Effect of dietary fish oil supplementation on cellular adhesion molecule expression and tissue myeloperoxidase activity in diabetic mice with sepsis

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This study investigated the effect of n-3 fatty acids on adhesion molecules and tissue myeloperoxidase (MPO) activity in diabetic mice with sepsis. Diabetes was induced by a streptozotocin injection. Mice with blood glucose levels exceeding 2000 mg/l were considered diabetic. Diabetic mice were assigned to two groups with a medium-fat (10 %, w/w) diet either provided by soyabean oil (SO, n 30) or fish oil (FO, n 30). n-3 fatty acids provided 4-3 % of the total energy and the n-3/n-6 fatty acid ratio was 1:2 in the FO diet. After feeding the respective diet for 3 weeks, all mice had sepsis induced by caecal ligation and puncture (CLP) and were killed at 0, 6 or 24 h after CLP, with ten mice at each time-point. The result showed that compared with the SO group, FO group had lower PGE\textsubscript{2} and TNF-\textalpha levels in peritoneal lavage fluid after CLP. Lymphocyte CD11a/CD18 expressions were higher at 6 h, whereas the percentage was lower at 24 h in the SO group than in the FO group. Neutrophil CD11b/CD18 expressions were significantly higher in the SO group than in the FO group at 0 h. The FO group had lower organ MPO activities at various time-points after CLP when compared with those of the SO group. The present findings suggest that compared with the diabetic mice fed SO, a low-dose n-3 fatty acid supplementation may attenuate leucocyte adhesion and infiltration into tissues in diabetic mice complicated with sepsis.

Diabetes: Sepsis: Fish oil: Cellular adhesion molecule: Myeloperoxidase

Diabetes mellitus was the fourth leading cause of death in Taiwan in 2005 (Department of Health, Taiwan, 2005). It is a metabolic disorder characterized by hyperglycaemia and dyslipidaemia. Many diabetic patients have an increased risk of CHD, peripheral vascular diseases and cerebrovascular diseases (Parillo & Riccardi, 2004). Endothelium dysfunction accompanied by upregulated inflammatory mediators is a major contributing factor to the pathogenesis of diabetic vascular complications (Nystrom \textit{et al.} 2006). Furthermore, the abnormalities in nutrient metabolism resulting from diabetes mellitus lead to impairment of wound healing and vulnerability to infection and sepsis.

Sepsis is a common clinical problem with extremely high mortality rates. Several components of the immune system are implicated in the process of sepsis, including the release of proinflammatory mediators and activation of endothelial cells and polymorphonuclear leucocytes (PMN; Shimizu \textit{et al.} 1992; Williams & Hellewell, 1992). On activated endothelium, members of the Ig family of adhesion molecules – intercellular adhesion molecules (ICAM) and vascular cell adhesion molecules (CAM) – are expressed. CAM are important in the adhesion of leucocytes to activated endothelium (Carlos & Harlan, 1994). CD11a/CD18 and CD11b/CD18 are members of the leucocyte adhesion molecules-\beta2 integrin. CD11a and CD11b are thought to play central roles in mediating the firm adhesion of leucocytes to endothelial cells (Henderson \textit{et al.} 2001). Overexpressions of adhesion molecules facilitate leucocyte–endothelial interactions which result in endothelial dysfunction and thus aggravate PMN accumulation and tissue damage (Ulbrich \textit{et al.} 2003; Nolte \textit{et al.} 2004). One study showed that plasma ICAM-1 levels increase in septic patients with multiple organ failure (Whalen \textit{et al.} 2000). Also, plasma ICAM-1 in diabetic patients was significantly higher than that in healthy controls (Glowinska \textit{et al.} 2005).

