Research Article

Photodynamic Therapy in the Extracellular Matrix of Mouse Lungs: Preliminary Results of an Alternative Tissue Sterilization Process

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Received 12 January 2021; Accepted 21 May 2021; Published 29 May 2021

Academic Editor: Umapada Pal

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Lung transplantation is one of the most difficult and delicate procedures among organ transplants. For the success of the procedure and survival of the new organ, the sterilization step for acellular lungs prior to recellularization is important to ensure that they are free of any risk of transmitting infections from the donor to the recipient subject. However, there are no available information concerning the lung mechanical parameters after sterilizing photodynamic therapy. The aim of this study was to evaluate the extracellular matrix (ECM) and lung mechanical parameters of decellularized lungs undergoing sterilizing photodynamic therapy (PDT). Besides, we also analyzed the lung after controlled infection with C. albicans in order to evaluate the effectiveness of PDT. The lung mechanical evaluation parameters, resistance ($R_L$) and elastance ($E_L$), exhibited no significant differences between groups. In addition, there were no PDT-induced changes in lung properties, with maintenance of the viscoelastic behavior of the lung scaffold after 1 h exposure to PDT. The ECM components remained virtually unchanged in the acellular lungs of both groups. We also showed that there was a reduction in fungal infection population after 45 minutes of PDT. However, more studies should be performed to establish and verify the effectiveness of PDT as a possible means for sterilizing lung scaffolds. This manuscript was presented as a master thesis of Nadua Apostólico at the postgraduate program in rehabilitation sciences, University Nove de Julho—UNINOVE.
1. Introduction

According to the World Health Organization (WHO), organ transplantation is frequently the only treatment for end-state organ failure, such as liver, lung, and heart failure. However, as a biomaterial, all implanted or transplanted material from human origin presents serious risks of several disease transmissions. Besides the biological risks, the extremely low availability of organ/tissue donors is the most important restriction for such procedures all over the world. In this scenario, tissue engineering has emerged as an alternative that is aimed at developing functional tissue substitutes with the goal of improving health and quality of life in terminal patients.

Tissue engineering has the specific target of promoting regeneration of structures and functions of tissues/organs compromised by disease or surgery. Research in the field of lung bioengineering has been particularly active in recent years due to the lack of viable lungs designated to transplant and the reduced long-term patient survival after transplantation [1, 2].

An important step in handling acellular lungs before they are subjected to recellularization is sterilization to suppress any risk of transmission of viruses or bacteria from the donor to the receiver of the transplanted tissue/organ. Indeed, potential transmission of infections, such as HIV and hepatitis C, has been reported in tissue engineering applications [3–5]. Therefore, the effects of different sterilization methods on a variety of tissue types have been studied to determine the optimal procedures. To this end, it is imperative to take into account that aggressive sterilization methods that ensure full elimination of pathogens may also deteriorate the structural components of the tissue, specifically its mechanical performance [6, 7].

Not all sterilization methods used in the health industry are applicable to bioscaffolds. At first, sterilization methods, such as those based on gamma irradiation, ethylene oxide, or other chemical agents, could be used. However, all sterilization procedures have their side effects, since any action to destroy infectious microorganisms at the same time can affect the different structures of the molecular scaffold.

It has been reported that both ethylene oxide and irradiation can interact with molecules and scaffolds and potentially degrade their function. It has recently been suggested that peracetic acid (PAA) can be a useful chemical agent for sterilizing scaffolds for tissue engineering [8]. However, it was not fully effective to sterilize pulmonary scaffolds [9].

Thus, appropriate sterilization procedures that preserve the mechanical properties of lung scaffolds after decellularization are essential for safe repopulation. Particularly, photodynamic therapy (PDT) could be a tool for obtaining viable lung scaffolds necessary for the repopulation process [10, 11].

The PDT was discovered in 1900 by Oscar Raab and Hermann Von Tappeiner [12], who found that Paramecium spp. protozoans were killed following acridine orange staining and subsequent exposure to bright light [13]. In the 1970’s, PDT was initially developed to be used in cancer therapy after it was discovered that porphyrins selectively localized in tumors [14]. Since then, PDT has been clinically used for the treatment of various malignancies and is an approved therapy for the destruction of choroidal neovascularization (CNV) in age-related macular degeneration. Recently, antimicrobial PDT has been proposed as an alternative approach for treating localized infections [12–16].

