fluid and macro-molecular exchange. It is a key "safety factor" against the formation of gross edema. Spontaneous contractions in collecting lymphatics of the rat intestine are necessary for the normal transportation of lymph. Hydrogen peroxide is one of the compounds released in inflammation. Therefore, the effects of $H_2O_2$ on the pumping activity of spontaneously contracting lymphatics were evaluated in the anesthetized rat (n=16). Diameter oscillations of the mesenteric collecting lymphatics were monitored before and after the application of $H_2O_2$ (4 and 37μM). The activity of the lymph pump was evaluated using: contraction frequency (F), stroke volume (SV), ejection fraction (EF), and lymph pump flow (LPF). These parameters were determined from the lymphatic diameter tracings. The following changes in lymphatic activity were seen after a 20 minute exposure to 37μM $H_2O_2$: 1) F declined 85%, from 11.6±1.5 to 1.9±1.9 cpm. 2) SV fell over 93%. 3) EF decreased 93%, from 0.57±.07 to 0.05±.04. 4) LPF fell dramatically (>95%) from 41.5±10.5 to 2.6±2.5 nl/min. In conclusion, $H_2O_2$ produced an intense inhibition of the active lymph pump and it is possible that the inhibition of the active pump contributes to the edema which occurs during inflammation.

The lymphatic system is the principal system by which plasma proteins that have crossed the capillary membrane are returned to the circulating blood. It is also one of the primary routes by which fluid is returned from the tissue space to the vascular space. It is thought that the lymphatic system is an important component responsible for the "margin of safety" against gross edema (1-3). Interstitial edema is one of the cardinal signs of an inflammatory reaction. One of the consequences of the inflammatory response is the production of oxygen-derived free radicals. These highly reactive species can be produced and secreted by activated leukocytes during the respiratory burst (4,5). Oxygen-derived free radicals can also be produced during ischemia-reperfusion injury (6). The list of oxygen-derived free radicals that can be produced during inflammation and ischemia-reperfusion includes superoxide anion, hydrogen peroxide, hydroxyl radical, singlet oxygen and hypohalous acids among others (5).

The lymphatic system transports fluid using "lymph pumps". Depending on the particular species and tissue in question, the lymph pumps can function via passive and/or active mechanisms. The mesenteric collecting lymphatics of the rat small intestine contract spontaneously and function as an active lymph pump. These vessels are approximately 100μm in diameter. They are separated by one-way valves approximately every 700-

1000μm. The vessel segments separated by these valves act as functionally individual pumps and have been defined as lymphangions (7). The lymphatics in the mesentery of the rat small intestine contract at a frequency of approximately 5 to 20 contractions per minute. Evaluation of the active lymph pump was accomplished using the analogy of the cardiac pump as applied to lymph vessels as previously published (8-11).

Previous studies have shown that oxygen-derived free radicals have a number of deleterious effects on the microvasculature (12-14). Recently we conducted a series of studies evaluating the effects of oxygen-derived free radicals generated using a hypoxanthine, xanthine oxidase mixture on lymphatic contractility (11). Using this system to generate oxygen-derived free radicals produced superoxide anion as well as hydrogen peroxide. The results of those studies indicated that superoxide anion, could significantly inhibit lymphatic contractile function. To further investigate the role of the different oxygen-derived reactive species on the active lymph pump, the present studies specifically investigated the effects of hydrogen peroxide on the spontaneous contractile activity of collecting lymphatics in the rat mesentery. We evaluated the hypothesis that hydrogen peroxide alters the contractile activity of lymphatic vessels, and thus affects lymphatic pumping function.

*MATERIALS AND METHODS*

*In situ Lymphatic Preparation*

Male Sprague-Dawley rats (Harlan, Houston, TX) were used for these studies (n=16). The mean weight of the rats was 223 grams with a range of 165 to 309 grams. The rats were housed in an environmentally-controlled, American Association for Accreditation of Laboratory Animal Care, approved vivarium. The rats were fasted for 18-24 hours before the experimental procedure. Water was available ad libitum

during this time. An intraperitoneal injection of sodium pentobarbital (50mg/kg) was used for anesthesia and supplemental doses were given intravenously as needed. After the rats were anesthetized, the trachea was intubated to prevent airway obstruction. The mean arterial pressure was measured and recorded throughout the experiment via an arterial catheter inserted into the femoral artery. Any experiments in which the mean arterial pressure fell below 80mmHg were terminated and discarded. A cannula was placed into the femoral vein to allow the delivery of fluids and drugs. The rats received an intravenous infusion of physiological buffer solution (PBS) at a rate of .004ml/min/100 gram body weight to make up for normal fluid losses.