Fish oils are rich sources of n-3 PUFA, especially EPA and DHA. A number of clinical trials have shown that fish oil has immune modulatory effects (Grimm \textit{et al.} 2002). The major advantages of n-3 fatty acids are related to their postulated reductions in proinflammatory effects. Several studies have shown that dietary fish oil has beneficial clinical effects on diseases including rheumatoid arthritis, inflammatory bowel diseases, multiple sclerosis and insulin-dependent diabetes mellitus (Calder, 1997, 2006). However, a previous study revealed that fish oil supplementation suppresses lymphocyte proliferation and has immunosuppressive properties (Virella \textit{et al.} 1991). An \textit{ex vivo} study also showed that n-3 fatty acids inhibit proliferative response and IL-2 production in lymphocytes obtained from diabetes mellitus patients (Alnajjar \textit{et al.} 2006). In accordance with such observations,
laboratory animals fed fish oil exhibited lower survival and higher viable bacteria numbers than those fed other types of fat when infected with bacteria (Chang et al. 1992; Puertollano et al. 2004). Conflicting results were also observed in septic conditions when n-3 fatty acids were administered (Fritsche et al. 1997; Lanza-Jacoby et al. 2001). Most animal studies done previously were seldom performed with co-morbidities, the studies concerned with the influence of fish oil on the inflammatory response focused exclusively on the condition of diabetes mellitus or sepsis. Studies investigating the effects of dietary fish oil on diabetes mellitus complicated with sepsis are rare. Therefore, we induced polymicrobial sepsis after treating diabetic mice with fish oil to investigate the effect of n-3 fatty acids on adhesion molecules and inflammatory cytokines in diabetic mice complicated with sepsis. Because oxyradicals released from leucocytes that accumulate in organs may damage organ cells and induce organ dysfunction (Klebanoff & Seymour, 2005), we analysed the myeloperoxidase (MPO) activities in organs as an indicator for identifying the extent of tissue injury resulting from diabetes mellitus with sepsis.

Materials and methods

Animals

Male ICR mice weighing approximately 25–30 g were purchased from the Animal Center of National Taiwan University, College of Medicine. Mice were maintained in a temperature-controlled (23 ± 2°C) and humidity-controlled (55 ± 15 %) room with a 12 h light–dark cycle. All mice were allowed free access to a standard Chow diet and water for 1 week before the study. Care of the laboratory animals was established by Taipei Medical University, and protocols were approved by the Animal Committee. Diabetes was induced in the mice by a single intraperitoneal injection of streptozotocin (Sigma Chemical Co., St Louis, MO, USA) at a dose of 150 mg/kg body weight as previously described by Oguri et al. (2003). Streptozotocin was dissolved immediately before use in saline to a concentration of 15 mg/ml. Three days later, blood was obtained by piercing a needle into the tail vein of the mice; it was directly applied on to a strip of a blood glucose monitor to determine the glucose levels. Mice were considered diabetic only if their blood glucose levels exceeded 2000 mg/l (Ackerman & Leibman, 1977). The average blood glucose levels for normal mice were 1195 (SD 124) mg/l (n = 8). Diabetic mice were not treated with insulin in the present study.

Experimental design and procedures

The diabetic mice were divided into two groups according to the weight and blood glucose of the animals to make average weights and blood glucose levels among groups as similar as possible. All mice were maintained for 3 weeks on a medium-fat (10 %, w/w) semi-purified diet. The diets fed to the two experimental groups were identical except for the sources of the fat (Table 1). The soyabean oil group (SO, n = 30) was exclusively fed soyabean oil (Taiwan Sugar Co., Taipei, Taiwan), while the fish oil group (FO, n = 30) had 23 % fish oil (Denofa Co., Fredrikstad, Norway) and 77 % soyabean oil (Table 1). The fish oil contained 34 % EPA, 27 % DHA and 72 % total n-3 fatty acids, while the mixed tocopherols was 2-4 mg/g. The soyabean oil contained 65 % n-3 fatty acids and 55 % n-6 fatty acids according to the manufacturer. The n-3/n-6 ratio in the FO diet was 1:2 in the present study. After feeding the respective diet for 3 weeks, polymicrobial sepsis was induced in the mice. Sepsis was induced by caecal ligation and puncture (CLP) according to the method of Ayala et al. (1994). Briefly, mice were lightly anaesthetized with diethyl ether and their abdomens were opened through a midline incision below the diaphragm. The caecum was isolated and ligated just below the ileocaecal valve. The caecum was then punctured twice with a 22-gauge needle and was slightly compressed until a small drop of stool appeared. After CLP was performed, the caecum was replaced into the abdominal cavity and the wound was closed in layers. Diabetes mellitus–sepsis mice were killed at 0, 6 and 24 h after the CLP, with ten mice at each time-point. Mice in 0h groups were killed immediately after CLP. All mice were anaesthetized with intraperitoneal pentobarbital sodium (50 mg/kg body weight) and were killed by heart puncture. The abdomen was opened and the peritoneal cavity was lavaged with 2 ml PBS. The peritoneal lavage fluid was collected for measurement of TNF-α and PGE2 analyses. Fresh blood samples were collected in tubes containing EDTA-Na2 for analysing leucocyte CD11a/CD18 and CD11b/CD18 expressions. Plasma samples were stored at ~80°C until glucose and ICAM-1 was measured. Tissues including the liver, kidneys, intestines and lungs were rapidly harvested and stored at ~80°C for the measurement of MPO activities.

