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Effects of Ischemia/Reperfusion on ß Cells of Pancreas and Protective Effects of Melatonin Treatment

Efectos de la Isquemia/Reperfusión sobre Células ß del Páncreas y Efectos Protectores del Tratamiento de Melatonina

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SUMMARY: Oxygen free radicals are considered to be important components involved in the pathophysiological tissue alterations observed during ischemia-reperfusion (I/R). In this study, we investigated the putative protective effects of melatonin treatment on pancreatic I/R injury. Sprague Dawley male rats were subjected to 30 min of pancreatic pedicle occlusion followed by 90 min reperfusion. Melatonin (10 mg/kg. s.c) was administrated 30 min prior to ischemia or I/R application. At the end of the reperfusion periods, rats were decapitated. Pancreatic samples were taken for transmission electron microscopy. The results indicated that ischemia created b cell damage as evidenced by dilatation between the nucleus inner and outer membrane and degeneration on islets of Langerhans cells, was reversed by melatonin treatment. As melatonin administration reversed these microscopic damage, it seems likely that melatonin protects pancreatic tissue against oxidative damage.

KEYWORDS: Pancreas; ß cells; Melatonin; Ischemia; Reperfusion.

INTRODUCTION

Ischemia/reperfusion (I/R) occurs when the blood supply to an organ or region is temporarily interrupted. Most often, I/R injury is discussed relative to the cardiovascular system, i.e. stroke or brain attack. While both these conditions can have serious debilitating consequences and often cause death, I/R in any organ is a matter of grave concern (Baydas *et al*., 2003; Borlongan *et al*., 2000).

That the molecular and cellular damage resulting from I/R involves destructive free radicals and related reactants is not contested. While interrupting the blood supply to an organ (depriving it of oxygen and essential nutrients) is very serious and leads to tissue death, it must be quickly relieved; re-establishing blood flow to the deprived tissue, i.e. reperfusion, is, however, also highly damaging. When oxygenated blood re-enters tissues that have been deficient in oxygen for even a brief period, numerous oxygen-based reactants are generated initiating damage beyond that caused by the ischemia. Thus, both ischemia and reperfusion contribute to tissue loss and organ dysfunction (Baydas *et al*.; Borlongan *et al.*; Cheung, 2003; Chung & Han, 2003).

Besides their direct damaging effects on tissues, free radicals seems to trigger the accumulation of leucocytes in the tissue involved, and thus cause tissue injury also indirectly through activated neutrophils. It has been shown that activated neutrophils secrete enzymes (e.g. myeloperoxidase, elastase, proteases) and liberate oxygen radicals (Borlongan *et al.*).

Recently the pineal secretory product melatonin was shown to have free radical scavenging ability and to reduce lipid peroxidation (Pierrefiche *et al*., 1993). Lipid peroxidation in cell membranes is devastating to the functional integrity of these structures and if the damage is severe, cell death is inevitable. Tan *et al.* (2002) had shown previously that melatonin scavenges the hydroxyl radical (OH), a radical that is certanly sufficiently toxic to initiate lipid peroxidation. Melatonin also has anti-inflammatory effects and inhibits the activation of neutrophils by free radicals (Reiter *et al.,* 2000).

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Beyond its anti-inflammatory activities, melatonin has been tested for an successfully used in other clinical situations. it was initially taken by transmeridian travelers to quell the severity of jet lag. Thereafter, it became popular as a sleep promoting agent and interest in its use in the suppression of growth of certain cancer types is supported by experimental and clinical observations of a number of scientists (Cardinali *et al*., 2002; Maestroni, 1999). More recently, melatonin has been used as an adjunct treatment in newborn infants suffering with gram-negative bacterial infections and respiratory distress syndrome (Gitto *et al.*, 2004). Both of these serious conditions are believed to be linked to massive toxic free radical generation and the associated tissue damage (Gitto *et al*., 2002).

The pathogenesis of the ischemia/reperfusion damage has been investigated intensively in many organs. As early as 1959 a lack of capillary reperfusion following ischemia was observed in the kidney (Sheehan & Davis, 1959).On the other hand, the protective effect of melatonin against oxidative damage caused by free radicals has been observed in a number of models both in vivo and in vitro (Pieri *et al*., 1994; Reiter *et al.*, 1997). It was shown in a model of liver I/R injury that exogenously administered melatonin protects against oxidative damage. It was suggested that melatonin provides an antioxidative protection in liver (Reiter, 1995; Sewerynek *et al*., 1996). Moreover, it has been shown both experimentally and clinically that the pancreas is highly sensitive to ischemia and hypoxia. However,damaging effect of I/R and the protective effect of melatonin in pancreas especially on b cells has not been studied electron-microscopica.