Access to the mesenteric lymphatics was accomplished by carefully exteriorizing a loop of the small intestine. A midline incision, 4 cm long was made through the skin, fascia and abdominal muscles of the abdomen. Through this incision a loop of the small intestine, approximately 7-8 cm long, was exteriorized. This loop and the accompanying mesentery were gently positioned over a semicircular viewing pedestal on a Plexiglass preparation board. A section of mesentery that contained a lymphatic which exhibited spontaneous contractions was centered over a viewing window in the preparation board. The exteriorized tissues were suffused with PBS (120mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1.2mM $MgSO_4$, 0.4mM $NaH_2PO_4$, 1.2mM $Na_2HPO_4$ and 20mM $NaHCO_3$) that was warmed to 37°C and bubbled with a mixture of 5% $CO_2$ and 95% $N_2$ to maintain the pH at 7.2. The temperature of the exteriorized tissue and the animal's core was maintained between 36 and 38°C. The preparation board was placed on the stage of an intravital microscope. The lymphatic was observed at a magnification of 100X-200X using a 80mm projective lens, a 10X water immersion objective and a variable Zeiss Optivar intermediate lens. The experiments were recorded onto a modified VHS video recorder using a CCD video camera. The images were

viewed on a high resolution video monitor.

After a suitable lymphatic was located, the preparation was suffused with normal PBS and allowed to equilibrate for 10-15 minutes. After the equilibration period, the experiment was designed with a 10-20 minute control period, a 20 minute test period, and a 10 minute recovery period. The data was acquired and analyzed in 5 minute intervals. During the control and the recovery periods, the suffusate was PBS. During the test period, the suffusate was PBS containing hydrogen peroxide at one of the following doses: 1) Hydrogen peroxide, 4μM (HPL, n=4). 2) Hydrogen peroxide 37μM (HPH, n=4). These concentrations of hydrogen peroxide fall within the range of hydrogen peroxide thought to be produced during experimental models of intestinal inflammation (15-16). A group of experiments was used for the corresponding time control studies (TC, n=8).

*Evaluation of the Lymph Pump*

To evaluate the pumping function of the lymphatics, we monitored the spontaneous lymphatic contractions that normally generate lymph flow in these vessels. The effects of hydrogen peroxide on the active lymph pump were determined using intravital microscopic techniques (8,11). Lymphatic diameters were measured at sampling frequencies of 10-15 samples per second from the videotape using techniques previously described in detail (11). We monitored the lymphatic diameter during each of the experimental periods. From these diameter changes, a lymphatic contraction cycle was discerned. This lymphatic contraction cycle was divided into periods of lymphatic systole and diastole. We then calculated parameters similar to those used to characterize cardiac pump function. The following indices of spontaneous lymphatic contractile activity were obtained: 1) End diastolic diameter (EDD, μm) of the lymphatic was defined as the vessel diameter immediately before the beginning of the lymphatic contraction. 2) The end systolic diameter

(ESD, μm) was the vessel diameter at the end of the lymphatic systole. 3) Lymphatic contraction frequency (F) was calculated as the inverse of the time that elapsed between consecutive contractions. 4-6) Lymphatic stroke volume, ejection fraction and lymph pump flow index were calculated from EDD, ESD and F (11) using the following equations: $SV=K \, (EDD^2-ESD^2)$, $EF=SV/(KxEDD^2)$, and $LPF=SVxF$. K is a constant that includes factors for: converting diameter to radius, the average lymphangion length and pi.

*Statistical Analysis*

The data was analyzed using Students t test and standard one-way ANOVA. Intergroup differences were evaluated using Fishers PSLD. Statistical significance was defined to be $p<0.05$.