Measurements and analytical procedures

Measurements of plasma glucose and intercellular adhesion molecule-1 concentrations. Glucose levels were determined by colorimetric methods after an enzymatic reaction with peroxidase (Randox Co., Antrim, Ireland). Procedures followed the manufacturer’s instructions. Concentrations of ICAM were measured by using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA). Antibodies specific

<table>
<thead>
<tr>
<th>Component</th>
<th>Soyabean oil group</th>
<th>Fish oil group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soyabean oil</td>
<td>100</td>
<td>77</td>
</tr>
<tr>
<td>Fish oil</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Maize starch</td>
<td>620</td>
<td>620</td>
</tr>
<tr>
<td>Salt mixture‡</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mixture§</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Methyl cellulose</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Methionine</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

† The salt mixture contained the following (mg/g): calcium phosphate diabasic, 500; sodium chloride, 74; potassium sulphate, 52; potassium citrate monohydrate, 20; magnesium oxide, 24; manganese carbonate, 3.5; ferric citrate, 6; zinc carbonate, 1.6; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenite, 0.01; and chromium potassium sulphate, 0.55.

§ The vitamin mixture contained the following (mg/g): thiamin hydrochloride, 0.6; riboflavin, 0.6; pyridoxine hydrochloride, 0.7; nicotinic acid, 3; calcium pantothenate, 1.6; d-biotin, 0.05; cyanocobalamin, 0.001; retinyl palmitate, 1.6; dl-α-tocopherol acetate, 20; cholecalciferol, 0.25; menaquinone, 0.005.
for mouse ICAM-1 were coated on to the wells of the microtitre strips provided. The minimum detectable dose for ICAM-1 was 17 pg/ml. The within-assay CV was 6·3 % in the present study.

**Measurement of TNF-α and PGE2 levels in peritoneal lavage fluid.** Concentrations of TNF-α were measured using a commercially available ELISA. Antibodies specific for mouse TNF-α were coated on to the wells of the microtitre strips provided (R&D Systems). PGE2 concentrations were also measured by ELISA. The surfaces of the microtitre plates were precoated with mouse monoclonal antibody. Acetylcholinesterase covalently coupled to PGE2 was used as the enzymatic tracer (R&D Systems). The detection limit for TNF-α was 5·1 pg/ml and for PGE2 was 8·5 pg/ml. The within-assay CV for TNF-α and PGE2 were 6·8 % and 7·2, respectively.