A photosensitizer plays a key role in the efficacy of PDT. Protoporphyrin IX (PpIX) is actively transported into the cell through an uptake mechanism induced by growth under nutritionally restrictive conditions. For instance, in C. albicans, the surface properties and composition are complex and dynamic and hydrophobicity is one of the many potential factors that may influence uptake. Previous studies demonstrated that filamentous forms and biofilms of Candida albicans were sensitive to PDT using porphyrin as a photosensitizer [17–19].

Understanding the mechanical consequences of lung scaffold sterilization is particularly relevant since the lung is an organ with high structural and mechanical complexity that is physiologically subjected to continuous deformation cycling during breathing. Therefore, the mechanical properties of the organ scaffold should be preserved as much as possible after sterilization to ensure optimal organ regeneration. However, studies concerning the efficacy of PDT in the sterilization of lung scaffolds and its repercussions on the mechanical properties are lacking. Thus, the aim of this study was to evaluate the extracellular matrix and lung mechanical parameters of decellularized lungs undergoing PDT with the purpose of sterilization and to perform a proof of concept of microbiological analyses of lungs infected with Candida albicans to determine the effectiveness of PDT.

2. Material and Methods

2.1. Experimental Animals. Male C57BL/6 mice (7–8 weeks old) were kept under standard housing conditions in ventilated rooms (12 h light–dark cycles, temperature at 23°C, and 60–70% humidity), with food and water provided ad libitum. Experimental procedures were carried out after submission and approvals by the Ethical Committee for Animal Research of the Universitat de Barcelona and by the Research Ethics Committee of Universidade Nove de Julho (UNINOVE) (protocol 0038/2011).

2.2. Experimental Setting. This study was divided into two phases: phase 1—decellularized mice lungs were mechanically evaluated and the extracellular matrix morphology was assessed before and after PDT and phase 2—decellularized mice lungs experimentally contaminated with C. albicans (ATCC 90028) were submitted to PDT; microbiological analyses were performed to evaluate the efficacy of PDT on C. albicans decontamination (Figure 1).

The animals were weighed, intraperitoneally anesthetized with 1.0 g/kg urethane, and sacrificed by exsanguination. Immediately, lungs were perfused via the pulmonary artery with phosphate-buffered saline (PBS) containing 50 U/mL heparin (Sigma-Aldrich, St. Louis, MO, USA) and 1 μg/mL sodium nitroprusside (SNP) (Fluka Analytical, St. Louis, MO, USA) to prevent the formation of blood clots. Then,
the lungs and heart were removed together with the trachea and esophagus. The whole block was stored at −80°C until the decellularization protocol was performed.

2.3. Lung Decellularization. At the day of the experiment, the tissues were thawed and decellularized as previously described [20]. Briefly, once the trachea and pulmonary artery were cannulated (Figure 2(a)) and placed into the experimental system, the following sequence of reagents was perfused: PBS for 30 min, deionized water (DI) for 15 min, 0.1% triton for 30 min, DI for 5 min, 1% sodium dodecyl sulfate (SDS) for 150 min, and a final wash of PBS for 30 min, resulting in a translucent acellular lung (Figure 2(b)).

2.4. Addition of a Photosensitizer and Sterilization with Red LED (660 nm). The first set of experiments consisted in a test to prove that PDT would be able to quell a controlled infection with *C. albicans*. A number of 12 decellularized mouse lungs were randomly divided into two groups of six animals: a group injected with protoporphyrin IX (group PpIX or GPpIX) and a control group injected with PBS (control). For the second stage, a total of eight decellularized mouse lungs were randomly divided into four groups, each containing two animals: a control group of infected mice (CG), a group treated with PpIX (group photosensitizer or GPS), a group treated with PDT for 15 minutes (group PDT 15 minutes or GPDT15), and a group treated with PDT for 45 min

![Flowchart of the study](image)

![Decellularization process of the lungs](image)

**Figure 1**: Flowchart of the study.

**Figure 2**: Decellularization process of the lungs. (a) Perfusion of the lungs with reagents through the trachea and the pulmonary artery. (b) Decellularized lungs.
After decellularization, GPpIX, GPS, GPDT15, and GPDT45 mice were tracheally injected with 1 mL PpIX (10 μM; Sigma-Aldrich), while both control groups were injected with 1 mL PBS. Each stage-one decellularized lung was irradiated for one hour using a light-emitting diode (LED) with a wavelength of 660 nm, controlled by a current source of 1400 mA at 7.5 V (Figure 3). For the second stage, decellularized lungs were irradiated for 15 minutes in GPDT15 and 45 minutes in GPDT45. The parameters of irradiation are showed in Table 1.