*RESULTS*

*Controls*

The rat mesenteric lymph pump was monitored before, during and after exposure of the lymphatics to hydrogen peroxide. *Table 1* contains a summary of the characteristics of the vessel contractions during control conditions. Lymph pump data from the control periods were in good agreement with previously published data (8,11). The average EDD of the vessels was 124μm and ranged from 40 to 220μm. The mean ESD of the vessels was 87μm. Average contraction frequency was 13.2 contractions per minute during the control period with a range of 8.2 to 19.1 contractions per minute. SV of these lymphatics was approximately 5nl. The EF of these vessels ranged from 0.35 to 0.73 with a mean of 0.50. The calculated average LPF was approximately 50nl/min.

To assure that the results observed in the hydrogen peroxide treated groups were not due to a time dependent decline in lymphatic contractile activity because of the preparation technique, we evaluated lymph pump function

# TABLE 1
## Lymph Pump Characteristics

| Parameter | Control | HPL | HPH |
|---|---|---|---|
| EDD (µm) | 124±19 | 137±33 | 127±21 |
| ESD (µm) | 87±14 | 112±31 | 110±10 |
| F (contractions/minute) | 13.2±1.4 | 3.5±1.2* | 1.9±1.9* |
| SV(nl) | 5.04±1.39 | 3.69±1.15 | 0.47±.47* |
| EF | .499±.046 | .373±.074 | .047±.047* |
| LPF (nl/minute) | 50.5±15.9 | 7.7±4.1* | 2.56±2.56* |

Values are mean±SEM. HPL, 4µM $H_2O_2$; HPH, 37µM $H_2O_2$; EDD, end diastolic diameter; ESD, end systolic diameter; F, contraction frequency; SV, stroke volume; EF, ejection fraction; LPF, lymph pump flow.
*significantly different from control p<0.05

for 45-50 minutes in a separate time control group, no significant alterations were observed in any of the experimental parameters that were monitored (data not shown) thus validating the use of our experimental model over this time frame.

*$H_2O_2$ Treatment*

The effects of hydrogen peroxide exposure on lymphatic contractile behavior can be seen in *Fig. 1*. This figure depicts an example of the lymphatic diameter records before and after exposure to 37µM $H_2O_2$. Each panel in *Fig. 1* represents a 30 second sample of a lymphatic diameter tracing. Panel 1A depicts the spontaneous lymphatic vasomotor activity during the control conditions and panel 1B shows the lymphatic contractions after 16 minutes of $H_2O_2$ exposure. In this example, the contraction frequency during the control segment was about 16 contractions per minute. The EDD and ESD for this vessel during the control period were about 130 and 80µ, respectively. This resulted in an EF of approximately 0.62 during the control conditions. After hydrogen peroxide exposure, these parameters were dramatically altered as can be seen in *Fig. 1B*. The lymphatic contraction frequency fell to about 6 contractions per minute. The lymph vessel

dilated to an EDD of about 150µm and a ESD of about 140µm. $H_2O_2$ exposure produced a dramatic decrease in the EF, falling to less than 0.13.

Hydrogen peroxide (at both concentrations) produced slight increases in the EDD (2-25%) and the ESD (32-33%), *Table 1*. However, these changes were not statistically significant. *Fig. 2* shows a temporal plot of the changes in lymph activity which occurred after $H_2O_2$ exposure. The data is plotted during control, 5, 10, 15, and 20 minute exposures to $H_2O_2$ at 4 and 37µM. The lymphatic contraction frequency decreased significantly upon exposure to either dose of $H_2O_2$. The contraction frequency in the HPL group fell to 29% of the control value. At the 37µM level of $H_2O_2$ exposure, the contraction frequency fell continuously over the 20 minute exposure period until it reached 15% of the control value. The strength of the contractions was also affected. Hydrogen peroxide exposure caused a decline in SV and EF at both doses. However, the fall in both SV and EF was significant only in the HPH group. At 4µM $H_2O_2$, both EF and SV decreased (12-17%) after 20 minutes of exposure, although this reduction in contraction strength was not statistically significant. SV and EF both fell to less than 8% of the control values in the HPH group. The effects of hydrogen peroxide

