Diabetes causes marked changes in function and metabolism of rat neutrophils

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Abstract
Several studies have shown impairment of neutrophil function, a disorder that contributes to the high incidence of infections in diabetes. Since glucose and glutamine play a key role in neutrophil function, we investigated their metabolism in neutrophils obtained from the peritoneal cavity of streptozotocin-induced diabetic rats. The activities of hexokinase, glucose-6-phosphate dehydrogenase (G6PDH), phosphofructokinase (PFK), citrate synthase, phosphate–dependent glutaminase, NAD+-linked and NADP+-linked isocitrate dehydrogenase were assayed. Glucose, glutamine, lactate, glutamate and aspartate, and the decarboxylation of [U-14C], [1-14C] and [6-14C]glucose; [U-14C]palmitic acid; and [U-14C]glutamine were measured in 1-h incubated neutrophils. Phagocytosis capacity and hydrogen peroxide (H2O2) production were also determined. All measurements were carried out in neutrophils from control, diabetic and insulin-treated (2–4IU/day) diabetic rats. Phagocytosis and phorbol myristate acetate (PMA)-stimulated H2O2 production were decreased in neutrophils from diabetic rats. The activities of G6PDH and glutaminase were decreased, whereas that of PFK was raised by the diabetic state. The activities of the remaining enzymes were not changed. Diabetes decreased the decarboxylation of [1-14C]glucose and [U-14C]glutamine; however, [6-14C]glucose and [U-14C]palmitic acid decarboxylation was increased. These observations indicate that changes in metabolism may play an important role in the impaired neutrophil function observed in diabetes. The treatment with insulin abolished the changes induced by the diabetic state even with no marked change in glycemia. Therefore, insulin may have a direct effect on neutrophil metabolism and function.


Introduction
Patients with diabetes mellitus have increased susceptibility to and severity of infections. Several studies have shown alterations in neutrophil function, an effect that contributes to the high incidence of infections in diabetic patients. The observed changes in neutrophils include impairment of the following: adhesion to endothelium and migration to the site of inflammation (Pereira et al. 1987), chemotaxis (Mowat & Baum 1971), bactericidal activity (Tan et al. 1974), phagocytosis (Nolan et al. 1978) and production of reactive oxygen species (ROS) (Sagone et al. 1983).

Neutrophil functions require energy (Mowat & Baum 1971, McMurray et al. 1990, Walrand et al. 2004), which is produced mainly by the metabolism of glucose to lactate (Beck & Valentine 1952, Borregaard & Herlin 1982). Only 2–3% of glucose is oxidized through the Krebs cycle in neutrophils (Beck 1958, Wood et al. 1963). These cells also utilize glutamine at high rates, which is mainly converted to glutamate, aspartate, lactate and CO2 (Pithon–Curi et al. 1997).

High levels of glucose and ketone bodies seem to influence neutrophil function through production of polyols (Wilson et al. 1986). Decreased rates of glycolysis and glycogen synthesis were observed in leukocytes of diabetic patients. These changes are abolished by in vivo insulin administration (Esmann 1983). Walrand et al. (2004) postulated that insulin may normalize neutrophil functions, not only by re-establishing the control of the intermediary metabolism, but also through a direct effect of the hormone on the cells. These authors postulated that changes in neutrophil metabolism might play a key role in the impaired function of these cells in diabetes.

In the present study, the metabolism of glucose and glutamine in neutrophils obtained from streptozotocin (STZ)-induced diabetic rats was investigated. Key enzyme activities of glycolysis (hexokinase and...
phosphofructokinase), the pentose-phosphate pathway (glucose-6-phosphate dehydrogenase), the Krebs cycle (citrate synthase) and glutaminolysis (phosphate-dependent glutaminase) were determined. In addition, the activities of NAD-linked and NADP-linked isocitrate dehydrogenase were also determined. Decarboxylation of [U-14C], [1-14C], and [6-14C]glucose; [U-14C]palmitic acid; and [U-14C]glutamine was measured in incubated neutrophils. Glucose and glutamine utilization, and production of lactate, glutamate and aspartate by incubated neutrophils were determined. Phagocytosis and production of hydrogen peroxide were also evaluated. The measurements were carried out in neutrophils from control, diabetic and insulin-treated diabetic rats.

