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Original Research

D-lactate as a marker of venous-induced intestinal ischemia: An experimental study in pigs

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ABSTRACT

Background: Intestinal ischemia is difficult to diagnose. The search for biomarkers has led to an increased interest in D-lactate. D-lactate measured in higher concentrations arises from bacterial fermentation in the gastrointestinal tract. Permeable intestinal wall is an early consequence of intestinal ischemia, which allows D-lactate to enter the portal circulation.
Methods: The superior mesenteric vein was clamped in eight pigs for two hours to induce ischemia of the intestine. Eight sham-operated pigs served as controls. Systemic and portal plasma D- and L-lactate, L-LDH and leukocytes were measured.
Results: Plasma D-lactate increased significantly and nearly simultaneously in the systemic and portal circulation. After 75 min, samples from the jugular vein showed concentrations of .019 ± .008 mmol/L in the sham group and .042 ± .022 mmol/L in the intervention group ($P = .023$). A similar significant effect was seen in the portal circulation after 90 min. L-lactate increased five minutes after clamping in the portal circulation, with values of 3.396 ± 1.119 mmol/L in the intervention group compared to 1.696 ± .483 mmol/L in the control group ($P = .006$). L-LDH increased significantly in the intervention group, while leukocytes were unaffected. L-LDH and L-lactate in plasma led to an overestimation of D-lactate if not handled.
Conclusion: Both systemic D- and L-lactate were markers of venous-induced intestinal ischemia. We speculate that D-lactate may be a valuable aid to the clinician in search of the anaerobic focus, because it may be more specific for mesenteric ischemia than L-lactate, in the sense that it is of bacterial origin.
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1. Introduction

Acute mesenteric ischemia, a condition primarily seen in elderly patients, is associated with high mortality and morbidity.[1] The condition ranges from reversible hypoperfusion to infarction of the intestine; the latter is lethal if left untreated. Mesenteric ischemia arises from several pathogeneses and can be divided in three groups: (1) arterial thrombo-embolic event, (2) venous thrombosis (up to 15% of all cases), and (3) non-occlusive causes.[2,3]

Venous-induced mesenteric hypoperfusion and ischemia are associated with cancer, coagulopathy, high body mass index, and smoking.[4] Superior mesenteric vein occlusion compromises the return of blood from the intestine, which again can lead to backward failure, oedema, and finally infarction. Superior mesenteric vein thrombosis often has a rather slow onset of symptoms. The symptoms can range from postprandial pain to severe constant pain and, in the early stages; there are often no signs of peritoneal reaction, bloody stools, or vomiting. These factors make the diagnosis of these patients very challenging.

During tissue ischemia, the metabolism changes from aerobic to anaerobic and cells depend on anaerobic glycolysis. The end-product of anaerobic glycolysis is mainly L-lactate. This metabolic pathway is common for all tissues in the body and, as such, an elevated L-lactate might be a marker of ischemia in various organs.

D-lactate is under normal conditions only seen in micromolar concentrations in humans, due to methylglyoxate metabolism.[5] D-lactate is thought to be metabolized to pyruvate in the liver by the enzyme D-2-hydroxy acid dehydrogenase, but at a much slower than the rate at which L-lactate dehydrogenase (L-LDH) metabolises L-lactate.[6] Some bacteria belonging to the normal gastrointestinal flora, such as *Escherichia coli*, *Lactobacillus* species, and *Klebsiella* species can produce D-lactate under anaerobic conditions.[5] The

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normal healthy mucosa prevents the D-lactate in the intestinal lumen from entering the circulation. During mesenteric ischemia, the barrier function of the mucosa is compromised, which facilitates translocation of bacteria into the intestinal wall and increases the capillary and mucosal permeability.[7,8] This allows D-lactate to enter the portal circulation.

The above-mentioned considerations prompted us to investigate D-lactate as a potential marker of acute mesenteric ischemia caused by venous occlusion, in a pig model.

2. Materials and methods

Sixteen fasting female Danish Landrace pigs were randomized in blocks of 2 to either intervention or control group. Mean weight was 63 kg (range, 46–82 kg). Anaesthesia was initiated with an intramuscular injection of 1 mL/10 kg Zoletil mixture (Virbac 50 Vet., BP27-06511, Camos, France). After tracheal intubation, anaesthesia was maintained by intravenous infusion of Propofol (Propofol, B. Braun Melsungen AG, Melsungen, Germany) at 10 mL/kg/h and Fentanyl (Haldid, Janssen Pharmaceutica, Beerse, Belgium), at .5 mL/kg/h throughout the operation. Pigs were mechanically ventilated in a (Dantex D-VHC14-23-02, Division of Instrumentarium Corp. Helsinki, Finland) respirator with 4 L of oxygen and 4 L of N_2O. A bladder catheter was placed to monitor urine production.

