Splenic autoimplantation in omentum and stomach, hematoimmunological follow-up and B-cell repertoire in the graft

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Summary

Introduction. *Splenic autoimplantation appears to be the only alternative to preserve splenic tissue after splenectomy; however, its relevance is still controversial. We intended to study splenic autoimplantation in the greater omentum and stomach wall of rabbits and analyze its hematoimmunological performance and the preservation of original structures.* ***Methods.*** *New Zealand rabbits were divided in two groups: autoimplanted (A) (n=13) and splenectomized (S) (n=4). The animals of group A underwent autoimplantation of splenic fragments in the greater omentum and gastric wall. Both groups were evaluated by hemocytological tests, scintigraphy, immunoglobulin and C3 dosages, before the surgery and 2 and 4 months afterwards. After 4 months, the grafts were removed and histological examination and gen rearrangement of B-lymphocytes receptors by polymerase chain reaction (PCR) were performed to assess the cellular diversity of clones.* ***Results.*** *The histological analysis demonstrated the presence of splenic tissue in 10 of the 13 cases (77%) with evident size reduction. The gastric location did not develop complications and demonstrated higher morphological correspondence to the autoimplanted tissue. Both groups showed significant decrease of IgM and increase of C3, without considerable differences between both of them during follow up. From the 8 grafts studied with PCR, 3 cases presented polyclonality and 5 oligoclonality.* ***Conclusions.*** *The revascularized grafts evidenced splenic regenerating tissue, probably associated to the oligoclonality detected by PCR. Consequently, we consider that autoimplantation is a reasonable alternative for splenectomized patients, even though the stomach placement and the high frequency of oligoclonality justify further investigation.*

Key words. *Splenic autoimplantation, PCR, rabbits, stomach, oligoclonality.*

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Autoimplante de bazo en epiplón y estómago, seguimiento hematoinmunológico y repertorio de linfocitos B en el injerto

Resumen

Introducción. *El autoimplante esplénico aparece como la única alternativa para preservar tejido esplénico luego de la esplenectomía, aunque su utilidad sigue siendo discutida. Nos propusimos investigar el autoimplante esplénico en epiplón y pared gástrica de conejos, sus efectos hematoinmunológicos y el mantenimiento de su estructura original.* ***Métodos.*** *Se emplearon conejos Nueva Zelanda los cuales fueron divididos en dos grupos: autoimplante (A) (n=13) y esplenectomía (E) (n=4). En el grupo A se realizó autoimplante de fragmentos esplénicos en epiplón mayor y estómago. Ambos grupos fueron sujetos a valoración prequirúrgica y postquirúrgica a los 2 y 4 meses mediante estudios hemocitológicos, centellografía, dosaje de inmunoglobulinas y C3. Al cuarto mes los injertos fueron sometidos a estudio histológico y genético mediante el rearreglo de genes de receptores de linfocitos B por reacción en cadena de la polimerasa (PCR) para valorar la diversidad de clones de esta célula.* ***Resultados.*** *El estudio histológico*

evidenció tejido esplénico en 10 de 13 casos (77%). El autoimplante en estómago no presentó complicaciones y demostró mayor correspondencia morfológica al tejido autoimplantado. Ambos grupos presentaron descenso significativo de IgM e incremento de C3, aunque sin diferencias significativas entre ellos. De 8 injertos estudiados por PCR, se confirmó policlonalidad en 3 y oligoclonalidad en 5. ***Conclusiones.*** *Los autoimplantes se revascularizaron mostrando tejido esplénico en regeneración, probablemente asociado a la oligoclonalidad hallada por PCR. Consecuentemente consideramos que el autoimplante sería una alternativa factible en pacientes esplenectomizados, aunque la ubicación gástrica de los implantes y la elevada frecuencia de oligoclonalidad hallada justifican mayor investigación.*

Palabras claves. *Autoimplante esplénico, PCR, conejos, estómago, oligoclonalidad.*

Abbreviations.
A: autoimplanted group.
S: splenectomized group.
PCR: polymerase chain reaction.
IgM: immunoglobulin M.
OPSI: overwhelming postsplenectomy infection.
NIH: National Institutes of Health.
MCV: mean corpuscular volume.
mCi: millicurie.
SD: standard deviation.
SPECT: single photon emission computed tomography.
bp: base pairs.