Materials and Methods

Animals

Male Wistar rats weighing 200 ± 20 g (about 2 months of age) were obtained from the Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo. The rats were maintained at 23 ± 2 °C under a cycle of 12-h light:12-h darkness, and were allowed free access to food and water. The animal ethical committee of the Institute of Biomedical Sciences approved the experimental procedure of this study.

Induction of diabetes

The experimental type 1 diabetes was induced by intravenous injection of 65 mg/kg STZ dissolved in citrate buffer (pH 4.2). Control rats were injected with buffer only. At 48 h after STZ injection, the diabetic state was confirmed by blood glucose levels above 200 mg/dl estimated with the aid of a glucose meter (Roche). Blood samples were obtained from the cut tip of the animal’s tail. One group of diabetic rats was treated with neutral protamine Hagedorn (NPH) insulin. The daily dose was 2–4 IU by unique s.c. injection at 0900–1000 h for 2 weeks. The NPH insulin treatment was not sufficient to restore the glucose blood levels to control values.

Experimental procedure

After 2 weeks of diabetes induction, fed rats were killed by decapitation without anesthesia at 1100–1200 h. Neutrophils were obtained by intraperitoneal lavage with 30 ml PBS, 4 h after the intraperitoneal injection of 10 ml 1% (w/v) glycogen solution (Sigma type II, from oyster) in PBS. The cell suspension was centrifuged at 4 °C (500 g for 10 min). The number of viable cells (>95% neutrophils) was determined in a Neubauer chamber under an optical microscope by Trypan blue exclusion (Pires de Melo et al. 1998, Pithon-Curi et al. 2002).

Phagocytosis

Neutrophils (1 × 10⁶) were incubated for 40 min at 37 °C in 1 ml RPMI 1640 medium with opsonized particles of zymosan. The particles (1 × 10⁷) were opsonized by incubation in the presence of control rat serum for 30 min at 37 °C. The cells that were capable of phagocytizing three or more particles were determined in a Neubauer chamber under an optical microscope by the violet crystal coloring method. The percentage of phagocytosis was expressed by the number of cells that had three or more particles of zymosan per total number of cells counted.

Hydrogen peroxide production

Hydrogen peroxide (H₂O₂) production was measured by the method of Pick and Mize (1981), which is based on horseradish peroxidase-dependent conversion of phenol red by H₂O₂ to a colored compound. Briefly, the cells were incubated in the presence of 5 mM glucose and a solution of phenol red (0.5%) and horseradish peroxidase (5 mg/ml) (222 units/mg) at 37 °C for 1 h. The production of H₂O₂ was measured at rest and after stimulation with phorbol myristate acetate (PMA) (20 nM). The reaction was terminated by addition of 10 µl 1 M NaOH solution, and the amount of product formed was measured by spectrophotometry at 620 nm.

Assay of the enzyme activities

The activities of hexokinase (E.C. 2.7.1.1), glucose-6-phosphate dehydrogenase (G6PDH) (E.C. 1.1.1.49), phosphofructokinase (PFK) (E.C. 2.7.1.11), citrate synthase (E.C. 4.1.3.7), phosphate-dependent glutaminase (E.C. 3.5.1.2), NAD⁺-linked isocitrate dehydrogenase (E.C. 1.1.1.41) and NADP⁺-linked isocitrate dehydrogenase (E.C. 1.1.1.42) were determined as previously described (Mansour 1963, Crabtree & Newsholme 1972, Curthoys & Lowry 1973, Bergmeyer & Bernt 1974, Sugden & Newsholme 1975, Alp et al. 1976). Enzyme activities were expressed as nmol of substrate utilized/min per mg protein. A similar procedure was used in our previous studies (Guimerais et al. 1993, Costa-Rosa et al. 1996, Otton et al. 2002).

Cell incubation for measurement of consumption and production of metabolites

Neutrophils (1·0 × 10⁶) were incubated for 1 h at 37 °C in PBS with glucose (5 mM) or glutamine (2 mM). After incubation, the cells were disrupted by the addition of 0·2 ml 25% (w/v) trichloroacetic acid solution. Protein was removed by centrifugation, and the supernatant fluid
was neutralized with a Tris (hydroxymethyl-amino-methane)/KOH (0.5–2.0 M) solution for the measurement of the metabolites.