**Analysis of the CD11a/CD18 distribution in lymphocytes and CD11b/CD18 in polymorphonuclear leucocytes.** Lymphocytes and PMN in blood were gated on the basis of the forward scatter and side scatter profiles by flow cytometry (Coulter, Miami, FL, USA), and were analysed for the expressions of CD11a/CD18 and CD11b/CD18, respectively. Fresh blood (100 μl) was incubated with 10 μl fluorescent isothiocyanate-conjugated rat monoclonal anti-mouse CD11a (I21/7) and phycoerythrin-conjugated rat monoclonal anti-mouse CD18 (C71/16; Serotec, Oxford, UK) for 15 min at 4°C. Fluorescent isothiocyanate-conjugated rat IgG2a and phycoerythrin-conjugated rat IgG2a were used for isotope control (Serotec). Subsequently, erythrocytes were lysed with lysing buffer (Serotec). The percentages of CD11a/CD18 expressed on lymphocytes were analysed by flow cytometry. Fluorescence data were collected and the results are presented as a percentage of CD11a-presenting cells in 1 × 10⁶ lymphocytes. To measure CD11b/CD18 expressions on PMN, fluorescent isothiocyanate-conjugated rat monoclonal anti-mouse CD11b (M1/70.15) and phycoerythrin-conjugated rat monoclonal anti-mouse CD18 (C71/16; Serotec, Oxford, UK) were added into 100 μl fresh blood. Fluorescent isothiocyanate-conjugated rat IgG2b and phycoerythrin-conjugated rat IgG2a were used for isotope control (Serotec). Fluorescence data were collected on 1 × 10⁵ PMN which were also analysed by flow cytometry. The results are presented as a percentage of CD11b/CD18 expression in 1 × 10⁵ PMN. Non-specific fluorescence was determined on cells incubated with isotype and fluorochrome-matched control antibodies (Hsu et al. 2006).

**Measurement of myeloperoxidase activity in organs.** The method of measuring MPO activity was modified as previously described (Hillegass et al. 1990). Tissue samples were homogenized in 50 mm-potassium phosphate buffer (pH 6.0), and centrifuged at 2000 g at 4°C for 15 min. After discarding the supernatant, the pellets were suspended in a solution containing 0-5 % hexadecyl-trimethyl-ammonium bromide dissolved in potassium phosphate buffer (pH 7.0) and centrifuged for 30 min at 15 000 g and 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1·6 mM) and 0·1 mM-H₂O₂. The absorbance at 650 nm was measured for 3 min and the rate of change in the absorbance was used to calculate the activities of MPO. MPO activity was defined as the quantity of enzyme degrading 1 μmol peroxide per min at 37°C and the data were expressed in units/g wet tissue.

**Statistical analysis**

Data are expressed as means and standard deviations. All statistical analyses were performed with SigmaStat version 3.1 software (SYSTAT Software Inc., Chicago, IL, USA). Differences among groups were analysed by two-way ANOVA using Fisher’s post hoc test. P<0·05 was considered statistically significant.

**Results**

**Body weights and plasma glucose levels**

There were no differences in the initial body weights and body weights after feeding the respective diets for 3 weeks between the two experimental groups (data not shown). There were no differences in fasting plasma glucose concentrations between the FO and SO groups at various time-points after CLP (Table 2).

**Plasma intercellular adhesion molecule-1 levels**

Plasma ICAM-1 levels were higher at 24 h than 0 and 6 h after CLP in both groups (P<0·001 for time effect). There were no differences in ICAM-1 concentrations between the SO and FO groups at various time-points after CLP (Fig. 1).

**PGE2 and TNF-α concentrations in peritoneal lavage fluid**

The PGE2 levels in the FO groups were significantly lower than in the SO groups at each time-point after CLP (P<0·001 for diet effect). The PGE2 levels were lower at 0 h than at 6 and 24 h after CLP in both groups (P<0·001 for time effect; Fig. 2(A)). Concentrations of TNF-α increased with the progression of sepsis in the SO group, whereas no differences were found among the FO groups at various time-points (P<0·001 for time effect). The SO group had higher TNF-α concentrations than the FO group 24 h after CLP (P=0·039 for diet effect, P=0·013 for diet and time interaction; Fig. 2(B)).