A catheter for blood sampling was placed in the left jugular vein (Avanti+, 7F, Cordis Corporation, Miami, FL, USA). A catheter (Super Torque Plus, 6F, 100 cm EGB III, Cordis Corporation), also for blood sampling, was placed in the right hepatic vein via the right jugular vein, guided by X-ray and controlled by contrast injection. This was to investigate a possible clearance of both lactate isomers in the liver.

A 25 cm long midline laparotomy was made, and a catheter for blood sampling was placed in the portal vein (Avanti+, 7F, Cordis Corporation). A 10 IU/mL heparin-saline solution was used to prevent clotting (Heparin, LEO Pharma A/S, Ballerup, Denmark). Finally, 5000 IU heparin was intravenously administered before blood sampling began.

In the intervention group, the superior mesenteric vein was dissected and clamped. In all animals, the abdominal wall was adapted to reduce evaporation and loss of body heat during the experiment. Blood was sampled in fluoride-citrate tubes (Venosafe, Terumo Europe, Leuven, Belgium) from both catheter sites prior to clamping and then in intervals of 15 min for a total of 120 min. Blood samples were immediately centrifuged at 2000 g for 12 min, and plasma was separated and stored at −20 °C for up to 1 month until analysis.

Throughout the experiment, the pigs were monitored by ECG, temperature, systemic blood pressure, oxygen saturation, and urine production. An isotonic saline solution (3 mL/kg/h) was infused to maintain normal renal function and circulation. After 2 h of clamping, one segment of small intestine, 100 cm from the ligament of Treitz's, and one segment of the spiral-shaped pig colon were resected and fixed in 10% neutral buffered formalin solution for later histological examination. At the end of the experiments, pigs were then euthanized by a pentobarbital overdose.

The experimental procedure was conducted under local project license J.nr.2007/561-1329 in conformity with Danish legislations regarding animal welfare and experimental surgery.

2.1. Biochemical assay

D-lactate in plasma was analyzed with the D-lactic Acid kit (Biocontrol Systems, USA) from Modular Analytics P (Roche Diagnostics, Mannheim, Germany), with use of a spectrophotometric method. Since the presence of L-lactate dehydrogenase (L-LDH) and L-lactate in plasma interfere with the estimation of D-lactate, inactivation or removal of L-LDH is necessary to measure the correct D-lactate concentration.[9] We prepared the samples by adding sodium hydroxide (NaOH) to the plasma, which inactivated L-LDH.[10] The activity of L-LDH was controlled and, after the preparation, all samples were less than 3 U/L. All samples were measured in duplicate. The dilution factor due to the addition of NaOH was taken into account in the final calculation. L-lactate and L-LDH were analysed using L-lactate and LDH kits both from Roche Diagnostics.

To illustrate the interference due to L-LDH and L-lactate, we measured the D-lactate with and without sample preparation in the samples taken from the jugular vein. We measured leukocytes and L-LDH at time zero minutes (T_0) and after 120 min of clamping (T_{120}).

2.2. Histological protocol

Tissue was embedded in paraffin and stained with haematoxylin and eosin. Sections were examined by a pathologist with light microscopy for evidence of ischemia. We graded the severity of ischemia from 0 to 4, based upon the extent of ischemic depth, infiltration of leukocytes and inflammation. All samples were examined twice by blinded evaluators. The mean of the two evaluations used the following to grade ischemia: grade 0: no evidence of changes; grade 1: oedema of the superficial epithelium; grade 2: denudation of the superficial epithelium; grade 3: denudation and homogenization of lamina propria; grade 4: injury deeper than half of the mucosa.

2.3. Statistics

Based upon the work by Assadian et al.[11], we wanted to detect a difference in means after 2 h by .013 with a standard deviation of .0085 with 80% power and 5% significance level after 2 h. Using power and sample size calculations versions 2.1.31 (Vanderbilt University), we calculated that 8 pigs were needed in each group.[12] The Student's *t*-test or One-way repeated measurement ANOVA was used as appropriate for comparison between groups. Results were presented as means ± SD and *P*-values <.050 were considered significant. The analyses were carried out using SPSS® software version 15 (SPSS Inc., Chicago, Illinois, USA).