*Fig. 1. Diameter tracings depicting the effects of hydrogen peroxide (HPH, 37μM) on spontaneous lymphatic contractions. Panel A depicts the lymphatic diameter during the control period. Panel B is the lymphatic diameter tracing of the same vessel approximately 16 minutes after the application of hydrogen peroxide to the preparation.*
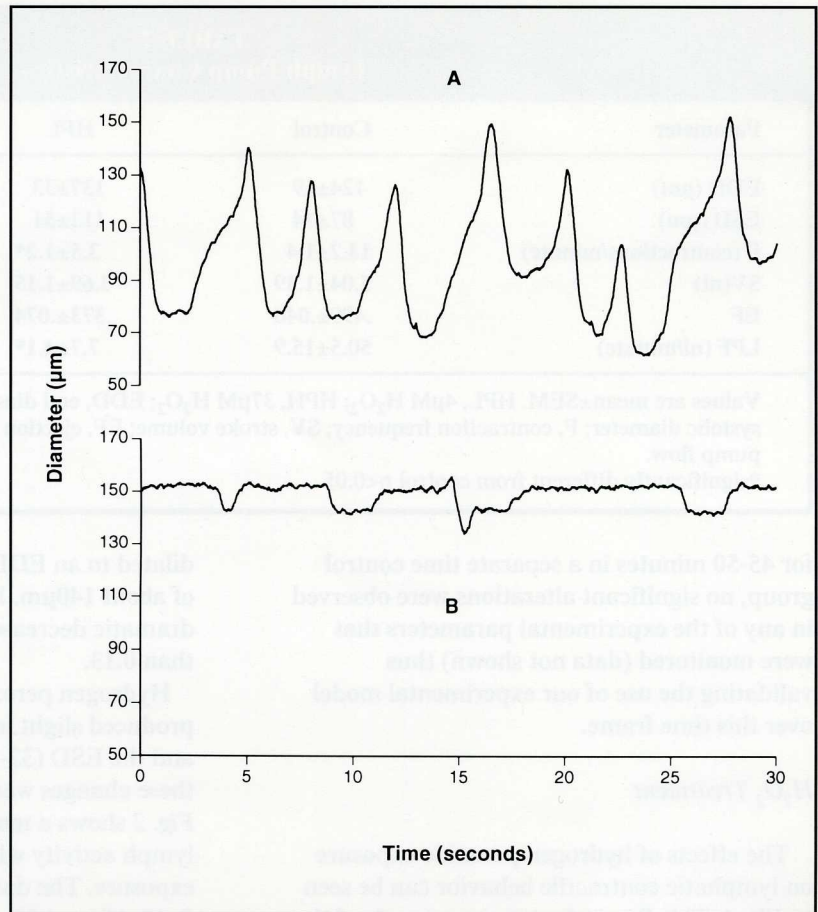


exposure on contraction frequency and SV combined to produce an intense inhibition of the calculated LPF. Lymph pump flow was severely depressed by exposure to $H_2O_2$ at both concentrations. Lymph pump flow was reduced 87 to 94% in the HPL and HPH groups, respectively. Again the inhibition of LPF was stronger at the 37μM $H_2O_2$.

Apparently at the rates of tissue damage produced by the bulk application of $H_2O_2$, the damage was not reversible over the 10 minute recovery period we used. No significant recovery was seen for any of the mean lymph pump parameters. However, when looking at the data from the individual experiments, some indications of a recovery from the $H_2O_2$ induced pump inhibition were evident, particularly in the HPL group. In the HPL

group 1 out of 4 vessels showed a recovery of the lymphatic contraction frequency and lymph pump flow. Three of the four lymphatics in the HPL group had a recovery in both the SV and EF. Only one of the vessels exposed to 37μM $H_2O_2$ showed any sign of recovery for any of the lymph pump parameters.