**Table 2. Plasma glucose concentrations (mg/l) of the fish oil group (FO) and the soyabean oil group (SO)†**

<table>
<thead>
<tr>
<th>Time</th>
<th>FO Mean</th>
<th>FO SD</th>
<th>SO Mean</th>
<th>SO SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>2512</td>
<td>477</td>
<td>3380</td>
<td>1082</td>
</tr>
<tr>
<td>6 h</td>
<td>2920</td>
<td>920</td>
<td>2803</td>
<td>779</td>
</tr>
<tr>
<td>24 h</td>
<td>2626</td>
<td>361</td>
<td>3047</td>
<td>1139</td>
</tr>
</tbody>
</table>

† For details of procedures, see Materials and methods section.
Expressions of CD11a/CD18 on lymphocytes and CD11b/CD18 on polymorphonuclear leucocytes

CD11a/CD18 expressions on lymphocytes were higher at 6 h, whereas the percentage was lower at 24 h in the SO group than in the FO group. SO groups had the highest CD11a expression at 6 h, while the highest CD11a percentage was found at 24 h after CLP in the FO group (P = 0.001 for time effect, P = 0.001 for diet and time interaction; Fig. 3(A)). The expressions of CD11b/CD18 on PMN decreased in the SO group as sepsis progressed. CD11b/CD18 expressions were significantly higher in the SO group than in the FO group at 0 h. There were no differences in the expressions of CD11b/CD18 among the various FO groups (P = 0.001 for diet effect, P = 0.006 for time effect, P = 0.001 for diet and time interaction; Fig. 3(B)).

Myeloperoxidase activities in the liver, lungs, kidneys and intestines

The activities of MPO increased as sepsis progressed and reached a peak at different time-points depending on the type of oil in various organs. The FO groups had lower MPO activities at 0 h in the liver, at 0 and 6 h in the kidneys, and at 24 h after CLP in the lungs compared with those in the SO groups (P < 0.05; Table 3).

Discussion

In the present study, n-3 fatty acids provided 4-3 % of the total energy in the FO diet. This amount of n-3 fatty acids is comparable to values used in studies with beneficial results, which showed that n-3 fatty acid supplementation reduced inflammatory-related mediators and improved survival in rodents in a CLP model (Johnson et al. 1993; Lanza-Jacoby et al. 2001). The n-3/n-6 ratio was adjusted to 1:2, because this ratio was...
considered to exert the most favourable modulation of lipid mediator synthesis (Morlione et al. 1997). In the present study, we used streptozotocin to induce diabetes, a model frequently used to stimulate non-insulin-dependent diabetes in animal studies (Chyi & Yeh, 2000), and CLP is a clinically relevant model of gut-derived sepsis in mice (Maier et al. 2004).

PGE2 is a product of arachidonic acid metabolism. Numerous studies have shown that an increased dietary intake of n-3 fatty acids suppresses PGE2 synthesis (Mayatepek et al. 1994; Calder, 2006). The previous results are in good agreement with the present finding that diabetic mice fed FO had lower PGE2 levels than those fed SO after CLP. TNF-α is an important mediator involved in the onset and regulation of inflammatory and immune response. Circulating TNF-α is associated with significant pathologic change, possibly leading to mortality (DiPiro, 1997), suggesting that the TNF-α synthesis must be controlled. A study by Blok et al. (1996) showed that the plasma TNF-α concentration was significantly increased in mice fed n-3 fatty acids. A previous study performed by our laboratory also showed that compared with the diabetic mice fed SO, FO administration resulted in lower CD11a and CD11b expressions in the early stage of sepsis. An in vitro study showed that endothelial cells treated with n-3 fatty acids inhibited cytokine-induced expression of adhesion molecules (De Caterina & Libby, 1996). A study by Miles et al. (2000) showed that dietary FO reduced CAM expression by murine peritoneal macrophages. The findings of the present study suggest that leukocyte adhesion and migration may be attenuated when FO is administered in a diabetes mellitus–sepsis condition.

ICAM-1 is a cell surface protein expressed on the vascular endothelium. ICAM-1 and its ligands CD11a and CD11b are important mediators of host defence localized in the earliest lesions of inflammation (Weber, 2003). In the present study, we observed that plasma ICAM-1 levels increased as sepsis progressed, however, the changes in plasma ICAM-1 were inconsistent with the alteration in the integrin expressed on leukocytes. Since ICAM-1 plays an important role in trans-endothelial migration of leukocytes, we speculate that blood leukocytes have transmigrated into the tissue especially at the late stage of sepsis so that only limited amounts of leukocytes can be measured in the blood.

