Background: Autogenic splenic implant (ASI) is one of the few alternatives for preservation of splenic tissue when total splenectomy is inevitable. The aim of this study was to determine the morphological and functional regeneration of ASIs, as indicated by the clearance of Howell–Jolly (HJ) bodies, in an experimental model.

Methods: Ninety-nine male Wistar rats were divided into three groups: sham-operated (group 1), total splenectomy alone (group 2), and total splenectomy combined with ASI (group 3). Animals in group 3 were further allocated to nine subgroups of nine rats each, and analysed at different time points (1, 4, 8, 12, 16, 20, 24, 28 and 32 weeks after surgery). Blood smears were prepared at predetermined times for detection of HJ bodies. Morphological regeneration of tissue in the ASI was analysed by histology.

Results: At 1 week, the regenerated mass corresponded to about 7 per cent of the tissue implanted, reaching approximately 54 per cent at 24 weeks. The HJ body levels were increased in groups 2 and 3 until 8 weeks after surgery, following which levels in the ASI group became similar to those in the sham-operated group. HJ bodies were difficult to detect when a level of 22·5 per cent of regenerated ASI mass was reached.

Conclusion: Functional regeneration of ASIs occurred from 8 weeks after surgery. When 22·5 per cent of regenerated ASI mass was reached almost no HJ bodies could be observed in the bloodstream, resembling a spleen in situ.

Surgical relevance
Splenectomy has been practised routinely, both in the emergency setting and as a therapeutic elective procedure. There is a correlation between asplenia/hyposplenia and the occurrence of fulminant sepsis, underlining the importance of developing surgical methods for preserving splenic function.

Both clinical and experimental studies have shown at least partial morphological and functional regeneration of autogenic splenic implants (ASIs). Experimental studies investigating the immunoprotective effect of ASIs, based mostly on exposure of animals to various bacteria, have demonstrated that ASIs can increase the rate of bacterial clearance and decrease mortality from sepsis. Clinical studies have shown their ability to remove colloidal substances and altered erythrocyte corpuscular inclusions, such as Howell–Jolly, Heinz and Pappenheimer bodies, from the bloodstream. In this experimental study the functional and morphological regeneration of ASIs was studied over time in rats.

Introduction
Total splenectomy has been practised routinely, both in the emergency setting, such as abdominal trauma, as well as in therapeutic or diagnostic elective procedures for splenic disorders caused by haematological, oncological, metabolic and immune disorders, and in portal hypertension. For a long time it was believed that removal of the spleen did not have any harmful consequences for the patient. It was only from the mid-20th century, with the recognition of overwhelming postsplenectomy infection, that the spleen was recognized to have multiple functions, notably those
related to the immune system and blood filtering. With increasing knowledge of these important splenic functions, alternative treatments were developed aimed at preservation of the spleen after trauma and elective procedures.

The active role of the spleen in functions related to immunity and phagocytosis is highlighted by the different types of cell found temporarily or permanently in the organ, by its anatomical and histological organization, which favours opsonization, phagocytosis and destruction of foreign antigens, among others, and by the abundance of its blood supply.

Blood filtration is one of the main functions of the spleen. The organ acts as a voluminous filter, through which up to 6 per cent of the total blood volume passes per minute (about 300 ml/min), removing senescent and altered erythrocytes, and particles such as Howell–Jolly (HJ), Heinz and Pappenheimer bodies, a procedure of great importance for maintenance of the morphology and function of red blood cells.

HJ bodies are basophilic DNA remnants of the erythrocyte precursor nucleus. Typically, erythrocyte precursors expel their nucleus, but some retain a small portion of DNA, usually observed as small cytoplasmic corpuscles (approximately 1 mm in size). The filtering function of normal spleen promotes the removal of cells containing these corpuscles from the bloodstream, but when the spleen is absent or is functioning inadequately they remain in the circulation. The presence of HJ bodies in peripheral blood smears, although not pathognomonic, is considered suggestive of splenic dysfunction or asplenia. Although HJ bodies may not be present in blood in patients with slight hyposplenism, when they are present they denote a degree of hyposplenism representing risk of onset of fulminant infection.