2.5. Evaluation of Pulmonary Mechanics. To evaluate the resistance and elastance of acellular lungs, a custom-built system was used. The trachea was cannulated, and the lung was vertically connected to the system. Flow signal was recorded as the pressure drop across a pneumotachograph with a differential pressure transducer (DCXL01DS, range ±2.5 cmH2O) and tracheal pressure (Pw) was measured using a pressure transducer (176PC14HD2, ±35 cmH2O) on a side port between the pneumotachograph and the cannula. Acellular lungs were subjected to conventional pressure-controlled ventilation with a quasi-sinusoidal flow pattern at a frequency of 80 breaths/min. A positive end expiratory pressure (PEEP) of 2 cmH2O was applied through the ventilator to counteract the absence of physiological negative pleural pressure. Flow and pressure signals were recorded with LabView (© 2011 National Instruments Corporation, Austin, Texas, USA) and postanalyzed with MATLAB (© 1994-2015 The MathWorks Inc., Natick, Massachusetts, USA). Effective lung resistance (Rr) and elastance (Er) were computed by linear regression of the recorded signals Pw (tracheal pressure), V′ (volume), and V (flow), using the conventional respiratory mechanics model Pw = Po + (Er · V) + (Rr · V′), where Po is a parameter to account for the external PEEP applied by the ventilator. For each decellularized lung, Rr and Er were computed from data including five ventilation cycles [21].

2.6. Immunofluorescent Microscopy. For conventional fluorescence microscopy analysis, decellularized lungs were fixed with a mixture of optimal cutting temperature compound (OCT) (Sakura Finetek, Torrance, CA, USA) and PBS in a 3:1 ratio and frozen at −80°C. Ten-micrometer sections of lung samples were obtained using an HM 560 CryoStar Cryostat (Thermo Scientific, Waltham, MA, USA). Then, sections were rinsed with PBS, fixed with 4% paraformaldehyde for 30 minutes at room temperature, and incubated for 10 min with 4′,6-diamidino-2-phenylindole (DAPI). To assess extracellular matrix proteins following PDT, cryosections were rinsed as previously described and incubated with a blocking solution (1% bovine serum albumin, 6% fetal bovine serum, and 0.5% triton in Tris-buffered solution) for 30 minutes, washed with PBS, and incubated overnight with the following primary antibodies: anti-collagen-I (Abcam, Cambridge, UK), anti-laminin (Sigma-Aldrich), anti-collagen-IV (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-elastin (Santa Cruz Biotechnology). The primary antibodies were detected using appropriate secondary antibodies. Images were taken using an Eclipse Ti fluorescence microscope (Nikon, Tokyo, Japan).

Table 1: Spectroscopic parameters used to irradiate the lungs with the LED.

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>660</th>
</tr>
</thead>
<tbody>
<tr>
<td>Operation mode</td>
<td>Continuous wave</td>
</tr>
<tr>
<td>Average radiant energy (mW)</td>
<td>2000</td>
</tr>
<tr>
<td>Beam area (cm²)</td>
<td>8.50</td>
</tr>
<tr>
<td>Intensity (mW/cm²)</td>
<td>236</td>
</tr>
<tr>
<td>Polarization</td>
<td>Aleatory</td>
</tr>
<tr>
<td>Beam profile</td>
<td>Multimode</td>
</tr>
<tr>
<td>Exposure time (min)</td>
<td>15, 45, 60</td>
</tr>
<tr>
<td>Radiant energy (J)</td>
<td>1808, 5424, 7232</td>
</tr>
<tr>
<td>Radiant exposure (J/cm²)</td>
<td>212.5, 637.5, 850</td>
</tr>
</tbody>
</table>

