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Original Article / Özgün Makale

Investigation of the preventive effect of proanthocyanidin in ischemia-reperfusion injury in lung transplantation: An experimental study

Akciğer naklinde proantosiyanidinin iskemi-reperfüzyon hasarını önleyici etkisinin araştırılması: Deneysel çalışma

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ABSTRACT

Background: This study aims to investigate the preventive effect of proanthocyanidin against ischemia-reperfusion injury after lung transplantation.

Methods: The study included 12 swines (weighing 35±5 kg) and separated into four groups. Groups 1 and 3 were identified as control groups and left upper lobectomy was performed. Groups 2 and 4 were identified as transplantation groups and left lower lobectomy and heterotransplantation were performed. Proanthocyanidin was only given to groups 3 and 4. Tissue samples were analyzed under light microscope and histopathological findings were recorded.

Results: There was no statistically significant difference between control groups in terms of the numerical values of histopathological findings that include congestion (p=0.565), alveolar edema (p=0.197) and peribronchial inflammation (p=0.444). However, numerical values of acute cellular rejection were statistically significantly different between transplantation groups (p=0.048). Mean oxidative stress enzyme levels were higher in group 2 compared to group 4; however, the difference was not statistically significant (p>0.05).

Conclusion: According to the findings of our experimental study, proanthocyanidin can be safely used in lung transplantation based on its preventive effect in ischemia-reperfusion injury that may lead to morbidity and mortality.

Keywords: Experimental; ischemia-reperfusion; lung transplantation; oxidative stress; proanthocyanidin.

ÖZ

Amaç: Bu çalışmada proantosiyanidinin akciğer nakli sonrasında iskemi-reperfüzyon hasarını önleyici etkisi araştırıldı.

Çalışma planı: Çalışmaya 12 domuz (ağırlık, 35±5 kg) dahil edildi ve domuzlar dört gruba ayrıldı. Grup 1 ve 3 kontrol grupları olarak belirlendi ve sol üst lobektomi uygulandı. Grup 2 ve 4 nakil grupları olarak belirlendi ve sol alt lobektomi ve heterotransplantasyon uygulandı. Proantosiyanidin sadece grup 3 ve 4'e verildi. Doku örnekleri ışık mikroskopu altında incelendi ve histopatolojik sonuçlar kaydedildi.

Bulgular: Kontrol grupları arasında konjesyon (p=0.565), alveolar ödem (p=0.197) ve peribronşiyal enflamasyonu (p=0.444) içeren histopatolojik sonuçların sayısal değerleri açısından istatistiksel olarak anlamlı farklılık yoktu. Fakat akut hücresel reddin sayısal değerleri nakil grupları arasında istatistiksel olarak anlamlı şekilde farklı idi (p=0.048). Ortalama oksidatif stres enzimi düzeyleri grup 2'de grup 4'e kıyasla daha yüksek idi, fakat farklılık istatistiksel olarak anlamlı değildi (p>0.05).

Sonuç: Deneysel çalışmamızın bulgularına göre, mobidite ve mortaliteye yol açabilen iskemi-reperfüzyon hasarını önleyici etkisine dayanarak proantosiyanidin akciğer kanserinde güvenle kullanılabilir.

Anahtar sözcükler: Deneysel; iskemi-reperfüzyon; akciğer nakli; oksidatif stres; proantosiyanidin.

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Lung transplantation (LT) is one of the most important treatment choices for patients with end-stage lung disease.[1-3] The outcomes of LT are worse when compared to the outcomes of other solid organ transplantations. The 30-day and three-year mortality rates were reported as 15% and 50%, respectively. Therefore, many conditions may arise in LT including ischemia-reperfusion injury (I-RI), which is the main cause of respiratory failure and organ dysfunction.[1,4,5] Improved surgical techniques, perioperative care and preservation methods may not be sufficient to prevent I-RI.[1,6] The 30-day mortality rate of patients increases about 40% when I-RI develops.[4]

Currently, the main purpose in this area is to prevent I-RI, which is mainly caused by inflammation and reactive oxygen species (ROS). Thus, use of the free radical scavenger proanthocyanidin (PC) may be promising with its anti-inflammatory and antithrombotic features.[7] In this study, we aimed to investigate the preventive effect of PC against I-RI after LT.