When a total splenectomy cannot be avoided, an autogenous splenic implant (ASI) is among the only alternatives for preservation of splenic tissue and function. This procedure is based on clinicopathological findings observed in spontaneous splenic implants or splenosis, after accidental or surgical severe splenic trauma, and has been further studied in an experimental setting.

Complete regeneration of autologous spleen slices implanted in the greater omentum of 6-week-old rats has been reported. After 16 weeks, the implanted splenic tissue appears to be morphologically indistinguishable from normal spleen. However, the functional regeneration and blood filtering function can be partial or may not occur, or take place over a different time interval. In experimental studies it has been shown that HJ bodies remain indefinitely in the blood of animals submitted to total splenectomy alone, but they disappear completely over the course of weeks in animals with an ASI. Similar findings were described in an experimental rabbit model after 12 weeks. At the same time, immunoglobulin M concentrations increased to normal levels, suggestive of functional regeneration of the implants.

Morphological and functional regeneration of ASIs, assessed by the presence of the bacterial phagocytic function of macrophages in young and adult rats of both sexes, was demonstrated by 16 weeks after implantation. In addition, the critical mass of ASI needed for the recovery of phagocytic activity in young adult rats was studied. If 26–100 per cent of the total splenic mass was regenerated, there was no difference in the bacterial index in the blood of rats undergoing total splenectomy combined with ASI compared with that in sham-operated animals.

The evidence that ASIs are effective for blood clearance of HJ bodies is of major importance, as it provides an indication of functional regeneration. The objective of the present study was to determine the evolution of morphological and functional regeneration of ASIs in young rats over time, especially regarding blood clearance of HJ bodies.

**Methods**

The study was approved by the Ethics Committee on Animal Research of the Biology Institute Roberto Alcantara Gomes, Rio de Janeiro State University, Brazil (protocol number 027/2008). All procedures rigorously followed Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines for animal experimentation.

Ninety-nine 21-day-old male Wistar rats weighing 45–48 g were allocated to three groups: sham-operated (group 1), total splenectomy alone (group 2), and total splenectomy combined with ASI (group 3). Animals in group 3 were subsequently allocated to nine subgroups of nine rats each, corresponding to the time after surgery at which they were analysed: 1, 4, 8, 12, 16, 20, 24, 28 and 32 weeks. The animals were housed in appropriate cages, five to a cage at most, under conditions of controlled temperature and humidity, on a 12-h light/12-h dark cycle. Free access to water and standard laboratory chow was allowed.

**Surgical procedures**

After fasting for 12 h, the animals were anaesthetized intramuscularly with 80 mg/kg ketamine and 12 mg/kg xylazine. The entire procedure was performed under aseptic conditions. In all animals, the procedure was started with a supraumbilical midline incision, approximately 3 cm
in length. In group 1 animals (sham-operated), the spleen was just mobilized to the surgical field, and subsequently returned to its usual place. In groups 2 and 3, the splenic vessels were ligated and the spleen was removed.

In group 3, after total splenectomy the spleen was weighed and cut transversely into three segments, each about 5 mm thick. The splenic slices were implanted into the greater omentum using continuous 4/0 polyglycolic acid sutures. Stitches were introduced alternately into the omentum and the splenic slices to permit interposition of omental tissue between the slices of spleen (Fig. 1). Finally, the abdomen was closed with continuous polyglycolic acid 3/0 sutures in all animals.

Postoperative monitoring
Rats in groups 1 and 2 were followed daily for 32 weeks. For those in group 3, the duration of monitoring depended on the subgroup to which the animal was allocated. At 1, 4, 8, 12, 16, 20, 24, 28 or 32 weeks after surgery, smears were prepared from peripheral blood obtained by tail vein puncture under anaesthesia. After collection of blood, the splenic implants were removed through a median laparotomy for macroscopic and microscopic studies, and the rats were then killed by overdose with ketamine and xylazine.