Assays of the metabolites

Samples of the incubation medium were used for measurements of glucose (Berham & Trinder 1972), glutamine (Windmueller & Spaeth 1974), lactate (Engel & Jones 1978), glutamate (Bernt & Bergmeyer 1974) and aspartate (Bergmeyer et al. 1974).

Spectrophotometric conditions of the enzyme assays and metabolite measurements

Activities of hexokinase, G6PDH, citrate synthase and PFK were assayed at 25 °C and activity of glutaminase at 37 °C. Citrate synthase activity was assayed by following the rate of change in absorbance at 412 nm and the remaining enzymes at 340 nm. The final volume of the assay mixtures in all cases was 1.0 ml. The production of NADH or NADPH was monitored in a Pharmacia Biotech spectrophotometer (model: Ultrospec 3000).

Incubation procedure for determination of metabolite decarboxylation

Neutrophils (1 × 10⁷) were incubated for 60 min at 37 °C in 1 ml PBS with the following labeled metabolites: [U-14C], [1-14C] or [6-14C]glucose (5.0 mM and 0.2 µCi/ml); [U-14C]palmitic acid (0.1 mM and 0.2 µCi/ml); or [U-14C]glutamine (2.0 mM and 0.2 µCi/ml). Palmitic acid was previously dissolved in ethanol (20 mM) for addition to incubation medium. The incubation was stopped by adding 0.2 ml chloridric acid. 14CO2 produced was neutralized with a Tris (hydroxymethyl-amino-methane)/KOH (0.5–2.0 M) solution for the measurement of the metabolites.

Protein determination

The total protein content of neutrophils was measured by the method of Bradford (1976), using BSA as standard.

Statistical analysis

All results are expressed as means ± s.e.m. Student’s t-test or ANOVA followed by the Tukey–Kramer test was used to assess the significance of differences between groups. Data were considered as statistically significant at P ≤ 0.05.

Results

Relative to controls, animals rendered diabetic by STZ injection exhibited a significant reduction in body weight gain during the experimental period (Table 1). Blood glucose levels were significantly elevated in comparison to controls (by 3.5-fold). The treatment of diabetic rats with NPH insulin (2–4 IU/day) did not restore the blood glucose levels to the values of the control group (Table 1).

Neutrophil counts were performed 4 h after the intra-peritoneal injection of 10 ml 1% (w/v) glycogen solution. In all groups, a similar number of cells had migrated into the peritoneal cavity. The mean values (×10⁷; ± s.e.m.) were as follows: 23.3 ± 2.7 (control, n=11), 21.1 ± 2.6 (diabetic, n=6) and 23.4 ± 2.5 (insulin treated, n=7).

Phagocytosis and PMA-stimulated hydrogen peroxide production by neutrophils from the diabetic group were decreased (by 17% and 31%, respectively) as compared with controls. The daily treatment with insulin restored phagocytosis and H2O2 production to the values found in neutrophils from control rats (Fig. 1A and B).

G6PDH activity was decreased (by 59%) in neutrophils from diabetic rats as compared with controls. The diabetic state also decreased phosphate-dependent glutaminase activity (by 22%) and increased PFK activity (by 32%) (Fig. 2). The treatment with insulin abolished the effect of diabetes on G6PDH, glutaminase and PFK activities. Hexokinase, citrate synthase and NADP+-linked and NADP+-linked isocitrate dehydrogenase activities were not changed by diabetes (Fig. 2).

Glucose and glutamine utilization by incubated neutrophils was not markedly affected by the diabetic state. Lactate production was decreased (by 24%) in neutrophils from diabetic rats as compared with controls, but the production of glutamate and aspartate was not.