MATERIALS AND METHODS

The study was conducted at Gülhane Military Medical Academy, Animal Research Facility between June 2009 and May 2010 after the approval of the Gülhane Military Medical Academy Ethics Committee for Experimental Animal Studies (Date: May 22, 2009, Nr: 09/42K). Twelve swines (German Landrace) weighing 35±5 kg were separated into four groups of five each. The upper lobes were removed to form control groups and lower lobes were removed to form transplantation groups to keep the number of swines limited. Design of the experimental study was shown in Figure 1.

No medication was given to Group 1, which was defined as the control group (CG). Left upper lobe was resected via left thoracotomy and 1 cm^3 specimen was obtained for oxidative stress parameters. Then, the lobe was placed into 10% formalin solution for histopathological evaluation.

No medication was given to Group 2, which was defined as the ischemia-reperfusion group (I-RG). Left lower lobe of the same animal was resected

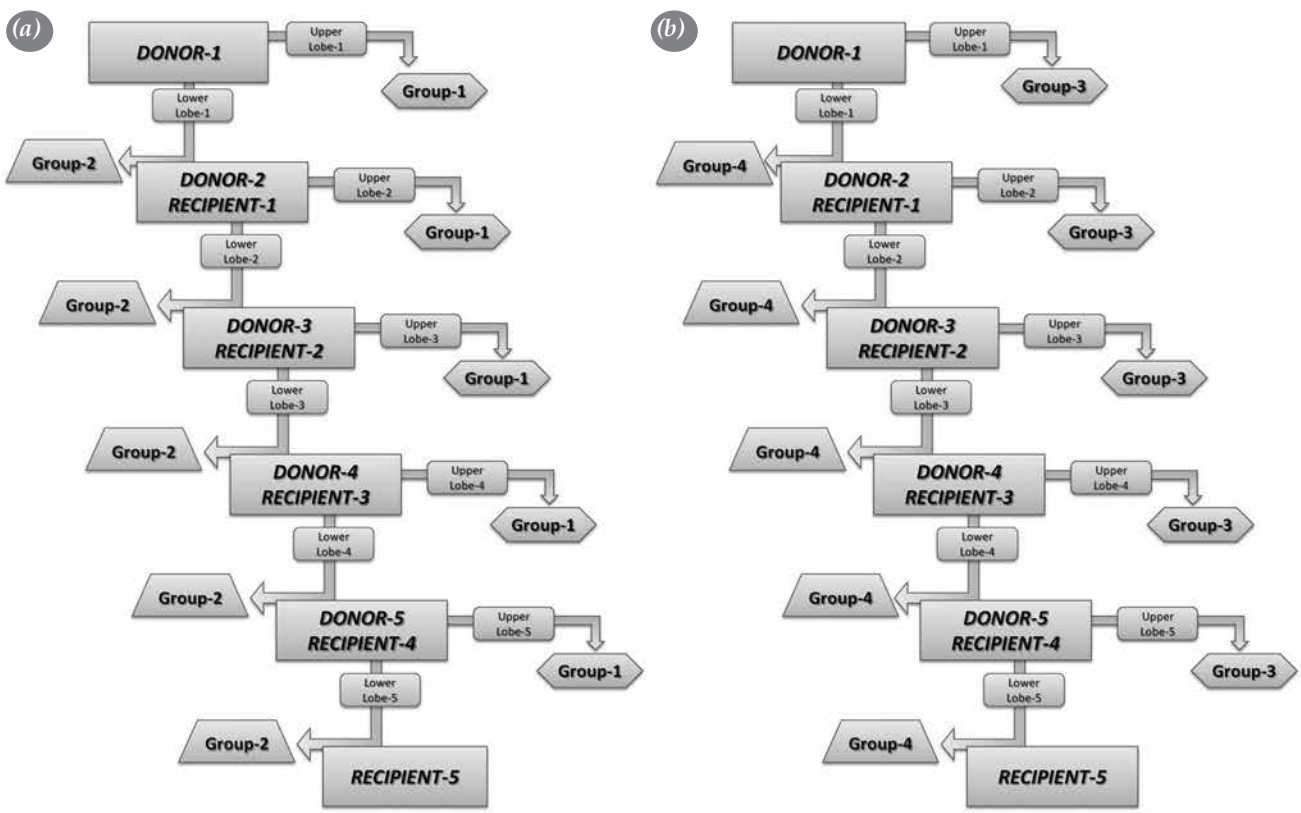

Figure 1. (a) Experimental design of control group and ischemia-reperfusion group. **(b)** Experimental design of proanthocyanidin control group and proanthocyanidin ischemia-reperfusion group.

in the same stage and the lobe was washed with 0.9% sodium chloride (NaCl) and 20 cm dihydrogen monoxide (H_2O) pressure. The lobe was placed into Euro-Collins solution (ECS) and cold ischemia (+4°C) was performed for 24 hours. The lobe was transplanted to next subject after its upper and lower lobes were removed. After two hours of reperfusion and reventilation, the same procedures for CG were carried out.