Staining of blood smears and counting of Howell–Jolly bodies
Blood smears were stained by the May–Grünewald–Giemsa method and examined under light microscopy. In each smear, ten microscopic fields were used for counting HJ bodies, with analysis of 100 erythrocytes in each, giving a total of 1000 red blood cells per animal. Results are reported as the percentage of erythrocytes with HJ bodies.

Histology
Splenic implants were removed and placed in a solution containing buffered 10 per cent formalin. The tissue was processed with increasing concentrations of alcohol and xylene, embedded in paraffin, and cut into 4-µm sections on to slides. The slides were stained with haematoxylin and eosin, and assessed by light microscopy.

Statistical analysis
The non-parametric Kruskal–Wallis test was used to compare weights of animals weekly during the experiment. Means(s.d.) and median values were calculated for implanted and regenerated splenic mass, as well as the percentage of regenerated mass relative to implanted
mass, for the different ASI subgroups of group 3. The Kruskal–Wallis test was used to compare changes throughout the course of the experiment. The Wilcoxon test was used to determine whether the mean regenerated splenic mass (absolute and relative) at each measurement from week 4 onwards was significantly higher than that of the previous measurement.

Mean percentages of erythrocytes with HJ bodies in the peripheral blood were compared at each time point during the experiment using Student’s t test. The sham-operated group was compared with the total splenectomy group, and each of these groups was compared with the ASI subgroups.

The level of significance was set at $\alpha = 0.05$. Analyses were performed using SAS® version 9.2 (SAS Institute, Cary, North Carolina, USA).

**Results**

Regeneration of the ASI was observed in all animals in group 3. At 8, 16 and 24 weeks after surgery, only two splenic slices could be recovered from two animals in each subgroup. These slices lacked interposed omental tissue, leading to the impression that fusion had occurred between two of the three slices originally implanted. No such fusion was observed at other time points, and three splenic slices were recovered from all remaining animals.

The masses of the ASI were similar at surgery. A significant growth of the ASI, expressed in both absolute terms and relative to the implanted mass, was observed until the 24th week after surgery (Table 1). One week after implantation, the mass of regenerated splenic tissue corresponded to about 7 per cent of the splenic tissue originally implanted, reaching 54 per cent at 24 weeks. From week 24 onwards, there was no further significant increase in regenerated splenic mass (regenerated mass, $P = 0.000$; regenerated mass as a percentage of implanted mass, $P = 0.954$).

**Morphological analysis of regenerated splenic implants**

Macroscopically, the regenerated ASIs were smaller than the original implants, and the splenic tissue was restricted to the periphery at 1 week after surgery. The centre of the implants became a large necrotic mass. From 4 weeks onwards, larger masses of regenerated splenic tissue were recovered, without any remaining signs of central necrotic tissue (Fig. 2). From 12 weeks onwards, the recovered ASI showed an anatomical conformation resembling that of the original slices implanted and resembled a portion of normal spleen *in situ*, especially when they were fused together. On palpation, their consistency was elastic and similar to that of normal splenic tissue.

Microscopically, at 1 week after implantation, lymphoid aggregates with a follicular aspect were observed, with a large amount of coagulation necrosis in the central part of the implanted fragments. There was structural disturbance, without clear demarcation of white and red pulp. Blood vessels with numerous red blood cells and other cell types dispersed in the interstitial spaces were already evident in the periphery of the implant. At 4 weeks, the focal concentrations of lymphocytes around blood vessels increased, with some already initiating the formation of follicles without central arterioles, and with sinusoids filled with red cells between them, resembling the outline of regenerating red pulp. At 8 weeks, these follicles were further developed, featuring regenerated white pulp, with central arterioles and a red pulp of better structure.