Table 2: Values of positive end-expiratory pressure (PEEP) and lung volumes of the control group and protoporphyrin IX group (GPpIX).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GPpIX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEEP (cmH2O)</td>
<td>1.95</td>
<td>1.93</td>
</tr>
<tr>
<td>Volume (mL)</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>PEEP (cmH2O)</td>
<td>1.93</td>
<td>1.93</td>
</tr>
<tr>
<td>Mean</td>
<td>0.15</td>
<td>0.12</td>
</tr>
<tr>
<td>SD</td>
<td>0.04</td>
<td>0.01</td>
</tr>
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</table>

Figure 3: (a) Setup of the irradiation system with the LED; (b) lungs being irradiated with the LED.
2.7. Scanning Electron Microscopy (SEM). Decellularized lungs were analyzed by SEM after PDT in both groups. Tissue samples were fixed with 2% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M cacodylate buffer for 2 h at room temperature. Then, samples were rinsed in cacodylate buffer, sliced, and dehydrated over an ethanol gradient. Samples were dehydrated in hexamethyldisilazane for 10 min, dried overnight, coated with a 14.4 nm layer of gold using a sputter coater (SC510; Fisons Instruments, San Carlos, CA, USA), and analyzed using a DSM 940A microscope (Zeiss, Oberkochen, Germany) with an acceleration of 15 kV.

2.8. Candida albicans Growth Conditions and Inoculum Preparation. Candida albicans (ATCC 90028) was cultivated in Sabouraud dextrose agar (SDA) at 37°C for 24 h. Cells were harvested, suspended in PBS, and homogenized in a vortex shaker. The concentration was estimated by the turbidity of the suspension at 15% transmittance [22]. The concentration of the inoculum was confirmed by culture on SDA and CFU/mL count, according to methodology proposed by Jett et al. [23].

2.9. Microbiological Evaluation. For microbiological analysis, we conducted a sagittal cut into the left lung. After slicing, half of the left lung was subjected to manual maceration. After maceration, serial dilutions were performed and plated on SDA and plates were incubated at 37°C for 24 h. After 24 hours, a count colony-forming unit of the plated content (CFU/g) was conducted.

2.10. Statistical Analysis. All rates are expressed as the mean ± standard deviation (SD). To verify the normality of groups, we used the Shapiro-Wilk test. One-way ANOVA was used to test for differences between groups. The group means were analyzed with the Tukey test and Levene test for comparison of variances. Comparisons between the values obtained for $R_t$ and $E_t$ measured in each group pre- and postirradiation were carried out using a paired Student’s $t$-test. The $p$ value was considered statistically significant at the 5% level. For these analyses, we used the Statistical Package for Social Sciences (SPSS) version 21.0 (SPSS Inc., Chicago, IL, USA).

3. Results

Pulmonary mechanics were evaluated in all 12 mice lung scaffolds divided in two groups: the control ($n = 6$) administered 1 mL PBS and GPpIX ($n = 6$) injected with 1 mL PpIX in the lungs, both of which were irradiated with a 660 nm LED. Mean of ventilation volumes and PEEP are described in Table 2.
There were no significant differences between the control and GPpIX groups, which represents an equal ventilation for both evaluations. The lung mechanical evaluation parameters, $R_L$ and $E_L$, exhibited no significant differences between both PDT intervals (Figure 4). Also, there were no changes observed over time of irradiation, representing the maintenance of the viscoelastic behavior of the lung scaffold after 1 h exposure to LED light.

Figure 5 demonstrates that extracellular matrix components responsible for the maintenance of the 3D lung structure, such as elastin, laminin, and collagens I and IV, remained virtually unchanged in the acellular lungs of both the control and GPpIX groups following application of the LED. The SEM analysis, observed in Figure 6, suggests that the microscopic lung structure has been maintained in both groups (control, Figure 6(a) and PpIX, Figure 6(b)), after application of photodynamic therapy. Also, the pulmonary scaffolds obtained from the decellularization process after irradiation LED compared to acellular lungs subjected to photodynamic therapy showed the absence of cell nuclei assessed by DAPI staining.