For centuries, the spleen was thought to be a rescindable organ for the normal human life. This idea has determined the resectionist approach implemented during decades.[1-4] At the beginning of the 20th century, Morris and Bullock [5] suggested the importance of the spleen for the immune system. This was the first evidence of the immune function of this organ and several researchers have described the negative effects of splenectomy since then.[1,6-9]

Currently, the overwhelming postsplenectomy infection (OPSI) has been recognized as the most severe consequence of the asplenia. This syndrome is characterized by a short disease period with high mortality rates (50% to 70%),[1,10,11] that can develop any time after spleen removal and implies a lifelong risk of approximately 5%.[10,11] Even though the incidence of OPSI is important in adults [1,11,12] the risk of sepsis is higher in children, where it reaches a 13.8% rate.[6,13,14] These patients are considered immunocompromised hosts and strategies for the appropriate management of these cases have been proposed.[15,16] However, current guidelines are scarce and not strictly or consistently followed.[9,16,17]

In order to avoid the risk of OPSI, the indications of splenectomy have been reduced in patients of any age.[2,4,17,18] In severe splenic lesions or diseases where splenic rescue is impossible, the autotransplant has been proposed as the only alternative to preserve the spleen functions.[3,4,17,19] Some studies have demonstrated that this procedure results in the return to almost baseline levels of some hematological and immunological parameters.[3,4,19-21] On the other hand, the possibility that the spleen autoimplantation fulfills protective functions against infections is still under discussion [2,4,22] since the single presence of spleen tissue cannot guarantee complete immunoprotection.

In this study, we intended to investigate the experimental results of spleen autoimplantation in young rabbits, analyze its hematological and immunological effects and evaluate graft viability, histological structure, vascularization and immunological repertoire of B-lymphocytes in the tissue.

Material and methods

Animals

Seventeen New Zealand male rabbits of 4-6 months of age (average body weight: 2.5 kg) were used for this study. They were randomly selected and separated in 2 groups: group A (n= 13), underwent autoimplantation and group S (n= 4) splenectomy. All procedures were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (NIH, publication Nº 85-23, revised 1996) and protocols approved by the Catholic University of Córdoba Ethics Committee. The animals received ordinary care, standard feeding ad libitum and had free access to water.

Anesthesia and surgical procedure

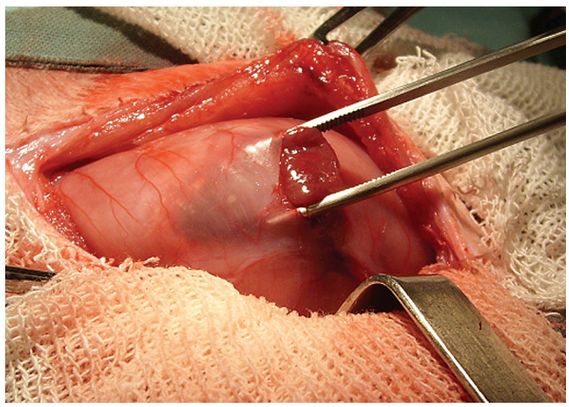
Anesthetic induction was performed with intramuscular Ketamine hydrochloride (30 mg/kg, *Fada Pharma*, Buenos Aires, Argentina) and Xylazine (5 mg/kg, *Lab. Koning*, Buenos Aires, Argentina) and maintained with intravenous ketamin hydrochloride.