3. Results

3.1. L-lactate

L-lactate levels in the portal vein increased significantly, to 3.396 ± 1.119 mmol/L in the intervention group and was 1.696 ± 483 mmol/L in the sham-operated group, 5 min after ligation of the upper mesenteric vein ($P = .006$). L-lactate levels continued to increase during the 2 h of ischemia (Fig. 1a). One-way repeated measurement ANOVA revealed significant differences between the groups during the experiment ($P = .004$). A similar increase, although with slight delay, was seen in the jugular vein (Fig. 1b). After 10 min, samples from the jugular vein showed concentrations of 1.658 ± 477 mmol/L in the sham group and 2.358 ± 520 mmol/L in the intervention group ($P = .018$). One-way repeated measurement ANOVA revealed significant differences between the groups for the jugular vein samples ($P < .001$). In the sham-operated group, L-lactate levels from the portal and jugular sampling sites were stable throughout the study period (Fig. 1a and b). There was no significant difference in L-lactate levels in the portal and the hepatic vein, suggesting little or no metabolic

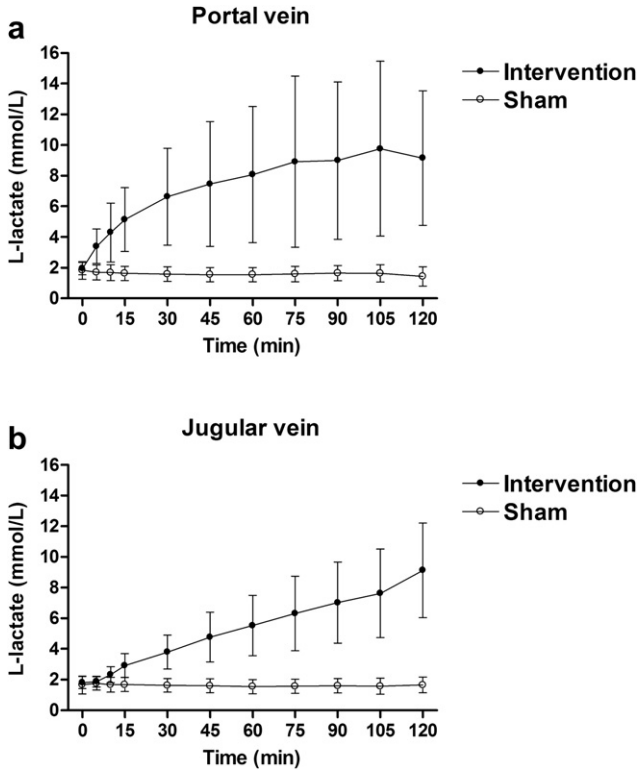

Fig. 1. Mean concentrations ± SD of L-lactate in plasma taken from the portal (a) and jugular (b) vein.

clearance. There were two missing samples in the intervention group at 120 min due to catheter clotting (results of the hepatic vein are not shown).

3.2. D-lactate

D-lactate started to increase at both sampling sites after approximately 30 min. This increase was significant in the jugular vein after 75 min: .019 ± .008 mmol/L in the sham group and .042 ± .022 mmol/L in the intervention group ($P = .023$) (Fig. 2b). The increase in D-lactate in the portal vein was significant after 90 min: .021 ± .008 mmol/L in the sham group and .121 ± .101 mmol/L in the intervention group ($P = .040$) (Fig. 2a). The mean concentration of D-lactate at T_0 was .019 mmol/L; this remained stable throughout the experiment in the sham group. One-way repeated measurement ANOVA revealed a significant difference between the groups for the jugular samples ($P = .038$). The portal samples were not significant ($P = .153$). There was no significant difference in D-lactate levels in the portal and the hepatic vein, suggesting little or no metabolic clearance. There were two missing samples in the intervention group at 120 min due to catheter clotting.

3.3. L-LDH and leukocytes

There was no difference in leukocyte levels measured prior to clamping and after 2 h of ischemia, for either of the two groups. In the sham group at 120 min, leukocytes were 10.12 ± .49 10^9/L and 11.09 ± 3.17 10^9/L in the intervention group ($P = .355$).

L-LDH activity remained the same in the sham group: 523.13 ± 146.54 U/L at 0 min and 491.75 ± 137.78 U/L at 120 min. In the clamped group, L-LDH activity increased from 471.63 ± 100.07 U/L to 1295.88 ± 1086.50 U/L after 120 min. A one-way repeated measurement ANOVA revealed significant different L-LDH activities between the intervention and sham group during the experiment ($P = .029$).