Group 3 was planned as the PC control group (pCG). Proanthocyanidin (GNC Bakara Ltd., PC: 100 mg, 90 capsules, Istanbul, Turkey) was given at a dosage of 100 mg/kg/day one day before the operation. The same procedures which were performed in CG were applied.

Group 4 was planned as the proanthocyanidin ischemia-reperfusion group (pI-RG). Proanthocyanidin was given at a dosage of 100 mg/kg/day one day before the operation. Left lower lobe of same subject was resected. This lobe was washed with 0.9% NaCl and 20 cm H_2O pressure. The lobe was placed into ECS and cold ischemia (+4°C) was performed for 24 hours. The lobe was transplanted to next subject after its upper and lower lobes were removed. After two hours of reperfusion and reventilation, the same procedures for CG were carried out.

Induction of anesthesia was achieved with intramuscular injection of 4.4 mg/kg tiletamine-zolazepam. Then, 10 mg/kg pentobarbital was given intravenously and 3% isoflurane was used to continue anesthesia.

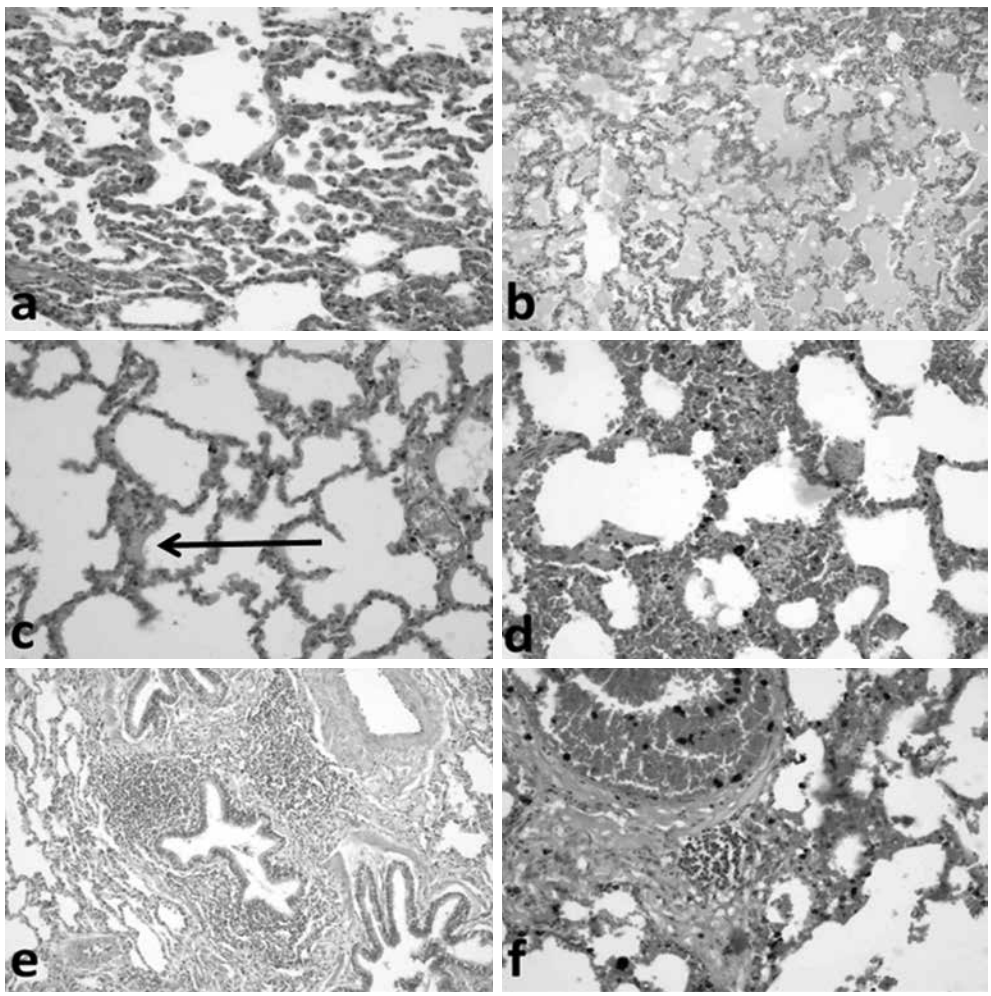

Figure 2. **(a)** Alveolar macrophage infiltration in lung parenchyma (H-E×200). **(b)** Alveolar edema (H-E×50). **(c)** Formation of hyaline membrane (arrow) (H-E×200). **(d)** Congestion finding in lung parenchyma (H-E×100). **(e)** Peribronchial lymphoplasmacytic infiltration (H-E×100). **(f)** Perivascular lymphoplasmacytic infiltration (H-E×200).

After left thoracotomy incision was performed, the subcutaneous tissue and muscles were divided and the pleural cavity was entered from fifth intercostal space. A dosage of 100 U/kg heparin was administered and left upper lobectomy was performed in control groups (CG and pCG). Left lower lobectomy was performed to the same subject to form the ischemia-reperfusion groups (I-RG and pI-RG). The left lower lobes were sustained to cold washing with 20 cm H_2O pressure from the main artery with 0.9% NaCl. The ventilation was also continued during cold washing. The lobes were kept in ECS at +4°C for 24 hours.

The transplanted lobes were administered reperfusion and ventilation for two hours and then these lobes were extracted. The planned procedures were performed according to groups.

Malondialdehyde (MDA) levels and catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities of tissue homogenate samples were measured. The same methods of measurement, which were described in our previous studies, were applied.[7,8]

After fixation, samples were routinely processed and embedded in paraffin. Four micrometer sections were obtained and stained with hematoxylin and eosin for histopathological examination.