The animals were placed in supine position and under sterile conditions; the surgical approach of

the spleen was performed by left subcostal laparotomy. In Group A, we performed spleen exeresis and autoimplantation of 60% of the total organ volume. The sample was divided into 6 equal segments of 5 x 5 x 8 mm, which were placed in a pouch performed between the 2 layers of the greater omentum. In 4 animals, a remnant segment of variable size was simultaneously implanted in a pouch of the anterior wall of the stomach, performed between the muscular wall and visceral peritoneum (Figure 1). These pouches were closed with 6-0 polypropylene monofilament suture (*Prolene*, *Ethicon Inc.*, Sommerville, NJ, USA), the abdominal cavity was subsequently washed with sterile solution and the abdominal wall closed by layers.

The rabbits of group S underwent radical splenectomy. All the animals received a pre-surgical intramuscular dose of Cephalotin (200 mg/kg, *Fada Pharma*, Buenos Aires, Argentina).

Figure 1. *Intraoperatory photo of the confection of an implant in the gastric wall, close to the gastro omental vascular beds.*



Laboratory monitoring and hemocatheretic activity

Blood samples were collected from rabbit's ear veins to perform pre and post-surgical routine laboratory tests at 8 and 16 weeks in both groups. Hematological exams consisted of red blood cell count, white blood cell count, hemoglobin and mean corpuscular volume (MCV). Immunological exams were dosage of serum immunoglobulin M (IgM), immunoglobulin G (IgG) and C3 fraction of the complement by immuno-turbidimetry with a Hitachi 917 analyzer (*Roche Diagnostics*, Buenos Aires, Argentina).The depurative function of the spleen was evaluated by detection of Howel Jolly bodies and abnormal red blood cells in peripheral blood smears with May Grunwald-Giemsa staining.

Scintigraphy

Pre-surgical scans and follow-up controls at weeks 8 and 16 after surgery were performed. Under sedation and anesthesia, an intravenous dose of 2.6 mCi Tc-99m phytate colloid solution was injected for hepatosplenic marking. Abdominal static views and dynamic reconstruction by single photon emission computer tomography (SPECT) were registered using a double-headed Picker Axis Camera gamma equipment (*Picker Axis*, *Marconi Medical Systems*, Cleveland, OH, USA).

Re-intervention and graft study

Four months after surgery, rabbits from Group A were re-operated and the grafts were extracted for macroscopic, histological and molecular evaluation.

Histological analysis

Samples were fixed in a 10% formaldehyde solution, dehydrated and wetted in paraffin. Slices of 5 µm width were sectioned and examined using hematoxylin-eosin staining. The analysis was performed in all cases by the same observer, comparing the structure of the autoimplanted tissue with normal spleen tissue.

Polymerase chain Reaction (PCR)

The gene rearrangement of B-lymphocyte receptors heavy chain variable regions was evaluated in order to assess the clone diversity of these cells in the graft. DNA was extracted from the autoimplanted tissue by digestion with proteinase K (*Promega*, Madison, WI, USA), followed by separation with phenol/chlorophorm (*Ambion Inc.*, Austin, TX, USA), precipitation in isoprophilic alcohol and afterwards resuspended in a storing buffer solution. The amplification was performed with specific oligonucleotides directed to the gene that codifies Ig HFR3 immunoglobulin heavy chain region, (conserved framework region FR3 size range 69-129 bp; sequence HR3C 5'-CTG TCG ACA CGG CCG TGT ATT ACT G-3', VLJH 5'-GTG ACC AGG GTN CCT CCT TGG CCC CAG-3'), free water of the nucleases, 10x buffer, Taq polymerase (*Promega*, Madison, WI, USA). For cycling, a thermocycler Perkin Elmer 9600 (*Perkin-Elmer Inc*, Waltham, MA, USA) was

used. For visualization, the amplified products were run in an electrophoretic 3% agarose gel, stained with ethidium bromide, and exposed to transillumination with ultraviolet light.

Statistical analysis

Results were expressed as means and standard deviation (SD). For the statistical analysis we used ANOVA of repeated measures and non-parametric Friedman test. Wilcoxon T test was used to compare preoperative and postoperative hematoimmunological parameters in both groups. P values equal to or lower than 0.05 were considered statistically significant.