For the second stage of this study, eight mouse lungs were decellularized and divided into four groups: CG ($n = 2$), GPS ($n = 2$), GPDT15 ($n = 2$), and GPDT45 ($n = 2$). In the CG, no intervention of any kind was performed. For the group-denominated GPS, there was no application of LED, just addition of the PpIX photosensitizer. We observed that the PDT protocol promoted a reduction in the fungal population by about 1.60 log$_{10}$ (CFU/g) (4.87 ± 0.16 and 3.27 ± 0.45 for GPDT15 and GPDT45, respectively) when compared to GPS (4.24 ± 0.23; $p < 0.05$). When comparing the CG (4.38 ± 0.31) with the GPS (4.24 ± 0.23), there was no significant reduction in microbial counts observed (Figure 7).

4. Discussion

Tissue engineering is a rapidly evolving field that aims at developing functional tissue substitutes with the goal of improving the quality of life in patients by promoting true regeneration of the structure and function of tissue compromised by disease or surgery. Hence, it is especially important that an appropriate sterilization method is chosen to ensure safety and maintain the mechanical properties of the lung scaffolds after sterilization for effective regeneration of the organ. Sterilization is required for bioengineering organs because there is a risk of contamination during the whole process of creation and due to the existence of potentially infectious organisms in the donor tissue. The organism microbiota considered benign in a healthy individual can become pathogenic to scaffolds when submitted to a bioreactor during the repopulation procedure.

In the past few years, researchers have explored alternative methods of sterilization due to the limitations of the conventional ones used for tissue engineering [8, 9, 24]. Sirrrientong et al. analyzed the effects of sterilization on scaffolds of polyvinyl-alcohol and freeze-dried sericin and came to the conclusion that gamma irradiation degraded the scaffolds by almost 70% after 24 hours, but it was the most appropriate method for sterilization [25]. Rainer et al. remarked that dry heat and autoclave treatments resulted in
an increase in crystallinity, while low-temperature UV and hydrogen peroxide plasma preserved the structural properties [26]. Shearer et al. verified that PAA and antibiotic solutions were effective in sterilizing hollow fiber and flat sheets of poly (lactide: glycolide) but obtained unfavorable changes in morphology [27]. The limitations found in these studies, mentioned previously, included a lack of uniform model organisms tested; many did not specify the source or the identity of the contaminating bacteria or another microorganism, making it difficult to compare sterilization method scaffolds for tissue engineering. Preliminary studies have shown that sterilization of scaffolds in tissue engineering is as much about maintaining the properties of materials and architecture, as about killing microbes [25–27]. Sterilization assumed major significance in scaffolds containing proteins or biological material, where the risk of denaturation is real and can result in the reduction or loss of important biological activity [8].

Finsen, Raab, and Von Tappeiner demonstrated that a combination of light and administration of drugs led to photochemotherapy emergence as a therapeutic tool and isolation of porphyrins and discovery of phototoxic effects on cancer cells led to the development of PDT [12, 28]. The ECM is important for tissue organization in multicellular organisms and is a complex network of macromolecules secreted by the cells, residing between cells as both a barrier and a scaffold on which tissues are built. Studies have demonstrated that PDT alters the extracellular matrix [29]. Pazos et al. demonstrated that ECM components are affected, even when they are not the primary targets [30]. However, we should also keep in mind that the action of PDT on specific molecular pathways depends on the fluence rate and photosensitizer used.

It has been reported that both ethylene oxide and irradiation can interact with molecules and scaffolds and potentially degrade their functions. It has recently been suggested that peracetic acid can be a useful sterilizing chemical agent in the tissue engineering of scaffolds [8, 31]. By contrast, in our study, we demonstrated that there were no apparent changes in the relevant extracellular matrix components of elastin, laminin, and collagens I and IV. These components remained almost unchanged in the acellular lungs of both groups following LED application.

Sterilization methods involving high temperature must obviously be discarded due to the denaturation of proteins and other biomolecules under these conditions. In a study on the impact of gamma radiation, the pulmonary valve scaffolds applied with a dose of 25–40 kGy exhibited great reduction in the collagen content fragmentation of the extracellular matrix, caused by oxidative damage following irradiation. This resulted in considerable biomechanical changes to tissue integrity, causing structural damage of collagen fibers and significant changes in the mechanical behavior [32]. Moreover, Uriarte et al. demonstrated that irradiation of acellular lungs with a typical $^{60}$Co gamma sterilization dose for health applications resulted in a significant increase in the mechanical resistance of lung scaffolds [33].