All samples were investigated for the presence of hyaline membrane, congestion, alveolar edema, alveolar macrophage, and type 2 cell hyperplasia. The histopathological features were illustrated in Figure 2. Reece's lung injury score was modified for the presence of hyaline membrane, congestion, alveolar edema, alveolar macrophage and type 2 cell hyperplasia.[9] Briefly, these histopathological findings were scored as 1 (minimal)=lower than 5%, 2 (mild)=between 6-25%, 3 (moderate)=between 26-50%, and 4 (severe)=over 50%.

The control groups and the transplantation groups were compared to each other separately. The numerical values of histopathological findings that include hyaline membrane, congestion, alveolar edema, alveolar macrophage, type 2 cellular hyperplasia, acute cellular rejection and peribronchial inflammation were compared between CG and pCG in order to determine the effect of PC on normal lung parenchyma and between I-RG and pI-RG in order to determine the preventive effect of PC on I-RI. Severity and localization of inflammation was also detailed according to the "Revised International Working Formulation" (Table 1).[10]

Statistical analysis

Data were analyzed using the SPSS for Windows 16.0 version (SPSS Inc., Chicago, IL, USA). The values of oxidative stress parameters were compared with t-test. Histopathological scoring was compared with Fisher's exact test. A chi-square test was performed for the comparison of histopathological values between the groups. A value of $p<0.05$ was considered statistically significant.

RESULTS

Numerical scoring scheme of the histopathological findings according to groups was shown in Table 2. Numerical scores of hyaline membrane, alveolar

Table 1. Scoring scheme of acute cellular rejection and bronchial infiltration

Acute cellular rejection	
0	No perivascular lymphoplasmacytic infiltration
1 (minimal)	Rare circumferential perivascular lymphoplasmacytic infiltration (2-3 cells thick; no eosinophils or endotheliitis)
2 (mild)	Perivascular lymphoplasmacytic infiltration (thicker than 3 cells, no eosinophilia or endothelialitis)
3 (moderate)	Wide perivascular lymphoplasmacytic infiltration adjacent to alveolar septae (alveolar macrophage, eosinophil and rare neutrophil)
4 (severe)	Diffuse lymphoplasmacytic infiltration (perivascular, interstitial, alveolar), macrophage, eosinophil, neutrophil, leukocyte infiltration diffuse alveolar injury, hemorrhage, parenchymal necrosis
Bronchial/infiltration	
0	No inflammation
1 (low grade)	Submucosal mononuclear cell infiltration may include eosinophilia, no epithelial injury, no intraepithelial infiltration
2 (high grade)	Intraepithelial infiltration, plasma cell infiltration and epithelial necrosis