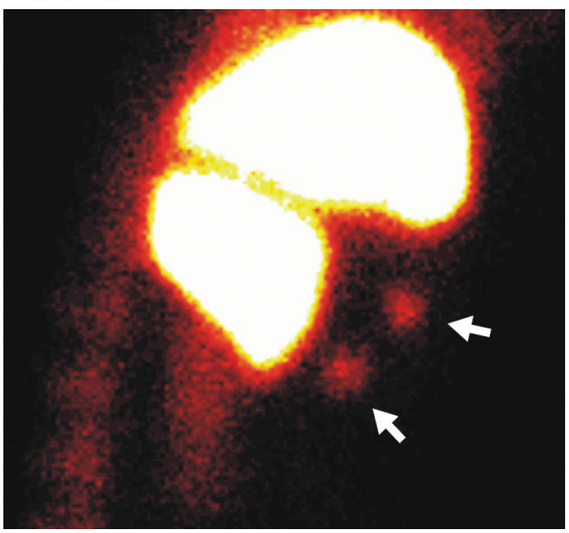
Results

All the animals survived to the surgical procedures without intraoperatory complications and with optimal recovery. They stayed free of disease until the moment of reoperation.

Scintigraphy

In 8 cases of Group A (8/13), images with spotlights of similar sizes were captured in static images and in SPECT method as well. In 2 animals, images with more than one implant-compatible spotlight were observed (Figure 2). There was accordance of the images obtained during follow up, without evidence of modifications of the implant's size in any case.

Figure 2. *Scintigraphy of autologous splenic tissue implanted in the omentum (arrows) 16 weeks after the surgery (right lateral view).*



Morphological evaluation

Macroscopic examination revealed that the omental implants were surrounded by adipose tissue forming a mesogastric spherical mass with several adhesions. After dissection, in 10 cases structures compatible with splenic tissue were found, with lower size than the original implants, representing 20% of the total spleen. In 1 of the 4 animals that simultaneously received implant in the gastric wall, sessile protruding splenic tissue near the greater curvature was also found (Figure 3). In the rest of the cases, we found fibrous scarring.

In the histological evaluation, 10 of the 13 animals of Group A (77%) presented autoimplanted splenic tissue (Table 1). In all the implants extracted from the omentum, red and white pulp were

Figure 3. *Macroscopic view of an implant on the anterior stomach wall. Autoimplant (arrow heads), gastric wall (arrow).*

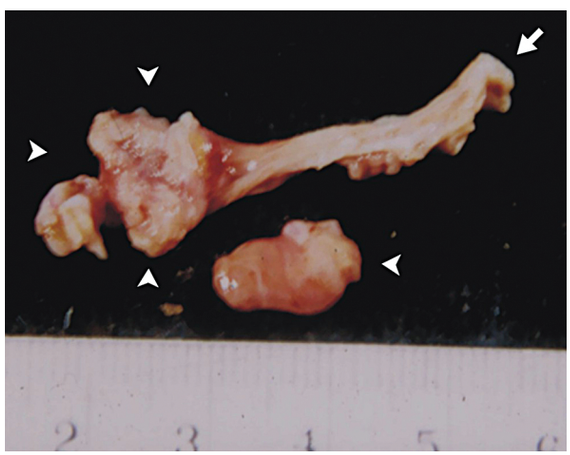


Table 1. *Histology, Scintigraphy and PCR results in Group A.*

Rabbit	Grafts site and histology	Scintigraphy	PCR
1	OP +	+	Oligoclonal
2	OP +	+	Oligoclonal
3	OP +	+	n/d
4	OP + / GW -	+	Policlonal
5	OP +	-	Policlonal
6	OP +	-	Oligoclonal
7	OP + / GW -	+	Oligoclonal
8	OP + / GW +	+	n/d
9	OP -	-	-
10	OP -	-	-
11	OP -	-	-
12	OP +/ GW -	+	Policlonal
13	OP +	+	Oligoclonal

OP, Omental Pouch; GW, Gastric Wall; n/d, not done.

