Peritoneal nitric oxide is a marker of peritonitis in patients on continuous ambulatory peritoneal dialysis

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Abstract
Nitric oxide plays an important role in mediating the inflammatory process. The aim of this study was to evaluate if nitric oxide production was increased during peritonitis in patients receiving continuous ambulatory peritoneal dialysis (CAPD), and the association with the prognosis. The study population comprised 21 patients with 22 episodes of peritonitis. Fifteen patients without peritonitis were controls. Nitrate was measured by HPLC and nitrite by the Griess method, to reflect nitric oxide production. Peritoneal dialysate effluent and plasma were collected from six patients during peritonitis and 1 week after treatment to study changes in dialysate:plasma ratio. In 15 patients, nitrite was measured during peritonitis and every 3 days for 2 weeks or until normalized for evolutional changes. The dialysate:plasma ratios of nitrate and nitrite during peritonitis were reduced 26% and 41.5%, respectively, after 1 week of treatment, indicating the peritoneal production of nitric oxide during peritonitis. In the evolutional study, a 5.1-fold increase of peak nitrite levels in bacterial peritonitis ($n=13$) and a 2.5-fold increase in fungal peritonitis ($n=3$) were observed compared to controls. Nitrite gradually declined to control levels ($9.3 \pm 7.2$ days) after effective antibiotic treatment, but took longer than to normalize leukocyte count in the peritoneal dialysate effluent ($3.9 \pm 1.9$ days). In four patients with refractory peritonitis (Candida infection in three, Acinetobacter infection in one), the nitrite levels remained elevated 2-fold despite treatment, and the catheters were removed. It is concluded that nitrite levels in peritoneal dialysate effluent may serve as a marker to assess treatment efficacy in CAPD patients with peritonitis.

Key words: continuous ambulatory peritoneal dialysis; nitric oxide; peritonitis

Introduction
While continuous ambulatory peritoneal dialysis (CAPD) is accepted as an adequate renal replacement therapy for chronic renal failure, peritonitis still remains a common and serious complication. Catheter loss due to refractory peritonitis represents the most frequent cause of discontinuation of CAPD. Assessment of treatment efficacy relies on the improvement of symptoms, clearance of dialysate, and disappearance of leukocytosis in peritoneal dialysate effluent. Normalization of leukocyte count has been regarded as a parameter of improvement, however, approximately 10% of peritonitis occurs with low dialysate white blood cell count [1]. Neither the clinical parameters nor the causative organism is useful in prediction of the outcome and relapse or reinfection may occur. Thus, a marker of peritonitis both in assessing the treatment efficacy and in predicting the outcome will be helpful in clinical practice.

Nitric oxide, a mediator of cytokine activation, plays an essential role in mediating inflammatory process and sepsis [2,3]. Nitric oxide and other reactive nitrogen intermediates produced by cytokine-activated cells are important components of inflammation and antimicrobial activity of these cells [4,5]. Urinary nitrite, an end product of nitric oxide, has been used as a screening test for urinary tract infection [6]. In peritonitis, increased peritoneal production of nitrate, another end product of nitric oxide, was found by an increased dialysate:plasma (D:P) ratio in CAPD patients. The reduction of D:P nitrate ratio after recovery indicated that nitric oxide production may originate from the inflamed peritoneum [7]. The aim of this study was to evaluate if nitric oxide, measured as nitrate or nitrite.
in the peritoneal dialysate effluent, may reflect the inflammatory status and be used as a marker for assessing treatment efficacy or to provide prognostic value.

Subjects and methods

Patients

From January 1995 to May 1996, 21 patients with 22 episodes of peritonitis (nine male and 12 female; mean age 52.7±12.1 years, range 31-72) and 15 patients without peritonitis (10 male and five female; mean age 56.9±13.3, range 26-74), all undergoing CAPD for more than 6 months, were enrolled in two consecutive studies. Evidence of peritonitis included abdominal pain, cloudy dialysate, and the presence of more than 100 white blood cells (WBC)/mm³ in which neutrophils exceeded 50%, and was confirmed by positive culture of micro-organisms.