As free radical scavengers are known to be protective in pancreatic I/R injury, the purpose of this study was to establish the putative protective effect of melatonin against oxidative stress during I/R injury of the pancreas using electron microscopic parameters.

MATERIAL AND METHOD

This study was performed on 60 young male Sprague-Dawley rats (weighing 300 to 400g). They were kept in individual cages in a controlled room (temperature, 20 to 25°C, humidity, 70% to 80 %; exposed to 12 hours of daylight).

The rats were fed with standard rat food and tap water until experimentation. In order for the rats to get used to the laboratory, they were kept waiting for 10 days. Ten hours before the experiment the rats were stopped feeding but allowed free access to tap water. The Dicle University Animal Research Committee approved all experimental procedures.

The rats were anesthetised with intramuscularly injected ketamine hydrochloride (29mg/kg). A midline laparotomy was performed after shaving and local cleansing with antiseptic solution. 60 rats were divided into 6 groups. Group 1 was control, group 2 was sham. Superior mesenteric artery, gastroduodenal artery, inferior splenic artery, left gastric artery and short gastric arteries of rats of group 3 were dissected carefully and then occluded the pancreatic artery by atraumatic microvascular clip (ischemia) for about 30 minutes. In the group 4, the circulation of the pancreatic artery was stopped for 30 minutes and after that a relaparotomy was performed and the clamp was removed. Therefore, circulation was restarted (reperfusion) and continued about 90 minutes. In group 5, 30 minutes before ischemia application 1O mg/kg melatonin was applied intraperitoneally. In the last group 30 minutes after the intraperitoneal melatonin application, ischemia was applied for 30 minutes too. After that 90 minutes reperfusion was performed and animals were sacrified. Pancreatic tissues samples were obtained from the experimental and control groups. The biopsy parts obtained were fixed with 2.5% glutaraldehyde solution with phosphate buffer for histopathological examination. Tissue samples were divided into 1-mm^3 pieces, and after being washed with phosphate buffer, placed in osmium tetroxide and kept for 1 h. In order to obtain dehydration, starting with 50% concentration processed in several alcohol series and in order to clean the specimen from alcohol, the specimens were kept in propylene oxide for 10 min. Then the tissues are placed in a solution containing araldyte and dodecenylsuccinic anhydrate for 12 h. After this process, the specimens obtained with an ultramicrotome were stained with toluidine blue, examined by electron microscopy (Carl Zeiss EM900, Jena, Germany), and photographed when necessary. Histopathologic examination in thin sections revealed the chromatin structure of the nucleus, unexpected shape of RER and degeneration of the mitochondria (loss of crista=cristalysis). Appearence of giant vacuoles, dilatation of nuclear membrane and RER cisternae, lipid and myeline deposition were taken as sign of degeneration in the b cells of pancreas. Evaluation was made by electron-microscopic examination of the pancreas specimens. If degeneration criteria were observed more or less during the evaluation of pancreas slices then degeneration was considered positive; For example, if the nuclear inner and outer membrane are separated from each other, degeneration was accepted to be present without considering the amount of dilatation. Also wiping out of the crista, completely or partially and disruptions in their configurations partially or completely were accepted as signs of degeneration.

RESULTS

The pancreas sections obtained from groups 2 to group 6 were compared with the results of the control group (group 1) in regard to degeneration parameters. The results are shown in Table I. The numbers of pancreas specimens meeting degenerative criteria are shown quantitatively in Table I. If there were no degeneration criteria observed, the result is shown as "0" (zero). The pancreatic tissues of control and sham rats were found to be completely normal (Figs. 1 and 2). There were significant differences in the appearence of giant vacuoles, dilatation in nuclear mebrane, occurrence of myeline figures, dilated GER cisternae which makes giant vacuoles in some areas, huge mitochondria and cristalysis in mitochondria when pancreatic samples of group 4 were compared to the control group. Although, some regenerative changes were seen, beta cells had large degenerative areas in ischemia group also. Dilatation between the nucleus inner and outer membrane were still prominent. Dilatation in GER cisternae, lipid droplet, myeline figures and giant mitochondria were observed in this group (Fig. 4). Melatonin applied before I/R application show degenerative changes but not as severe as in groups 3 and 4. There was still dilatation between the nuclear inner and outer membrane and there were some vesicular structures between perinuclear space. GER cisternae were still dilated in some areas, mitochondria had crystolysis, myeline figures in dilated GER cisternae were seen also (Fig. 5). There were no significant changes in group 5, indicating there was almost no degeneration in beta cells. The electron-microscopic findings in beta cells as shown in figure 6 serve an minimal dilated areas in GER sisternaes and showed very little miyeline figures.