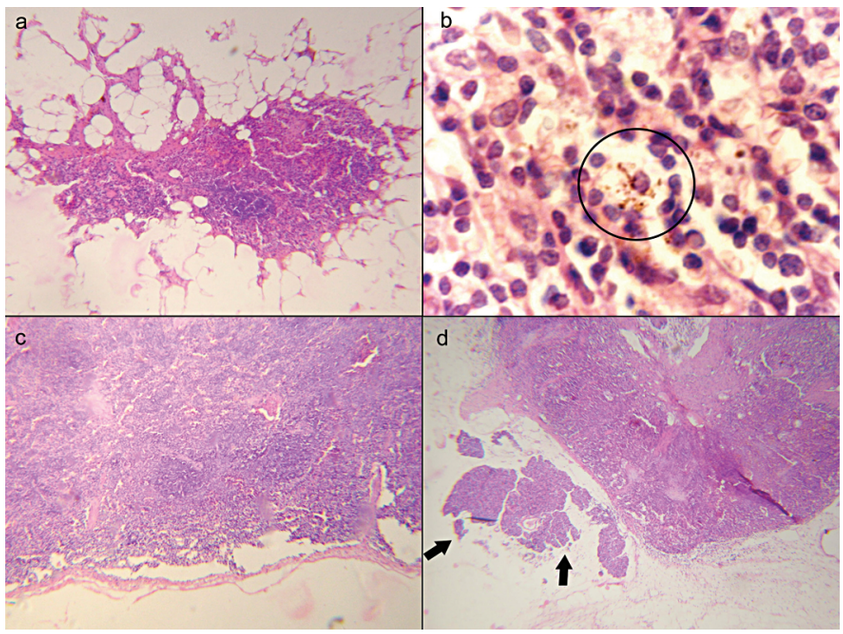


Figure 4. *Microscopic photograph of autoimplanted splenic tissue (H&E).* ***a)*** *Omental implantation surrounded by fat with diffuse borders.* ***b)*** *Hemosiderin pigments in the cytoplasm of an implant macrophage (circle).* ***c)*** *Autoimplant on the gastric wall that shows normal tissue with intact capsule, white and red pulp with preserved architecture and proportions.* ***d)*** *Omental autoimplant with some scares and perigraft pancreatic tissue (arrows). Original magnification: 10x (a), 100x (b), 10x (c) and 4x (d).*

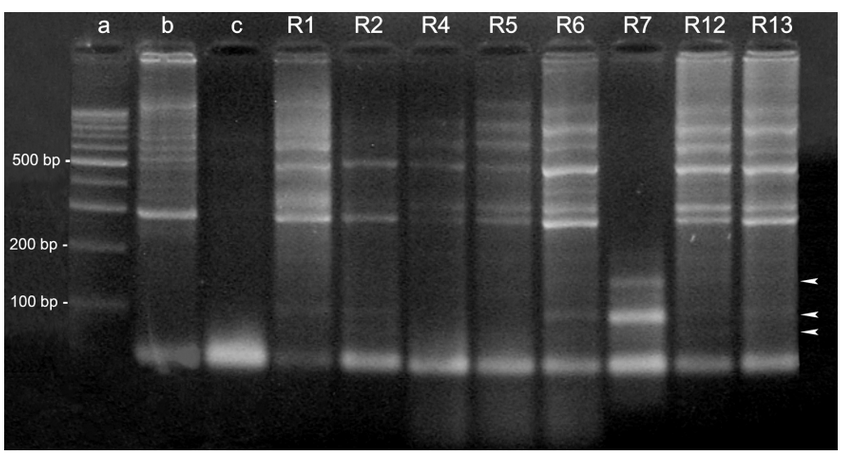


Figure 5. *Electrophoretic run of DNA amplified with PCR (gene Ig HFR 3).* ***a)*** *Molecular weight (base pairs marked on left),* ***b)*** *Polyclonal normal control (spleen),* ***c)*** *Negative control (omentum),* ***R****: Rabbits. In rabbits 1,2,6, 7 and 13, an oligoclonal pattern of bands can be observed under 150 bp (right side arrows).*