Table 1 Body weight gain and blood glucose levels of the control, diabetic and insulin-treated diabetic groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight gain (g)</th>
<th>Blood glucose levels (mg/dL)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>52 ± 5</td>
<td>81 ± 4</td>
</tr>
<tr>
<td>Diabetic</td>
<td>25 ± 4</td>
<td>365 ± 25</td>
</tr>
<tr>
<td>Insulin-treated diabetic</td>
<td>44 ± 2</td>
<td>301 ± 13</td>
</tr>
</tbody>
</table>

Rats were rendered diabetic by streptozotocin injection (65 mg/kg, i.v.) and the measurements were carried out 2 weeks afterward. NPH insulin (2–4 IU/day) was subcutaneously administered during the experiment period. Values are presented as means ± s.e.m. n indicates the number of rats used in each group. *P<0.001 as compared to diabetic and insulin-treated rats; †P<0.05 as compared to insulin-treated rats.
modified. The treatment of diabetic rats with insulin restored the production of lactate by neutrophils to the values of the control group (Table 2).

Diabetes decreased the decarboxylation of [1-14C]glucose (by 31%) and [U-14C]glutamine (by 22%) by 1-h incubated neutrophils. On the other hand, [6-14C]glucose (by 55%) and [U-14C]palmitic acid (by 83%) decarboxylation was higher in neutrophils obtained from diabetic rats. The treatment with insulin abolished the changes induced by diabetes (Fig. 3). Similar results for palmitic acid decarboxylation (data not shown) were obtained by measuring the decrease of [U-14C]palmitic acid in the medium and cell preparation; there was an increase of 61% (P<0.01) in neutrophils obtained from diabetic rats, and the treatment with insulin abolished the changes induced by the diabetic state. The decarboxylation of [U-14C]glucose was not affected by the diabetic state; glucose decarboxylation by neutrophils of the control and diabetic groups was 14.9 ± 0.6 and 14.0 ± 1.1 nmol/h per 10⁷ cells (mean ± S.E.M. of two determinations from six animals in each group) respectively.

Discussion

Neutrophils constitute the first line of defense against bacterial and fungal infections. Phagocytosis stimulates the respiratory burst of neutrophils as a result of activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Bellavite 1988). The superoxide anion (O₂⁻) is the first metabolate generated through reduction of molecular oxygen by NADPH oxidase (Tauber et al. 1985, Bellavite 1988). The O₂⁻ formed dismutates spontaneously or via superoxide dismutase to H₂O₂, which is converted to hypochlorous acid by myeloperoxidase released from neutrophil granules (Tauber et al. 1985).

Conflicting results have been reported about the production of H₂O₂ by neutrophils in diabetes. In unstimulated neutrophils from diabetic patients, no significant effect on H₂O₂ production was found (Noritake et al. 1992, Inoue et al. 1996), while Zozulinska et al. (1996) found it to be increased. After incubation with PMA, neutrophils from diabetic patients have shown low (Noritake et al. 1992, Inoue et al. 1996) or unchanged (Zozulinska et al. 1996) production of H₂O₂ as compared with cells from healthy subjects. Neutrophils from diabetic patients with nephropathy have shown increased production of H₂O₂ as compared with those without kidney complications (Watanabe 1992). In our study, neutrophils from diabetic rats showed low production of H₂O₂ after PMA stimulation was abolished by treatment with insulin. There was no other study on H₂O₂ production by rat neutrophils in diabetes. An increase in H₂O₂ production by nonactivated neutrophils in the insulin-treated group was observed. Some studies have shown that insulin activates a plasma membrane enzyme system that presents properties of a Nox (NADPH oxidase enzymes). The enzyme system catalyzes the reduction of oxygen to superoxide (2 O₂⁻ +NADP⁺+H⁺), which is spontaneously or by action of superoxide dismutase converted to H₂O₂ (Mukherjee et al. 1978, Rhee et al. 2003, Goldstein et al. 2005).

Impairment of phagocytosis and decreased release of lysosomal enzymes in neutrophils from diabetic patients have been observed by many researchers (Bybee & Rogers 1964, Bagdade et al. 1972, Sagone et al. 1983, Wilson & Reeves 1986). Studies with diabetic rats and mice also showed a decreased neutrophil phagocytosis capacity (Canturk et al. 1998, Panneerselvam & Govindasamy 2003). The lowering of blood glucose levels by insulin treatment has been reported to have significant correlation with the improvement of phagocytosis capacity by neutrophils (Jakelic et al. 1995, Delamaire et al. 1997).
In our study, the decreased capacity of neutrophils from diabetic rats to phagocytize zimosan particles was reversed by insulin treatment even under high blood glucose levels.