Experimental design

Study A: Measurement of nitrate and nitrite in peritoneal dialysate effluent and in circulation.

Peritoneal dialysate effluent and plasma were collected simultaneously at the appearance of peritonitis and 1 week after treatment for calculation of D:P ratio of nitric oxide production.

Study B: Assessing evolutional changes of nitrite levels in the peritoneal dialysate effluent after treatment.

Peritoneal dialysate effluent was collected at the appearance of peritonitis, and daily, where possible, for the first 4 days, then every 3 days for 2 weeks or until normalized. For each collection, peritoneal dialysate effluent, which was in the peritoneum for at least 4 h, was taken for total cell and differential count, and another 10 ml was saved at -20°C for nitrite measurement. The levels of nitrite were measured and compared among patients with or without peritonitis, and followed as peritonitis evolved after treatment. Peritoneal dialysate effluent was also collected at 30 and 60 days after treatment in a follow-up study of those patients with cured peritonitis.

High performance liquid chromatography

Nitrate (NO₃⁻) concentrations were measured by HPLC using a method modified by Witter et al. [8]. One ml of plasma or peritoneal dialysate effluent was diluted with 3.5 ml double-distilled, deionized water, deproteinized with 0.25 ml Carrez II solution (30% ZnSO₄-7H₂O) and 0.25 ml Carrez I solution (15% K₂Fe(CN)₆·3H₂O) and centrifuged at 11,000g for 5 min. The supernatant was passed through a 0.45-μm syringe-mounted filter. Fifty μl samples were injected onto the HPLC for analysis. The HPLC system was equipped with a pump (Model 600E, Waters, Bedford, USA), a U6K injector (Waters) and a programmable multiwavelength detector (model 490E, Waters) set at 205 nm. Guard column was a Lichrospher 100 RP-18 column (125 mm, I. D. 4 mm, particle size 5 μm; Merck, Darmstadt, Germany). Separation was achieved by a Hypersil ODS (C18) column (150 mm, I. D. 4.6 mm, particle size 3 μm; Alltech, Deerfield, USA). The mobile phase was 0.01 M o-cymylamine added with 2% acetonitrile. pH was adjusted to 6 with sulphuric acid. The flow rate was 1.2 ml/min. Nitrate concentration was determined by measuring the peak area under the curve with an integrator (Chem-lab, SISC, Taiwan).

Greiss method

Accumulation of nitrite (NO₂⁻) in the peritoneal dialysate effluent and plasma was determined by the Griess reagent and was taken as an index of nitric oxide production [9]. All samples were kept for 20°C until analysis. To a 500 μl sample was added 100 μl 35% sulfosalicylic acid and 0.25 ml Carrez II solution (30% ZnSO₄-7H₂O) and 0.25 ml 5% NaOH was added for nitrite measurement. The Griess reagent (1 ml) comprised equal amount of 0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% concentrated H₃PO₄, which were added immediately before reaction. The Griess reagent reacted with nitrite in the peritoneal dialysate effluent-treated sample to form a pink to dark pink colour after incubation for 15 min. This was read by spectrophotometer at 546 nm. NaN₃ was included as a standard.

Statistical methods

The results of nitrite measured in individual subjects were averaged for each study period, with group data expressed as means±SEM. Student's t-test was used for comparing the mean differences for those data with normal distribution, and Wilcoxon's signed rank test was used for comparing differences for those data with small sample size and which were not normally distributed.

Results

Study A: Decreased dialysate/plasma ratio of nitrate, nitrite after treatment.