Table 2. Numerical scoring scheme of histopathological findings of study groups

	Subject no	Hyaline membrane	Congestion	Alveolar edema	Alveolar macrophage	Type 2 cell hyperplasia	Acute cellular rejection	Peribronchial inflammation
Group 1 (CG)	1	0	2	1	0	0	0	0
	2	0	2	1	0	0	0	0
	3	0	3	0	0	0	0	1
	4	0	2	0	0	0	0	1
	5	0	2	0	0	0	0	0
Group 2 (I-RG)	1	0	2	1	0	0	0	0
	2	0	2	1	0	0	0	0
	3	0	2	0	0	0	0	0
	4	0	2	1	0	0	0	0
	5	0	0	0	0	0	0	0
Group 3 (pCG)	1	0	2	1	2	0	1	1
	2	0	2	1	0	0	1	1
	3	0	2	0	0	0	1	1
	4	1	2	2	1	0	1	1
	5	1	2	2	1	1	1	1
Group 4 (pI-RG)	1	0	2	1	0	0	0	1
	2	0	2	1	0	0	1	0
	3	0	2	0	0	0	0	1
	4	0	1	0	0	0	0	0
	5	0	1	0	0	0	0	0

CG: Control group; I-RG: Ischemia-reperfusion group; pCG: Proanthocyanidin control group; pI-RG: Proanthocyanidin ischemia-reperfusion group.

macrophage, type 2 cell hyperplasia and acute cellular rejection were the same in CG and pCG. There was no statistically significant difference between CG and pCG in terms of the numerical scores for congestion (p=0.565), alveolar edema (p=0.197) or peribronchial inflammation (p=0.444).

Numerical scores of acute cellular rejection (p=0.048) were statistically significantly different while the rest of the scores did not differ statistically significantly (p>0.05) between I-RG and pI-RG (Table 3).

Oxidative stress parameters of the CG and pCG were compared to determine the effect of PC on

Table 3. Comparison of histopathological values between study groups

	Group 1 vs Group 3 (CG vs pCG)	Group 2 vs Group 4 (I-RG vs pI-RG)
	p	*p*
Hyaline membrane	-	0.444
Congestion	0.565	0.114
Alveolar edema	0.197	0.223
Alveolar macrophage	-	0.117
Type 2 cell hyperplasia	-	0.292
Acute cellular rejection	-	0.048
Peribronchial inflammation	0.444	0.167

CG: Control group; pCG: Proanthocyanidin control group; I-RG: Ischemia-reperfusion group; pI-RG: Proanthocyanidin ischemia-reperfusion group.

Table 4. Comparison of the oxidative stress parameters between the groups

	Group 1	Group 3	
	Mean±SD	Mean±SD	*p*
Malondialdehyde (nmoL/g)	57.8±5.3	56.2±7.7	0.719
Glutathione peroxidase (U/g)	41.3±13.1	43.1±8.7	0.809
Superoxide dismutase (U/g)	117.3±29.4	123.6±43.6	0.794
Catalase (KU/g)	36.8±21.2	30.2±10.0	0.549
	Group 2	Group 4	
	Mean±SD	Mean±SD	*p*
Malondialdehyde (nmoL/g)	67.9±11.2	49.8±17.3	0.086
Glutathione peroxidase (U/g)	40.4±15.1	31.3±8.2	0.267
Superoxide dismutase (U/g)	136.3±26.3	100.5±71.8	0.326
Catalase (KU/g)	34.2±10.1	32.5±6.2	0.746

SD: Standard deviation; Group 1: Control group; Group 2: Ischemia-reperfusion group; Group 3: Proanthocyanidin control group; Group 4: Proanthocyanidin ischemia-reperfusion group.

normal lung tissue. The result was not statistically significant (p>0.05), suggesting that PC did not have any injurious effect on normal lung tissue.

Oxidative stress parameters of the I-RG and pI-RG were compared to determine the preventive effect of PC on I-RI after LT. Although the differences were not statistically significant (p>0.05) (Table 4), mean enzyme levels were higher in I-RG.

DISCUSSION

Ischemia-reperfusion injury is one of the most common causes of morbidity and mortality after LT.[6,11] It occurs approximately in 10-25% of LT patients despite the advances in surgical and lung preservation techniques.[2] Moreover, there is a strict correlation between the severity of I-RI and the development of bronchiolitis obliterans.[12]

Variables of surgical techniques, inflammation, oxidative stress, duration of ischemia, preserving temperature, and preserving solutions are commonly investigated to prevent I-RI. Particularly, oxidative stress, which we aimed to decrease in our experimental study, is one of the main causes of I-RI.