## DISCUSSION

Normally, fluid flow in these lymphatics is thought to be primarily generated by the intrinsic contraction of lymphatic smooth muscle found in the vessel wall. The effects of a number of mediators of inflammation on active lymphatic pumping have been published (17,18). Previous *in situ* studies in
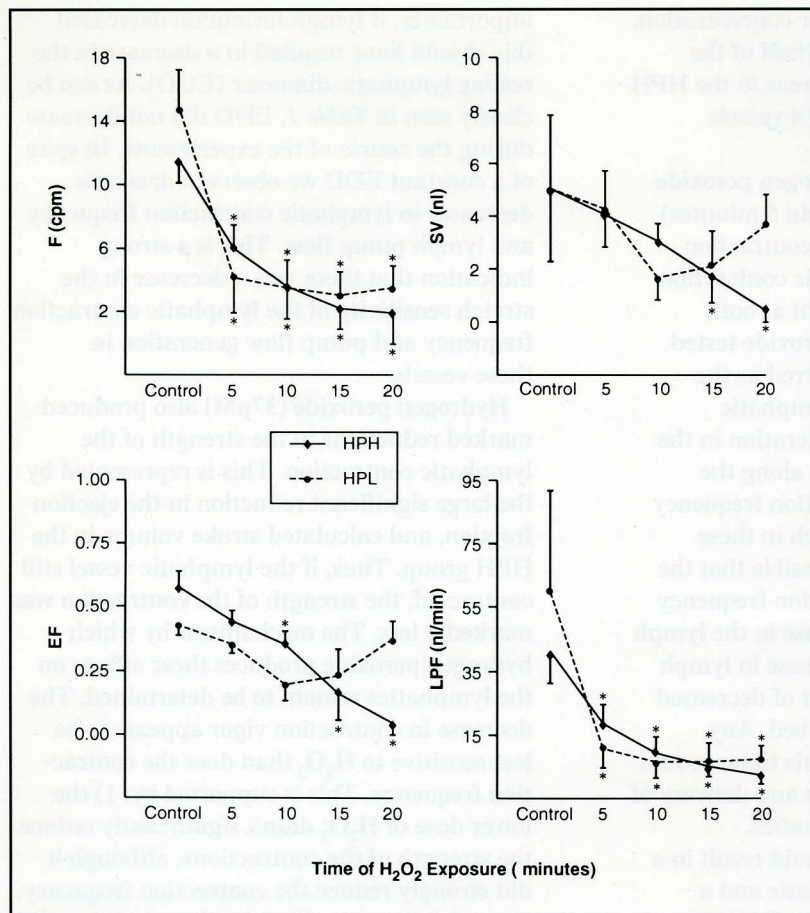
F (cpm)

18
14
10
6
2

Control 5 10 15 20

SV (nl)

10
8
6
4
2
0

Control 5 10 15 20

HPH
HPL

EF

1.00
0.75
0.50
0.25
0.00

Control 5 10 15 20

LPF (nl/min)

95
75
55
35
15

Control 5 10 15 20

**Time of H₂O₂ Exposure ( minutes)**

Fig. 2. Temporal pattern of the effects of hydrogen peroxide (HPL, 4µM and HPH, 37µM) on lymphatic contraction characteristics. Each point represents the mean response from 4 different experiments. The upper left panel depicts the lymphatic contraction frequency in contractions per minute (F, cpm) while the upper right panel shows the lymphatic stroke volume (SV) in nanoliters. The lower left panel represents the ejection fraction (EF) and the lower right panel depicts the lymph pump flow (LPF in nl per minute). The x-axis in each panel represents the five minute period ending the control and 5, 10, 15 and 20 minutes after continuous exposure to $H_2O_2$.

our laboratory had shown that exposure of similar vessels to an oxygen-radical generating solution of hypoxanthine and xanthine oxidase produced a similar inhibition of the lymph pump (11). In those studies, the effects of oxygen-radicals were partially inhibited by the addition of superoxide dismutase. This implied that superoxide anion was directly responsible for at least part of the lymph pump inhibition. Thus questions about the effectiveness of hydrogen peroxide as an inhibitor of the active lymph pump arose. To address these questions, we evaluated the effects of hydrogen peroxide on the spontaneous contractions of the mesenteric lymphatics of the rat small intestine. These studies were conducted in situ. It is well established that changes in fluid filtration and

thus lymph formation can have powerful influences on lymphatic contractile activity in situ (8). Thus, caution must be used when interpreting the direct effects of various protocols on lymphatic contractile activity. Knowledge of how the lymph formation in the intestine is affected by these protocols is important in the evaluation of lymphatic contractile effects.