In six patients who had peritonitis (two with Streptococcus viridans infection, one with Acinetobacter anitatus, two with Hemophilus influenza, and one culture negative), plasma nitrate and nitrite levels were similar before and 1 week after treatment. The peritoneal dialysate effluent showed reductions in nitrate levels (64.9±24.4 vs 51±15.6 μM, NS) and in nitrite levels (86.8±18.6 vs 52±8 ng/ml, P<0.05). The D:P ratios of nitrate and nitrite levels at the appearance of peritonitis, calculated by the method described previously [7], were reduced 26% (P=0.105) and 41.5% (P<0.05), respectively, after 1 week of treatment (Fig. 1). This result confirmed previous observations of increased production of peritoneal nitric oxide during peritoneal inflammation.

Study B: Peritonitis

Nitrite was measured from peritoneal dialysate effluent in 15 patients who had 16 episodes of peritonitis. Thirteen episodes were due to bacterial infection (five coagulase-negative Staphylococcus, one Streptococcus...
Fig. 1. The dialysate:plasma ratio of nitrate (NO₃⁻) and nitrite (NO₂⁻) levels are reduced 26% (P=0.105) and 41.5% (P<0.05), respectively, after 1 week of treatment.

viridans, two Staphylococcus aureus, two Acinetobacter anitatus, two Staphylococcus epidermidis, and one Pseudomonas aeruginosa), and three episodes were due to fungus. Treatment with antibiotics was effective in 12 patients, in whom peritoneal dialysate effluent WBC count was normalized in 3.9±0.5 days (range 3-7 days) for a standard 2-week treatment. No relapse was observed in patients after effective treatment. A diabetic patient experienced two episodes of peritonitis 60 days apart. The first episode was due to coagulase-negative Staphylococcus and the second was caused by Staphylococcus aureus. Catheters were removed in four patients with peritonitis because of refractory peritonitis. The catheter was removed in one patient with Acinetobacter infection and in three patients infected with Candida after 20±5.1 treatment days.

Study B: Evolutional changes of increased nitrite levels in peritonitis

The mean nitrite levels in peritoneal dialysate effluent were elevated in patients with peritonitis. At the appearance of peritonitis, the nitrite levels reached a peak within the first 3 days after cloudy dialysate or peritoneal symptoms occurred. In bacterial peritonitis, the peak nitrite levels were increased 5.1-fold (132.9±17.8 ng/ml, n=13) compared with those from

Fig. 2. (A) The peak nitrite level in peritoneal dialysate effluent is increased 5.1-fold in patients with bacterial peritonitis (n=13, P<0.001) and 2.5-fold in patients with fungal peritonitis (n=3, P<0.01). (B) The lowest nitrite levels in peritoneal dialysate effluent after treatment are similar to controls in patients with effective treatment (n=12, P=0.03), but remain 2-fold higher in patients where treatment was ineffective (n=4, P<0.01). P, peritonitis.

Fig. 3. Evolutional changes of nitrite levels (A) and WBC count (B) in peritoneal dialysate effluent in patients (n=12) with effective antibiotic treatment. The peak nitrite levels coincide with the peak WBC in the peritoneal dialysate effluent but are sustained longer than the elevated WBC count.
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control patients (25.1 ± 2.2 ng/ml, n = 15; P < 0.001). In fungal peritonitis, the peak nitrite levels were 2.5-fold elevated in the peritoneal dialysate effluent (61.7 ± 9.1 ng/ml, P < 0.001) compared with controls (Fig. 2A). Nitrite measured in patients with refractory peritonitis before catheter removal was 2-fold (56.7 ± 3.7 ng/ml; P < 0.001) higher than the control levels (Fig. 2B). When nitrite levels were normalized after antibiotic treatment, in a mean of 9.3 ± 7.2 days (range 4–30 days), to control levels (30.9 ± 4.76 ng/ml, P = 0.03), the rate was slower than the disappearance of leukocytosis and peritoneal symptoms (Fig. 3). In four patients with refractory peritonitis, the mean peak nitrite levels were 66.6 ± 8.5 ng/ml and remained elevated despite prolonged treatment (Fig. 4). In the diabetic patient who experienced two episodes of peritonitis, vancomycin 2 g intraperitoneally effectively eradicated cloudiness and leukocytosis of the peritoneal dialysate effluent 7 days following peritonitis. A second dose of vancomycin was given a further 7 days later. The initial nitrite level was 247.9 ng/ml, which was reduced after treatment but still remained 2-fold higher than controls. Exit site infection was discovered at the 56 days and full-blown peritonitis recurred at 60 days. The nitrite level was 181 ng/ml at the second episode of peritonitis (Fig. 5).