Proanthocyanidin is a natural powerful antioxidant, which is classified in the polyphenols.[13,14] The antioxidant effect of PC in the grape seed is superior than vitamin C, vitamin E and beta-carotene.[15,16] We preferred PC since it is highly available, causes no injury on normal lung parenchyma, constitutes a new study area and has evidence for preventing I-RI in other tissues.[13,15,17] To our knowledge, the preventive effect of PC in I-RI after LT was investigated for the first time in our study. We determined the dosage of PC according to studies of Yucel et al.[7,8] According to this study, we administered PC one day before the operation to the pCG and pI-RG at a dose of 100 mg/kg orally mixed with daily meal. However, to our knowledge, there is no concordance in the literature about the application dose and time of PC. This ambiguity is a question mark for further studies.

We used 12 swines (German Landrace) in our study parallel to studies of Sommer et al.[18] and Gohrbandt et al.[19] We subjected the extracted lobectomy specimen to cold ischemia (+4°C) for 24 hours as its benefits were indicated in the studies of de Perrot et al.[20] and Clavien et al.[21]

The numerical scoring scheme which we used for histopathological evaluation was adapted from the studies of Reece et al.[9] and Maxey et al.,[22] and was modified according to the "Revised International Working Formulation for the Standardization of Nomenclature for the Diagnosis of Lung Rejection".[10] A statistical comparison of the CG and the pCG showed that PC had no injurious effects on normal lung tissue (Table 3).

Although free radical reactions are necessary for immune cells like neutrophils and macrophages, excessive production of free radicals may cause tissue injury and cellular death.[23] Reactive oxygen species attack proteins, nucleic acids (break down the deoxyribonucleic acid chain) and lipids (causes peroxidation which damages cell membrane or destroys the cells).[24] A great number of studies have shown that ROS cause I-RI on tissues.[25-28] We compared

the hyaline membrane, congestion, alveolar edema, alveolar macrophage, type 2 cell hyperplasia, acute cellular rejection and peribronchial inflammation values to determine the effectiveness of PC and detected a statistically significant difference when we compared the acute cellular rejection (p=0.048) values between I-RG and pI-RG (Table 3). These results suggested that PC can be used to prevent oxidative stress-related I-RI.

Superoxide dismutase, CAT, glutathione, allopurinol, deferoxamine and N-acetylcysteine are known for their free radical scavenger (antioxidant) features that reduce the free oxygen molecules to the more harmless variants.[13] Reactive oxygen species usually occur after ischemia, cause lipid peroxidation (the final product is MDA) and activate the inflammatory cells for proliferation of neutrophils. Therefore, lipid peroxidation can be used as a candidate measurement to evaluate the oxidative injury on lung tissue after ischemia-reperfusion.[5] Catalase, GPx and SOD enzyme activities were used to determine the severity of oxidative stress and MDA levels were used to detect the severity of tissue injury by Yousef et al.[14] and Yucel et al.[7,8] In our study, CAT, GPx and SOD enzyme activities and MDA levels were measured to show the effects of PC on both normal lung tissue and transplanted lung tissue. We detected no statistically significant difference in terms of the GPx, SOD, CAT and MDA levels between the CG and the pCG (p>0.05). These results were also confirmed with histopathological findings which revealed that PC has no harmful effects on normal lung tissue. A comparison between the I-RG and the pI-RG in terms of GPx, SOD, CAT and MDA levels did not reveal any statistically significant difference (p>0.05). However, mean SOD, GPx and MDA levels were relatively lower in pI-RG compared to the I-RG (Table 4). We concluded that the dose of PC was not sufficient to reveal the effect of PC on antioxidants and MDA levels.

A limitation of our study was that, due to its high cost, we were unable to administer the perfadex solution that is used in human lung transplantation. Therefore, we were required to perform our study with the easily-obtained Euro-Collins solution.

In conclusion, proanthocyanidin can be safely used in lung transplantation because of its preventive effect on ischemia-reperfusion injury which is the major cause of morbidity and mortality. However, further studies are required to determine the optimal dosage for proanthocyanidin in lung transplantation.

Declaration of conflicting interests

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