Hydrogen peroxide produced an intense inhibition of the spontaneous contractions in the collecting lymphatics of the mesentery of the rat small intestine. Exposure of these lymphatics to $H_2O_2$ inhibited both EDD did not the frequency and the strength of the spontaneous contractions. This resulted in a dramatic reduction in lymph pump flow. $H_2O_2$ did not significantly change the EDD or

ESD of the lymphatics at either concentration. In the HPL group, the EDD of half of the lymphatics was decreased whereas in the HPH group, the EDD of 3 out of the 4 vessels increased.

Lymphatic exposure to hydrogen peroxide solutions produced rapid (within 5 minutes) reductions in the spontaneous contraction frequency. The fall in lymphatic contraction frequency was highly significant at both concentrations of hydrogen peroxide tested. This decrease could have occurred as the result of an inhibition in the lymphatic pacemaker activity or as an alteration in the propagation of the contraction along the lymphatic. Lymphatic contraction frequency is sensitive to pressure or stretch in these vessels (8-10,19). Thus, it is possible that the decrease in lymphatic contraction frequency occurred as a result of a decrease in the lymph pressure. This theoretical decrease in lymph pressure could occur as a result of decreased fluid filtration in this vascular bed. Any decrease in fluid filtration in this tissue would result in a decreased formation and delivery of lymph to the mesenteric lymphatics. Decreased lymph pressures would result in a decreased stretch of the lymphatic and a smaller lymphatic diameter (8). This series of events could also produce a decrease in lymphatic contraction frequency and lymph pump flow. However, this theoretical sequence of events is not a likely explanation of the observed results for a number of reasons. The first of which is that other studies that have been conducted under roughly similar conditions have indicated that intestinal permeability and fluid filtration goes up not down (16,20-21). Thus, these studies would produce conditions that tend to increase the formation of lymph in the intestine. Secondly, these same studies indicate that these changes occur over a much longer time frame than the very rapid results we observed (20). Thus, it is not likely that any changes in lymph forma-tion occurred as rapidly as the observed decrease in lymphatic contractile activity (<5 minutes of $H_2O_2$ exposure). Lastly, and most

importantly, if lymph formation decreased this should have resulted in a decrease in the resting lymphatic diameter (EDD). As can be clearly seen in *Table 1*, EDD did not decrease during the course of the experiments. In spite of a constant EDD we observed dramatic decreases in lymphatic contraction frequency and lymph pump flow. This is a strong indication that there was a decrease in the stretch sensitivity of the lymphatic contraction frequency and pump flow generation in these vessels.

Hydrogen peroxide (37µM) also produced marked reductions in the strength of the lymphatic contraction. This is represented by the large significant reduction in the ejection fraction, and calculated stroke volume in the HPH group. Thus, if the lymphatic vessel still contracted, the strength of the contraction was markedly less. The mechanisms by which hydrogen peroxide produces these effects on the lymphatics remain to be determined. The decrease in contraction vigor appears to be less sensitive to $H_2O_2$ than does the contrac-tion frequency. This is supported by: 1) the lower dose of $H_2O_2$ didn't significantly reduce the strength of the contractions, although it did strongly reduce the contraction frequency. 2) the fall in contraction frequency occurred more rapidly (typically within 5 minutes) than did the decline in either SV or EF.

Together the reductions in the contraction frequency and strength of the contraction resulted in a large decrease in the lymph pump flow. Calculated lymph flow fell to less than 15 and 5% of the control values in the HPL and HPH groups respectively by the end of the 20 minute exposure period. This was visually observed as a stasis of lymph flow, as judged by the movement of the cells within the lymph. If the insult to which the lymphatics were exposed in these studies is representative of the situation that exists during pathological states, these studies imply that lymphatic exposure to hydrogen peroxide during the course of inflammation would severely inhibit the lymphatic pumping ability. This inhibition of the lymphatic pump occurs despite large

increases in the transvascular flux of fluid and protein seen during inflammation. Therefore, the role of the lymphatics as a "safety factor against edema" is compromised. In fact the inhibition of the lymphatic pump during inflammation could be a major factor contributing to the formation of edema.

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