The levels of nitrite did not correlate significantly with any specific micro-organism nor with the duration it took to normalize nitrite levels in the peritoneal dialysate effluent. However, three patients who had elevated nitrite for more than 2 weeks were diabetic. The first diabetic patient had normalized leukocyte count after 3 days of treatment, and nitrite normalized at 14 days. In the second diabetic patient, leukocyte counts normalized after 4 days and nitrite normalized after 30 days. Follow-up nitrite levels in these two patients at 60 days showed normal values.

Discussion

A marker of peritonitis may be helpful to evaluate the treatment efficacy while peritonitis is still a major cause of morbidity in CAPD patients. In this study, increased nitric oxide production was found in the peritoneal dialysate effluent. The levels coincided with the acute phase of peritonitis, and were sustained much longer than the leukocyte count in the peritoneal dialysate effluent. The peak peritoneal nitrite level was elevated 5.1-fold in patients with peritonitis. The nitrite levels remained elevated when leukocyte count was normalized and clinical symptoms and signs of peritonitis had disappeared after effective treatment. The mean duration of nitrite elevation in the peritoneal dialysate effluent under effective treatment was 2.4 times longer than the duration of leukocyte elevation. Nitrite levels were higher in patients with bacterial peritonitis than with fungal peritonitis. In 11 patients where nitrite levels were normalized after effective treatment, no relapse of peritonitis was found 30–60 days after peritonitis and nitrite remained at control levels. Persisting elevation of nitrite in four patients indicated refractory peritonitis, and catheter removal was inevit-

Fig. 4. Nitrite levels in refractory peritonitis. Persistent elevated nitrite levels in peritoneal dialysate effluent were found in four patients with refractory peritonitis where catheter removal was inevitable (A). The WBC count was mild elevated in these patients (B).

Fig. 5. Two episodes of peritonitis were encountered in a diabetic patient 60 days apart. The nitrite levels did not normalize after clinical symptoms subsided. Exit site infection was discovered at 56 days and peritonitis recurred at 60 days.
able due to persistent inflammation. Normalized nitrite levels indicated resolved peritoneal inflammation, whereas persisting elevation of nitrite levels indicated unresolved peritoneal inflammation. Thus, nitric oxide levels provided more information than white cell count alone.

Nitric oxide plays an essential role in mediating cytokine activation in the inflammatory process [3]. The production of nitric oxide and other reactive nitrogen intermediates by cytokine-activated cells including macrophages is an important component of antimicrobial and/or antineoplastic activity of the cells [4,5]. By mass transfer area coefficient (MTAC) study, dialysate concentrations of nitrate in stable CAPD patients are determined by diffusion from the circulation [7]. During peritonitis, an increased nitrate D:P ratio indicates that the production is originated from the inflamed peritoneum rather than the peripheral circulation [7]. In this study, we confirmed that local production of nitric oxide is likely to occur during peritonitis, and that the D:P ratio is reduced after treatment. Nitric oxide is formed from the terminal guanidino nitrogen of L-arginine, in a reaction catalysed by the enzyme nitric oxide synthase. Both constitutive and inducible (iNOS) forms of this enzyme have been described, but from an immunopathological perspective, attention has been focused on the inducible enzyme, which is expressed in macrophages a few hours after activation in a sustained action [4,10,11]. The sustained action of iNOS may explain the phenomenon observed in this study, where nitrite was elevated longer than the leukocyte count in the peritoneal dialysate effluent. However, the sources of production of iNOS in the peritoneum await further study.