<table>
<thead>
<tr>
<th>Time after implantation (weeks)</th>
<th>Implanted mass (g)</th>
<th>Regenerated mass (g)</th>
<th>Regenerated mass/implanted mass (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean(s.d.)  Median</td>
<td>Mean(s.d.)  Median</td>
<td>Mean(s.d.)  Median</td>
</tr>
<tr>
<td>1</td>
<td>0.21(0.02)  0.21</td>
<td>0.02(0.00)  0.02</td>
<td>7.09(0.95)  6.99</td>
</tr>
<tr>
<td>4</td>
<td>0.21(0.02)  0.21</td>
<td>0.03(0.00)*  0.03</td>
<td>11.99(1.23)*  12.07</td>
</tr>
<tr>
<td>8</td>
<td>0.21(0.02)  0.21</td>
<td>0.05(0.00)*  0.05</td>
<td>22.48(1.47)*  22.37</td>
</tr>
<tr>
<td>12</td>
<td>0.22(0.02)  0.22</td>
<td>0.07(0.01)*  0.07</td>
<td>33.97(2.10)*  33.88</td>
</tr>
<tr>
<td>16</td>
<td>0.21(0.02)  0.20</td>
<td>0.09(0.01)*  0.09</td>
<td>40.47(2.86)*  41.34</td>
</tr>
<tr>
<td>20</td>
<td>0.21(0.02)  0.21</td>
<td>0.10(0.00)*  0.10</td>
<td>45.05(3.00)*  45.00</td>
</tr>
<tr>
<td>24</td>
<td>0.21(0.02)  0.21</td>
<td>0.12(0.02)*  0.12</td>
<td>54.04(5.85)*  55.28</td>
</tr>
<tr>
<td>28</td>
<td>0.21(0.02)  0.21</td>
<td>0.12(0.02)  0.12</td>
<td>54.12(6.02)  53.56</td>
</tr>
<tr>
<td>32</td>
<td>0.21(0.02)  0.22</td>
<td>0.12(0.02)  0.12</td>
<td>54.28(7.00)  54.48</td>
</tr>
</tbody>
</table>

$P < 0.050$ versus previous measurement (Wilcoxon test); †Kruskal–Wallis test.
Fig. 2 Histological sections of autogenic splenic implants, showing a lymphocytic aggregate of follicular aspect (arrow) at 1 week; b lymphocytic infiltrate (arrow) at 4 weeks; c lymphoid follicle with central arteriole (arrow), and the outline of red and white pulp at 8 weeks; and d well defined red and white pulp (arrow and asterisk respectively) and a marginal zone (MZ) at 12 weeks (haematoxylin and eosin stain, scale bar 50 µm)

At 12 weeks, the focal concentrations of lymphocytes around blood vessels increased, although they were slightly smaller than those observed at 8 weeks. The red and white pulp, as well as the marginal zones, were now more clearly defined. Numerous macrophages containing cytoplasmic haemosiderin pigments were observed in the splenic parenchyma. Blood vessel walls were preserved without signs of vasculitis or thrombosis. From 16 weeks onwards, developed lymphoid follicles with precise delimitation of the white and red pulp, and the marginal zone were observed, with the implant resembling a neospleen (Fig. 2).

Blood clearance of Howell–Jolly bodies

The percentage of erythrocytes containing HJ bodies was similar in the total splenectomy (group 2) and ASI (group 3) groups at weeks 1 and 4, with a high frequency of HJ bodies compared with sham-operated animals. From week 8 onwards, however, these two groups began to differ significantly, with the ASI group beginning to resemble the sham-operated group (Fig. 3).

In the ASI group, analysis of the percentage of erythrocytes with HJ bodies in relation to the percentage of regenerated splenic mass showed that the HJ body level
was close to zero when 22.5 per cent of the regenerated mass (measured relative to the implanted mass) was achieved.

**Discussion**

Besides its immune function, the predominant functional activity of the spleen is blood filtration, removing senescent and abnormal erythrocytes, as well as foreign particles. Thus, it is of importance to demonstrate that an ASI is effective in blood clearance of HJ bodies, as this is an indication of the functional regeneration of the spleen.