3.4. Interference

To elucidate the interference due to L-LDH and L-lactate when measuring D-lactate, we ran the D-lactate assay with and without sample preparation, as described.

The effect of choosing not to eliminate the contribution from L-LDH and L-lactate during venous ischemia is shown in D-lactate from the eight intervention pigs were measured with and without inactivation of L-LDH (Fig. 3).

3.5. Histology

All pigs in the intervention group showed macroscopic signs of intestinal oedema, as compared to none in the sham group. Histological examination revealed two pigs with moderate, four with light, and two without signs of small-intestine ischemia in the intervention group. In the sham group one had signs of light small-intestine ischemia and seven had normal histology. There were no microscopic signs of intestinal injury in colon specimens.

4. Discussion

In the present pig model of venous induced mesenteric ischemia, we found significant elevations of D- and L-lactate in both the systemic and portal circulation. The significant D-lactate values after 90 min and non-significant results after 120 min in the portal vein could be explained by 2 missing samples in the intervention group after 2 h, due to catheter clotting. The increase in D-lactate was seen simultaneously at all sampling sites, indicating little or no hepatic clearance. We expected if anything an earlier increase in L- and D-lactate in the portal vein compared to the systemic circulation. The fact that D-lactate increased earlier in the systemic circulation we think could be by chance as the differences in fact were minor. All pigs in the intervention group showed macro- and microscopic signs of ischemia. Leukocytes did not change significantly during 2 h of ischemia, which may not be enough time for a response in this condition.

Lobo et al. demonstrated a reduction in mortality from 82% to 18% if the diagnosis was made within 12 h of onset, compared with a delayed diagnosis of more than 24 h after onset.[13] In that perspective the difference in time to significant elevations for L- and D-lactate are of no clinical significance.

No other studies have been preformed to investigate D- and L-lactate levels in an experimental environment with venous

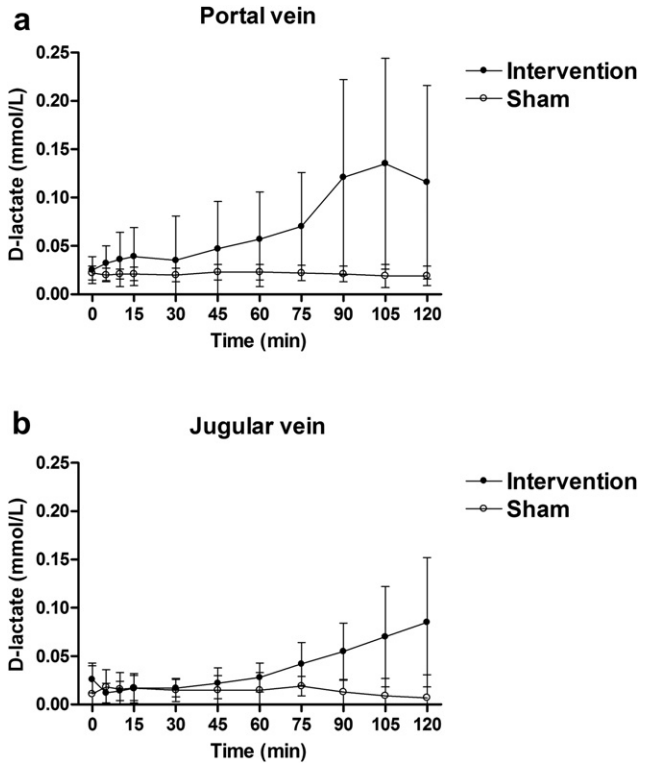

Fig. 2. Mean concentrations ± SD of D-lactate in plasma taken from the portal (a) and jugular (b) vein.

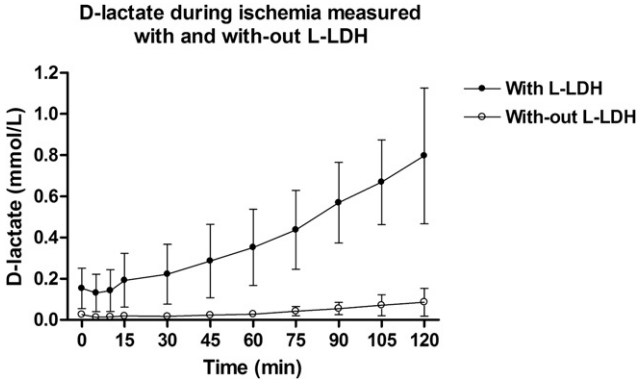

Fig. 3. Mean concentrations ± SD of D-lactate from the jugular vein during venous clamping with and without sample preparation.