Overproduction of nitric oxide can rapidly react with superoxide to form peroxynitrite, which is a powerful oxidant and causes tissue damage [12], and has been implicated in a variety of immunopathological situations including septic shock [2]. These findings suggest that nitric oxide may contribute to, on one hand, the anti-microorganism activity, and on the other hand, morbidity associated with advanced inflammation such as sepsis. Nitric oxide synthase inhibition may improve the efficacy of conventional antimicrobial treatment of severe infections, and inhibition of nitric oxide synthesis improves survival in a murine peritonitis model of sepsis that is not cured by the enzyme nitric oxide synthase. Both constitutive and inducible (iNOS) forms of this enzyme have been described, but from an immunopathological perspective, attention has been focused on the inducible enzyme, which is expressed in macrophages a few hours after activation in a sustained action [4,10,11]. The sustained action of iNOS may explain the phenomenon observed in this study, where nitrite was elevated longer than the leukocyte count in the peritoneal dialysate effluent. However, the sources of production of iNOS in the peritoneum await further study.

In conclusion, nitrite measurement provides a simple and rapid method of detecting peritoneal inflammation, to assess treatment efficacy. Measurement of nitrite is recommended in CAPD patients with peritonitis at the disappearance of clinical symptoms and before discontinuation of antibiotics, to detect possible residual peritoneal inflammation. Persisted elevation of nitrite mandates the intensive search of underlying inflammation, and longer duration of antibiotic treatment may be indicated.

References

3. Marcinkiewicz J, Grabowska A, Chain B. Nitric oxide without peritonitis, in which Staphylococcus aureus induced the highest levels and Escherichia coli the lowest [19]. In this study nitric oxide is added to the list of inflammatory mediators studied, and it may serve as an indicator for assessment of peritoneal inflammation. The main difference from the cytokines studied was that nitric oxide levels dissociated from the polymorphonuclear cell count in the peritoneal dialysate. Thus, normalized levels of nitrite in the peritoneal dialysate effluent ensure the effective treatment of peritonitis. On the other hand, elevated levels of nitrite mandate prolonged treatment and possibly poor outcome. The practical use of nitrite to aid assessment of treatment efficacy is to check the nitrite levels before discontinuation of treatment to assure resolution of peritoneal inflammation. Prolonged treatment is indicated when nitrite level is elevated. There is no role of nitrite in differentiating relapse or reinfection, since nitric oxide is only a marker of inflammation.

Diabetes mellitus seems to be a co-morbid condition that retards the recovery of peritonitis. Two diabetic patients had longer durations of nitrite elevation in the peritoneal dialysate effluent, 14 and 30 days, compared with the mean duration of 9.3 days. Nitrite level in another diabetic patient did not return to control levels after two doses of vancomycin treatment, and persisted for 2 months when exit site infection occurred followed by a second episode of peritonitis. The elevation of nitrite in the peritoneal dialysate effluent was however not specific to peritonitis. In a recent observation, a CAPD patient with acute gastroenteritis had high levels of nitrite in peritoneal dialysate effluent, indistinguishable from peritonitis. Thus, peritoneal nitrite production may be an indicator of inflammation in the peritoneal cavity.

In conclusion, nitrite measurement provides a simple and rapid method of detecting peritoneal inflammation, to assess treatment efficacy. Measurement of nitrite is recommended in CAPD patients with peritonitis at the disappearance of clinical symptoms and before discontinuation of antibiotics, to detect possible residual peritoneal inflammation. Persisted elevation of nitrite mandates the intensive search of underlying inflammation, and longer duration of antibiotic treatment may be indicated.

Acknowledgements. The authors would like to thank Su-Fen Chen, Mei-Ur Chen, Su-Man Wong, Mai-Gi Liang, Su-May Chin, Hsiao-Mei Yo, Mon-Wei Li, Yi-Chin Ger for their excellent technical support.
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Received for publication: 6.2.96
Accepted in revised form: 29.7.96