Previous studies on the effect of diabetes on glucose metabolism in neutrophils are controversial. Esmann (1972) and Munroe & Shipp (1965) did not observe differences in glucose utilization by neutrophils from healthy and diabetic patients. Nevertheless, decreased utilization of glucose by neutrophils from diabetic patients was found by others (Martin et al. 1954, Esmann 1983).

Figure 2 Enzyme activities of neutrophils. The results are expressed as nmol/min per mg protein, and the values are presented as means ± S.E.M. of 10 determinations from five animals in each group. *P<0·01 as compared to control, bP<0·05 as compared to diabetic rats, cP<0·05 as compared to control and dP<0·01 as compared to diabetic rats.
Munroe & Shipp (1965) did not observe alteration, whereas Martin et al. (1954) and Esmann (1983) found decreased production of lactate by neutrophils from diabetic patients. In the present study, there was no alteration in glucose utilization by neutrophils from diabetic rats, whereas lactate production was decreased. These findings support the proposition that the diabetic state reduces glycolysis activity in neutrophils.

Neutrophils from diabetic rats had no significant change in glucose oxidation, citrate synthase, and NAD+-linked and NADP+-linked isocitrate dehydrogenase activities, suggesting that the flux of substrates through the Krebs cycle was not altered. Decreased citrate synthase activity was observed in lymphocytes from diabetic rats (Otton et al. 2002), whereas no significant change was found in macrophages (Costa-Rosa et al. 1996). There was no study on glucose oxidation, or NAD+-linked and NADP+-linked isocitrate dehydrogenase activities by rat neutrophils in diabetes.

The pentose-phosphate pathway oxidizes glucose-6-phosphate to intermediates of the glycolytic pathway, generating NADPH and ribose-5-phosphate for fatty acid and nucleotide synthesis respectively (Casazza & Veech 1986). NADPH is important for NADPH oxidase activity and for glutathione reductase to recycle oxidized glutathione in neutrophils (Pithon-Curi et al. 1998, 1999).

Table 2 Consumption and production of metabolites by 1-h incubated neutrophils from the control, diabetic and insulin-treated diabetic groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Glucose consumption</th>
<th>Lactate production</th>
<th>Glutamine consumption</th>
<th>Glutamate</th>
<th>Production of aspartate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>855 ± 170</td>
<td>945 ± 65</td>
<td>1110 ± 95</td>
<td>390 ± 5</td>
<td>515 ± 50</td>
<td>215 ± 5</td>
</tr>
<tr>
<td>Diabetic</td>
<td>5</td>
<td>755 ± 150</td>
<td>700 ± 65+</td>
<td>1015 ± 70</td>
<td>380 ± 10</td>
<td>555 ± 65</td>
<td>230 ± 40</td>
</tr>
<tr>
<td>Insulin-treated diabetic</td>
<td>6</td>
<td>815 ± 150</td>
<td>935 ± 35</td>
<td>1115 ± 85</td>
<td>390 ± 5</td>
<td>510 ± 60</td>
<td>230 ± 35</td>
</tr>
</tbody>
</table>

Consumption of glucose and glutamine and production of lactate, glutamate and aspartate were determined in 1 h incubated neutrophils. The cells were obtained from the peritoneal cavity of the rats, and the results are expressed as nmol/h per mg protein. The values are presented as means ± S.E.M. n represents the number of rats per group. *P>0·05 as compared to control and insulin-treated diabetic rats.

Figure 3 Decarboxylation of labeled metabolites by 1 h incubated neutrophils. The values are presented as means ± S.E.M. of eight determinations from four animals in each group. *P<0·01 as compared to control, *P<0·05 as compared to diabetic rats and *P<0·05 as compared to control and insulin treated diabetic rats.
Similarly, glutamine is also very important in neutrophils to provide glutamate for glutathione synthesis (Newsholme et al. 2003).