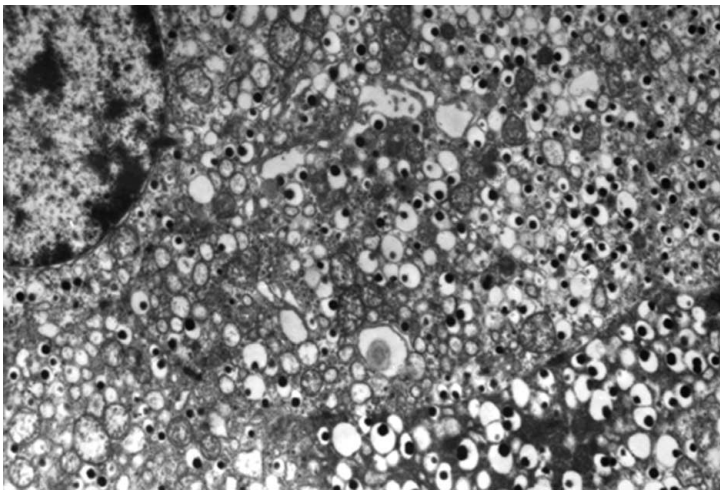

Fig. 1. Normal appearance of beta cells. (Uranyl acetate Lead citrate, Original magnification X 3000).

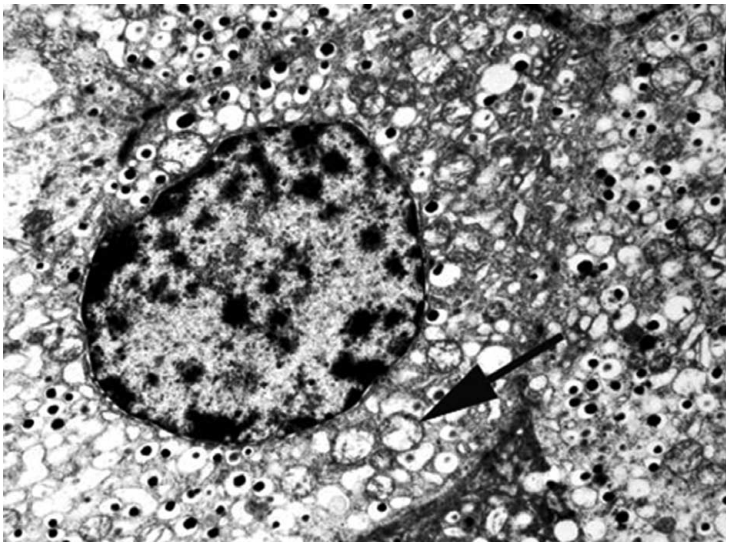

Fig. 2. Electron micrography of a sham-treated beta cell shows minimal crystalysis in mitochondria (Uranyl acetate Lead citrate, Original magnification X 3000).

Table I. Total results from the study group, G1 group1, G2 group 2, G3 group3, G4 group 4, G5 group 5, G6 group 6, n number of rats.

Electron-microscopic criteria	G1 (n=10)	G 2 =10)	G3 (n=10)	G4 (n=10)	G5 (n=10)	G6 (n=10)
Big mitochondria	0	0	2	3	0	0
Giant vacuoles	0	0	0	5	0	0
Dilated RER cisternae	0	1	3	4	2	2
Nuclear membrane dilatation	0	0	2	4	0	2
Lipid granules	0	0	2	2	0	1
Myelin figures	0	0	1	3	2	2
Crystolysis	0	0	0	1	0	3

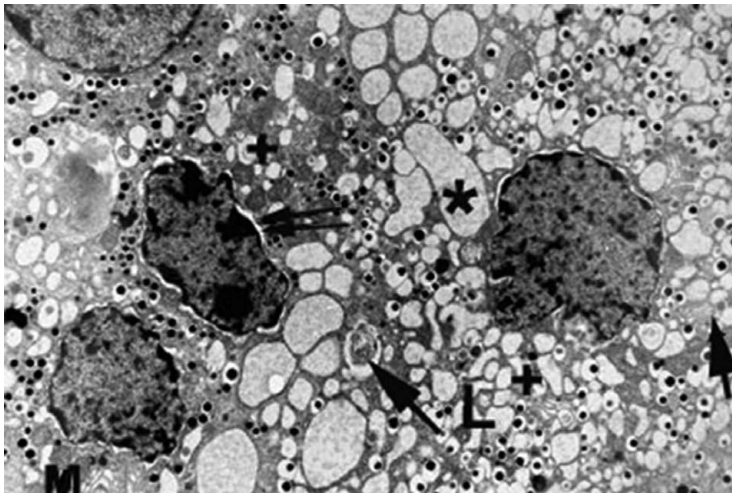

Fig. 3. In I/R group, separation between inner and outer membrane of nucleus (double arrow) and large vacuoles filled with some electron dense material (*), myelin figures (Æ), dilated GER cisternaes (+), big mitochondria (M) and lipid granules (L) were seen (Uranyl acetate Lead citrate, Original magnification X 3000).