induced intestinal ischemia. Kurimoto et al. investigated L-lactate as a marker of colon ischemia induced by arterial clamping. The authors found a significant elevation of L-lactate in the upper mesenteric vein after 2 h of superior mesenteric artery clamping and after 4 h in the systemic circulation.[14] These results are in agreement with our results; i.e., L-lactate is produced under intestinal ischemic conditions and can be measured in the systemic circulation. Such measurements reveal a slight delay, indicating hepatic clearance. Other studies have reported positive[11,15,16] and negative[1,17] results, respectively, for use of D-lactate as a biomarker for intestinal ischemia induced by arterial occlusion, high intra-abdominal pressure, and ischemia/reperfusion injury.

Assadian et al. showed a higher D-lactate concentration in 4 patients with colon ischemia after abdominal aorta aneurysm (AAA) repair, as compared to non-ischemic patients ($n = 8$).[11] These findings were confirmed by Poeze et al., who also saw increased D-lactate in 11 patients with ischemic bowel after ruptured AAA repair, as compared to 21 controls.[16] Murray et al. reported significant D-lactate increase in ischemic patients compared to patients with other intra-abdominal catastrophes, as well as admitted patients with central vein catheters.[15] Murray et al. found that D-lactate was significantly higher in rats with clamped superior mesenteric artery, as compared to controls.[18] Collange et al. did not find increased D-lactate in an experimental rat model, where 8 animals were treated with 40 min of supraceliac aortic clamping and one hour of reperfusion and compared to eight controls, even though histological examination of the intestine afterwards revealed a 30% decrease in villus height.[17] Günel et al. found D-lactate increases in a similar ischemia- reperfusion rabbit model.[19] Block et al. saw 71 consecutive patients above 50 years of age, with severe abdominal pain, 10 of whom also had intestinal ischemia. The authors found significantly higher L-LDH in the ischemic group, but did not find higher levels of D-lactate.[1] Düzgün et al. investigated D-lactate as a predictor of intestinal ischemia due to high intra-abdominal pressure in a rat model. The authors also found significantly higher D-lactate in rats with intra-abdominal pressure of 25 mmHg, as compared to rats with lower pressures.[20] However, there is no indication that L-LDH was removed prior to D-lactate measurement in the last three mentioned studies, which could interfere with the results.

Neglect of the interference due to L-LDH in the interpretation of D-lactate assay results leads to an overestimation of D-lactate levels. In the spectrophotometric assay, the contributions from plasma L-LDH and L-lactate can be considerable.

Block et al. reported higher L-LDH activity in patients with acute mesenteric ischemia compared to healthy controls,[1] which suggests that the overestimation of D-lactate increases with the length of an anaerobic condition, as observed in this study.

We eliminated the interference of L-LDH and L-lactate prior to D-lactate measurement. We saw macroscopic signs of ischemia in all the pigs in the intervention group and of microscopic ischemia in all but two, which indicates that the present model induces venous mesenteric ischemia. In conclusion, the present study in a pig model of venous induced mesenteric ischemia showed that both D- and L-lactate were markers of intestinal ischemia. The present study was designed to investigate L- and D-lactate as markers of mesenteric ischemia in a pig model. The exact mechanisms of venous induced mesenteric ischemia are not fully understood but extrinsic occlusions and thrombosis can be aetiologies for this condition., and the outcome might be cause specific. Clinical studies as well as experimental studies of other aetiologies of mesenteric ischemia need to be conducted before D-lactate can be relied upon as a clinical marker of mesenteric ischemia. The present results allow us to speculate that D-lactate could support the diagnosis of intestinal ischemia because it is not cleared by the liver and because it is a metabolic product of bacterial metabolism in the intestine and, as such maybe more specific for mesenteric ischemia than L-lactate. D-lactate measurements are easy accessible and the D-lactate assay provides a result after less than 30 min. The present study also revealed the importance of removing L-LDH when estimating D-lactate in plasma.

Conflict of interest

There are no conflicts of interest.

Fundings

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Ethical approval

The experimental procedure was conducted under local project license J.nr.2007/561-1329 in conformity with Danish legislations regarding animal welfare and experimental surgery.

Contributors

Casper Nielsen: Study design, data collection, analysis, and writing, Jes S. Lindholt: Study design, analysis, and writing. Erland J. Erlandsen: Data collection, analysis, and writing. Frank V. Mortensen: Study design, analysis, and writing. Lena Pedersen: biochemical analysis. Gorm Soendergaard: histological analysis.

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