observed with mild structural disorders, without capsule, with diffuse limits and surrounded by omental fat (Figure 4). The grafts presented regeneration with abundant neovascularization and abundance of blastoid lymphocytes with indented voluminous nuclei. Rests of hemosiderin in the cytoplasm of splenic macrophages were also observed, demonstrating phagocytic activity (Figure 4). On the other side, the implant of the gastric wall demonstrated higher correspondence in histological structure and volume with the splenic implanted tissue, compared with those performed in the omentum, being indistinguishable to the normal tissue, free of fat and with a defined capsule (Figure 4). Normal diffuse pancreatic tissue was frequently observed near the omental implants, as well as areas of cystic steatonecrosis with fibrosis (Figure 4). The 3 animals with unsuccessful implants were excluded from the study.

Gen rearrangement of B-lymphocyte receptors

In 5 of the 8 processed grafts (62.5%), a pattern of oligoclonal bands was found under the 150 base pairs (bp), and the other 3 were polyclonal with intense bands under 150 bp (Figure 5), as occurs in the normal spleen.

Table 2. *Hematological and immunological parameters.*▲

	Hematological parameters				Immunological parameters, mg/dl		
Group	RBC x106/mm3	WBC x103/mm3	MCV (fl)	Hematocrit (%)	IgM	IgG	C3
A Basal	5.371 ± 0.172	6.078 ± 0.590	64.2 ± 1.9	34.3 ± 0.9	34.2 ± 7.3	363.7 ± 55.9	3.7 ± 0.8
8 weeks	5.449 ± 0.169	7.090 ± 0.660*	64.0 ± 0.9	34.7 ± 0.9	26.8 ± 3.8	365.8 ± 35.6	12.7 ± 0.6*
16 weeks	4.931 ± 0.507	6.370 ± 0.790	64.2 ± 1.3	35.0 ± 0.8	12.8 ± 2.8*	376.4 ± 22.4	23.9 ± 1.8*
S Basal	5.507 ± 0.219	5.625 ± 0.679	63.7 ± 0.8	35.1 ± 1.0	33.2 ± 11.9	289.5 ± 68.5	4.7 ± 1.4
8 weeks	5.835 ± 0.213	7.575 ± 1.227	65.5 ± 0.5*	38.1 ± 1.2	31.7 ± 1.5	363.0 ± 19.2	13.7 ± 0.2*
16 weeks	5.450 ± 0.307	6.550 ± 0.202	66.2 ± 1.5*	36.2 ± 2.0	17.7 ± 4.1*	405.7 ± 43.2	29.2 ± 3.9*

RBC, Red Blood Cells; WBC, White Blood Cells; MCV, Mean Corpuscular Volume
▲Values are mean ± SEM
**P < 0.05 vs Basal*

Hematological and immunological findings

Regarding the hemocatheteric activity, both groups presented a significant increase of Howell Jolly bodies during the postoperatory period (P = 0.001). However, the value was significantly higher in Group S compared to Group A at weeks 8 (A: 0.2±0.02 vs. S: 1.3±0.3%, P = 0.002) and 16 (A: 0.6±0.07 vs. S: 1.4±0.2%, P = 0.0001).

None of the groups presented statistically significant variations in leukocyte or erythrocyte counts, and hematocrit during the study period. MCV in Group S significantly increased at weeks 8 (P = 0.03) and 16 (P = 0.05) compared with baseline levels. In addition, MCV and hematocrit were higher in Group S than in Group A at weeks 8 and 16, even though they did not reach significant differences in any case.

Serological levels of IgG did not present significant changes in any group during the postoperatory period, but a significant decrease of IgM was noted in both groups (P = 0.01). In contrast, C3 values significantly increased along the study in both groups (Group A: P = 0.0001 and Group S: P = 0.02). In spite of these variations, there were not significant differences in IgM and C3 levels between both groups in any of the controls. The results of immunological parameters in both groups are presented in Table 2.