To examine the flux of glucose through the pentose-phosphate pathway of neutrophils from diabetic rats, G6PDH activity and decarboxylation of [1-14C] and [6-14C]glucose were determined. The difference between 14CO2 production from [1-14C] and [6-14C]glucose estimates the flux of glucose through the pentose-phosphate pathway (Larabee 1989). The difference for neutrophils (107; ± S.E.M.) from the diabetic group was 2.35 ± 0.2 nmol/h compared with 3.79 ± 0.15 nmol/h for the control group (difference of 38%; P<0.001). Insulin treatment abolished the difference between the decarboxylation of [1-14C] and [6-14C]glucose. The activity of G6PDH was decreased in neutrophils from diabetic rats, as was also found in macrophages and lymphocytes (Costa-Rosa et al. 1996, Otton et al. 2002). These findings support the proposition that the activity of the pentose-phosphate pathway is decreased in leukocytes from diabetic rats. Leukocytes with deficiency of G6PDH activity present impaired phagocytosis, bactericidal capacity and superoxide production (Gray et al. 1973, Roos et al. 1999). Moreover, decreased flux of glucose through the pentose-phosphate pathway is expected to reduce the production of NADPH and ribose-5-phosphate. Therefore, reduced pentose-phosphate pathway activity may be related to impaired neutrophil function in the diabetic state. Although the production of NADP-linked isocitrate dehydrogenase activity was not affected by the diabetic state, this may not be sufficient to compensate for the reduced flux of substrates in the pentose-phosphate pathway.

Although decreased lactate production was observed in neutrophils from diabetic rats, the PFK maximal activity was increased. Similar results were observed in mesenteric lymph nodes and thymus lymphocytes from diabetic rats (Moreno-Aurioles et al. 1996, Otton et al. 2002). The activity of PFK is stimulated by fructose 2,6-bisphosphate (Wegener & Krause 2002) and inhibited by ATP at low fructose 6-phosphate content, but not at high fructose 6-phosphate concentration (Mansour 1963). In this study, we did not observe changes in the glucose consumption and oxidation in neutrophils from STZ-induced diabetic rats, but lactate production and pentose-phosphate pathway activity were markedly reduced. Therefore, it is expected that the content of intermediates of glycolysis, such as fructose 6-phosphate and fructose 2,6-bisphosphate, are elevated in this condition. This may partially explain the increase in the PFK activity. In accordance with this proposition, Moreno-Aurioles et al. (1996) found increased fructose 2,6-bisphosphate content and PFK activity in neutrophils from STZ-induced diabetic rat.

The production of glutamate and aspartate was not altered in neutrophils from diabetic rats incubated in the presence of 2 mM glutamine. Nevertheless, glutamine oxidation and glutaminase activity were significantly decreased in neutrophils from diabetic rats. Glutamine plays an important role in protein (as amino-acid source), lipid (through NAD(P)H production) and nucleotide synthesis (through purine and pyrimidine production), and in NADPH oxidase activity (Newsholme et al. 2003, Curi et al. 2005). Glutamine raises the in vitro bacterial killing activity and the rate of ROS production by neutrophils (Ogle et al. 1994, Pithon-Curi et al. 1998, 2002). Pithon-Curi et al. (2003) showed that glutamine has a protective effect on neutrophil apoptosis. Therefore, decreased glutamine utilization may contribute to the impaired function by increasing the occurrence of apoptosis in neutrophils from diabetic rats. This issue remains to be investigated.

Diabetes leads to an increase in plasma levels of free fatty acids and triglycerides (Boden 1999, Kelley & Mandarino 2000). We have previously demonstrated increased fatty acid oxidation in lymphocytes from diabetic rats (Otton et al. 2002). Herein, we observed that palmitic acid decarboxylation is also elevated in neutrophils from diabetic rats.

In summary, neutrophils from diabetic rats present impaired metabolism of glucose and glutamine. On the other hand, palmitic acid oxidation is increased, and this may compensate for the reduction in glucose and glutamine utilization for ATP production. These metabolic changes may be related to impaired functions of these cells, such as phagocytosis and hydrogen peroxide production. The changes in metabolism and function of neutrophils from diabetic rats are fully abolished by insulin treatment, even with no marked changes in glycemia. These findings show that insulin may have a direct effect on neutrophil metabolism and function.

Acknowledgements

The authors are indebted to the constant assistance of E P Portioli and G de Souza. This research is supported by FAPESP, CNPq and CAPES. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 17 October 2005
Accepted 10 November 2005