Remarkably, in the present study, we observed that there were no changes in the values of pulmonary elastance ($E_L$) and pulmonary resistance ($R_L$) after irradiation by LED for 60 min with addition of photosensitizer. This was in contrast to previous reports on gamma irradiation, which causes degradation and fragmentation of the peptide chain and also damages collagen fibers, resulting in changes to lung mechanical properties [34].

PDT has been used successfully to treat different localized infections [35, 36], and our results are a proof of concept that PDT is also a suitable method to promote the reduction of $C.\ albicans$. Our study provides evidence that the photosensitizer PpIX does not exhibit any antifungal activity when used alone without the application of LED. It is also well described in the literature that the use of lasers alone, without the addition of a photosensitizer, similarly does not reduce fungal populations. We observed that there was a reduction in the fungal population after 45 min of PDT when compared to only 15 min of PDT. It is supposed that from the moment at which PDT acts, $C.\ albicans$ disintegrates when a part of the cell detaches, forming another cell, which may be confused with a yeast recolonization. Teichert et al. demonstrated that the total reduction in fungal load was dependent on the concentration of the photosensitizer and light parameters, using red and methylene blue lasers in the oral mucosa of a mouse model [37].

5. Conclusion

In summary, this is the first study evaluating the mechanical behavior of lung scaffolds subjected to LED irradiation with a photosensitizer, with the objective of sterilizing lung scaffolds. We found that irradiation doses that effectively can reduce infection do not modify the structural and mechanical properties of decellularized lungs. This study opens the door to further research to confirm the suitability of PDT as a tool for routinely sterilizing lung scaffolds in the process of lung biofabrication.

Data Availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Additional Points

*Highlights*. The following were highlighted: behavior of the mechanical properties of pulmonary scaffolds submitted to photodynamic therapy, application of photodynamic therapy in the sterilization of pulmonary scaffolds, and use of LED in the process of sterilization of lung scaffolds in regenerative medicine.

Disclosure

This manuscript was presented as a master thesis of Nadua Apostólisco at the postgraduate program in rehabilitation sciences, University Nove de Julho—UNINOVE.
Conflicts of Interest

The authors declare no financial or other conflict of interest regarding this article. This manuscript presents the results of the research of a master’s thesis by the author Nadua Apostolico defended in the master’s degree program in rehabilitation sciences at Universidade de Nove de Julho (UNINOVE), São Paulo (SP), Brazil [38]. The results of this study are original and have not been previously published in any manuscript.

Authors’ Contributions

LVFO, RF and DN: Conceptualization of the study, project administration and, supervision; NA, JJJU, RKP, RABLM, LRF, RAP, AMD, LRF and, RPV: Investigation, Methodology, Data collect and, Formal analysis; RPV, MGOJ, RABLM, RPK, LVFO, RF and, JPPRA Interpretation of the data; RKP, RABLM, JJJU, LVFO and, NA: wrote the original version of the manuscript; RF, DN, JJJU and, LVFO: reviewed the final version of the manuscript; All authors read and agreed with the final version of the manuscript.

Acknowledgments

We would like to thank the technical support offered by the Unidad de Biophyscis i Bioenginyeria, Facultat de Medicina, Universitat de Barcelona (UB), Barcelona, Spain. We would like to express a special thanks to Prof. Dr. Paulo de Tarso Camillo de Carvalho for his friendship, opportunity, learning, and participation in this study. This is in memoriam of Paulo de Tarso Camillo de Carvalho, m.d., 1962–2020. This work was supported in part by the Spanish Ministry of Economy and Competitiveness (SAF2011-22576). Luis Vicente Franco de Oliveira receives grants by CNPq Research Productivity modality—PQID protocol number 313053/2014-6. Nadua Apostolico receives national support from “Fundaçao de Amparo à Pesquisa do Estado de São Paulo,” protocol number 2013/21765-5, and was also covered with a “Bolsa de Estágio de Pesquisa no Exterior (BEPE/FAPESP) protocol number 2015/01649-6.” The co-authors Nadua Apostolico, Luis Rodolfo Ferreira, and Manoel Carneiro de Oliveira Júnior received support from the Coordination for the Improvement of Higher Education Personnel (local acronym CAPES).

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