Discussion

In spite of the great number of studies that support that splenic autoimplantation is a relatively safe and easy procedure,[4] there is not current consensus regarding its functionality. Probably for this reason it has not yet become a routinely performed procedure worldwide.

In this study, we confirmed that splenic autoimplantation is a feasible technique for splenic tissue preservation, since our grafts revascularized and survived for 4 months in 10 of 13 animals (almost 80%). However, we found a moderate architectural distortion and marked reduction of the size of the omental implants, fact previously reported in rabbits.[22] We believe that this finding can be attributed to the animal model, since in spite of the initial ischemic period with partial necrosis described in other animals,[4,22-24] the presence of diffuse pancreatic tissue in the omentum of rabbits could have prejudiced the development and survival of the implants. This hypothesis is supported by the histological findings of cystic steatonecrosis with fibrosis replacing the non-viable implants and close to viable grafts.

According to previous reports, the autotransplants have been experimentally placed in several locations.[4,25] The most frequently used technique involves the greater omentum, and this has been suggested as the best site for implantation.[3,4,21,25]

Nunes et al [20] and Liaunigg et al [26] found that the histological morphology of the grafts did not differ significantly between the sites of implant. Surprisingly, in our study, we found that the implant extracted from the gastric wall 4 months after surgery had better regeneration and conservation of the original histological structure. In addition, the animals that underwent postoperatory complications neither developed abdominal adhesions. Furthermore, this location also allows maintaining the natural venous drainage to the liver through the portal vein. We discarded as possible cause of failure in non viable implants a prolonged preoperatory shock, as mentioned by Weber et al,[27] since the omental implants simultaneously performed in these animals were viable. Instead, we do consider that the failure could be due to the reduced size of the grafts, considering as an important issue the fact that the viable autoimplant had greater volume at the moment

of transplant (3 x 1 x 0.5 cm). In spite of the mentioned benefits, we are not able to state if this improved tissue regeneration could also be accompanied by a better functional reorganization.

Previous reports have mentioned the determinant role of B lymphocytes of the marginal zone of the splenic tissue in the immunological response against T-cell independent type 2 antigens, like the polysaccharides of encapsulated bacteria,[28] which are the most frequently found responsible agents of OPSI.[1-3,7,11] It is important to remark that not only the histological presence of these cells but also their ability and immunological repertoire are determinant factors in the defensive function of the autoimplanted splenic tissue.

This study is the first one where the diversity of B-lymphocyte clones of the autoimplanted tissue is evidenced using the molecular biology technique. Surprisingly, we found that in 5 of 8 studied cases (62.5%), this tissue presented oligoclonality, which is far away from the normal polyclonality and was manifested by the low variability of genomic rearrangements in areas that encode for variable regions of immunoglobulin heavy chains, which are the functional antigen receptors of these cells and represent the unique specificity of each B lymphocyte. This oligoclonality, translated into a reduced repertoire for antibodies response against different antigens is probably associated to tissue immaturity during the regenerative process, histologically demonstrated in this and other reports.[4,22,23]

Regarding hematoimmunological effects, we did not find significant differences in any of the evaluated parameters between both groups during the postoperatory follow-up.

Probably, the absence of significant beneficial effects in the group of the autoimplanted animals was a consequence of an insufficient amount and quality of the volume of the tissue regenerated in 4 months. Skipa et al[29] studied rats that underwent autoimplantation with a remnant tissue equivalent to a 10-15% of the original spleen and demonstrated a significantly higher level of IgM, compared to the splenectomized animals. However, this was manifested only 8 months later.

We can conclude that the two surgical techniques proved to be effective for the preservation of splenic tissue. However, the reduction of the implanted tissue mass, the quality of its components and a modification of the blood flow could compromise the important depurative function of the spleen in case of septicemia, at least during the initial months of the follow up.

Based on the high incidence of oligoclonality and the encouraging results of the autoimplantation in the gastric wall found in our reduced number of experiences, we would like to emphasize that our observations deserve further research to determine the actual significance of these findings.

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