MPO is an enzyme synthesized by neutrophil and monocyte precursor cells. MPO plays an important role in leukocyte-mediated vascular injury responses in inflammatory vascular diseases (Klebanoff & Seymour, 2005). Previous studies showed that MPO activities increased in vessels of diabetic rats (Zhang et al. 2004) and in type 2 diabetic patients (Moldoveanu et al. 2006). Also, a study performed by our laboratory found that MPO activities increased in the lungs,

Table 3. Myeloperoxidase activities (U/g tissue) in organ homogenates of the fish oil group (FO) and the soyabean oil group (SO)‡

<table>
<thead>
<tr>
<th></th>
<th>Liver</th>
<th>Kidneys</th>
<th>Intestines</th>
<th>Lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>0h FO</td>
<td>2.62*</td>
<td>0.98</td>
<td>6.30†</td>
<td>1.49</td>
</tr>
<tr>
<td>0h SO</td>
<td>4.75</td>
<td>0.95</td>
<td>9.01†</td>
<td>1.69</td>
</tr>
<tr>
<td>6h FO</td>
<td>4.05†</td>
<td>1.26</td>
<td>9.30*</td>
<td>2.04</td>
</tr>
<tr>
<td>6h SO</td>
<td>4.96</td>
<td>1.44</td>
<td>12.5</td>
<td>1.85</td>
</tr>
<tr>
<td>24h FO</td>
<td>2.31</td>
<td>0.75</td>
<td>13.4</td>
<td>3.99</td>
</tr>
<tr>
<td>24h SO</td>
<td>2.75†</td>
<td>0.62</td>
<td>12.4</td>
<td>2.69</td>
</tr>
</tbody>
</table>

Mean values were significantly different from those of the SO group at the same time-point: *P<0.05.
Mean values were significantly different from those of the other time-points in the same groups: †P<0.05; ††P<0.001.
‡ For details of procedures, see Materials and methods section.

CD11a/CD18 are exclusively expressed on leukocytes and CD11b/CD18 are abundant in PMN (Henderson et al. 2001). We analysed lymphocyte CD11a/CD18 expression in the present study, because the function of T lymphocyte subsets is important on influencing the type of immunity and the inflammatory response to diabetes mellitus–sepsis (DiPiro, 1997). CD11a is important for lymphocyte trafficking and activation. Although lymphocytes constitute a relatively small population of the total lymphocyte pool in normal conditions (Westermann & Pabst, 1990), the total numbers of lymphocyte subsets in blood were greatly increased under inflammatory conditions. In the present study we found that compared with the diabetic mice fed SO, FO administration resulted in lower CD11a and CD11b expressions in the early stage of sepsis. An in vitro study showed that endothelial cells treated with n-3 fatty acids inhibited cytokine-induced expression of adhesion molecules (De Caterina & Libby, 1996). A study by Miles et al. (2000) showed that dietary FO reduced CAM expression by murine peritoneal macrophages. The findings of the present study suggest that leukocyte adhesion and migration may be attenuated when FO is administered in a diabetes mellitus–sepsis condition.
liver, intestines and kidneys at an early stage of sepsis (Hsu et al. 2006). In the present study, we found that FO administration resulted in lower MPO activities in the liver, kidneys and lungs at 0, 6 or 24 h after sepsis in diabetic mice. The present finding may indicate that diabetic mice with FO administration have less neutrophil infiltration in these organs after sepsis. A previous study showed that proinflammatory cytokines upregulate CAM expression (Myers et al. 1992). It is possible that FO decreased proinflammatory cytokine production and thus decreased CAM expression and neutrophil migration.

In summary, the present study demonstrated that diabetic mice with low-dose n-3 fatty acid supplementation resulted in lower PGE2 and TNF-α levels after CLP. Also, leucocyte CD11a and CD11b expressions and MPO activities in various organs were decreased at different time-points after sepsis when FO was administered. The present findings suggest that dietary FO supplementation may attenuate leucocyte adhesion and infiltration into tissues, thus producing a favourable effect in diabetic mice complicated with sepsis.

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References


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