Studies in humans with ASIs have shown restoration of some splenic functions. These include the ability to remove colloidal substances and altered erythrocyte corpuscular inclusions, such as HJ, Heinz and Pappenheimer bodies, from the bloodstream. ASIs also result in normalization of the production of antibodies against pneumococcal polysaccharides, along with levels of immunoglobulins, complement, platelets and lymphocytes. How this splenic regeneration evolves over time is not well known.

Experimental studies investigating the immunoprotective effect of ASIs have been based mostly on exposing animals to various bacterial species, then assessing the level of clearance of these microorganisms from the bloodstream, and death from bacterial sepsis. Blood filtering and the potential benefits of immunization combined with ASI have also been investigated, especially after exposure to pneumococci; this combination increased the rate of bacterial clearance and decreased mortality from sepsis. In general, the regenerative capacity of an ASI varies according to animal species and age. In young animals, ASIs seem to regenerate better, with well developed splenic compartments resembling the structure of a normal spleen, and showing better recovery of functional activity than in adults.

Although analysing blood clearance of HJ bodies in rats seems to be a suitable model for monitoring the functional regeneration of an ASI, there is some controversy about the reliability of its use as an indicator of splenic function, especially in cases of low-grade hyposplenism, because the number of cells with HJ bodies is very low (0.1–1.1 per cent of erythrocytes in splenectomized patients). However, others have reported that blood clearance of HJ bodies is a good marker of functional splenic regeneration. A small percentage of erythrocytes with HJ bodies has been found in the peripheral blood of healthy young rats (21 days after weaning). This is in line with findings of HJ bodies in neonates, especially in premature infants, suggestive of splenic dysfunction during the first weeks of life.

Previous experimental studies demonstrated morphological regeneration and phagocytic activity of macrophages in ASIs implanted on the greater omentum. It was concluded that ASIs provide protection against bacteraemia. Subsequently, a similar experimental model, with thin splenic slices sutured to the greater omentum and intercalated omental tissue, was used to allow better vascularization. In this model the critical mass needed to achieve efficacious phagocytic activity in macrophages in adult rats occurred when 26.0 per cent of the total splenic mass had regenerated.

As ASI regeneration starts in a centripetal way from the periphery to the centre of the graft, the present experimental model also had three thin splenic slices sutured to the greater omentum with omental tissue intercalated between them. Omental implants led to more complete morphological and functional regeneration than implants at other sites, showing the relevance of maintaining venous drainage into the portal vein, resembling the spleen in situ.
All implants showed morphological regeneration with the presence of red and white pulp, lymphoid follicles and a marginal zone from 12 weeks after surgery onwards. The mass of implanted spleen was the same in each subgroup, but the regenerated mass as a percentage of the implanted mass increased significantly with each experimental week. Regeneration occurred in a centripetal direction and gradually replaced the central necrotic zone, in agreement with previous findings\textsuperscript{33, 41}. One week after completion of the ASI, the regenerated mass was only 7 per cent of the implanted mass, but significant linear growth was subsequently observed, reaching 40 per cent at 16 weeks. By week 24, 54 per cent regeneration was attained, after which no further significant regeneration was found.

From week 8 of the experiment, HJ bodies in the peripheral blood of animals with an ASI decreased significantly, to reach levels comparable to those in sham-operated animals; when 22.5 per cent of regenerated mass was achieved, the proportion of erythrocytes with HJ bodies tended to approach zero. At this point, a more structured morphological regeneration was also seen. This indicates that, besides a requirement for a critical minimal structural morphological regeneration was also seen. This was achieved, the proportion of erythrocytes with HJ bodies in the peripheral blood of animals with an ASI decreased significantly, reaching 40 per cent at 16 weeks. By week 24, 54 per cent regeneration was attained, after which no further significant regeneration was found.

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