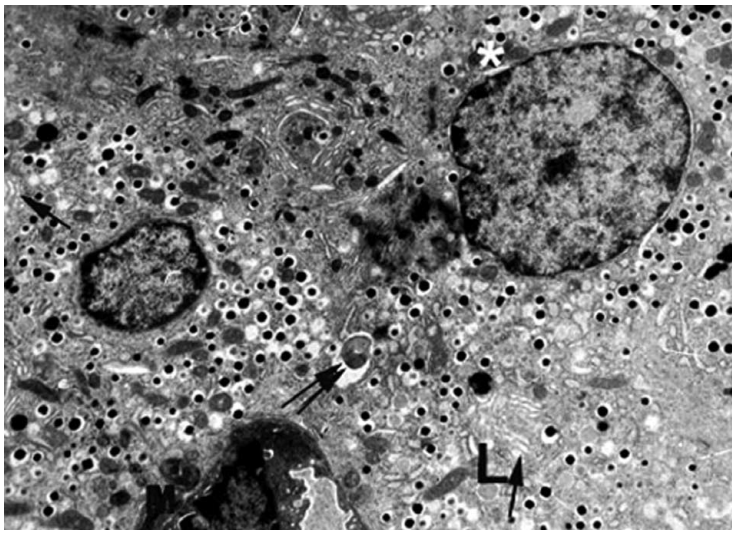

Fig. 4. Dilate RER of Ischemia group (Æ), lipid granules (L), myeline figures (double arrow), giant mitochondria (M) and dilated nuclear membrane (*) were seen in ischemia group (Uranyl acetate Lead citrate. Original magnification X 3000).

DISCUSSION

It is well known that patients were undergone pancreas transplantation and cardiac surgery with cardiopulmonary bypass and aortic aneurysm frequently have tried to elucidate the effects of hypoperfusion and ischemia plus reperfusion on the pancreas by using various experimental rat models such as the injection of microspheres into pancreatic arteries, the temporal ligation of the coeliac trunk and superior mesenteric artery and the clamping of the gastroduodenal, splenic, left gastric artery and short gastric arteries. In the current study, a rat model of ischemia reperfusion was prepared by selectively blocking the inferior splenic artery with nontraumatic vascular clamp and removing the clamp under the microscope.

Ligation of the coeliac and superior mesenteric arteries and the clamping of the gastroduodenal, splenic, left gastric and short gastric arteries may produce complete ischemia of the whole pancreas; however, they might also induce the ischemia on the liver, stomach and small intestines. The impairments of blood supply on those organs may secondarily have a harmful influence on pancreatic function. Therefore, we produced the ischemia to the splenic lobe pancreas by selectively clamping the inferior splenic artery which feeds the splenic lobe of pancreas (Hochachka, 1986; McCord, 1985).

Despite distinct interorgan differences concerning pathophysiology and manifestation of ischemia/reperfusion injury some general characteristic have been identified. Ischemia and hypoxia of tissue result in a depletion of tissue energy stores (energy rich phosphates) due to insufficient oxygen supply (Hochachka). Hemorrhagic shock leads to a drastic decrease of ATP reserves and an accumulation of the matebolite hypoxanthin in liver, intestine and pancreas. Further reduction of hypoxanthin to uric acid is prevented due to lack of O_2. Ischemia results not only in catabolization of ATP, but also in conversion of NAD-reduced xanthine hydrogenase, into the oxygen radical producing xanthine oxidase. Calcium dependent activation of proteases is responsible for the conversion of the enzyme.These reactions have been proven to occur in acute experimental pancreatitis (McCord). Reperfusion and reoxygenation of tissue in the presence of hypoxanthine and xanthine oxidase leads to production of aggressive oxygen radicals (Cunningham & Keaveny, 1977; Zimmerman & Granger, 1994; Sanfey *et al*., 1985). This phenomenon has also been demonstrated in experimental and clinical pancreatitis (Schoenberg *et al.*, 1994; Granger *et al.,* 1981).

Free oxygen radicals and the resultant activation of leucocytes, which has been shown in many different organs such as gastrointestinal tract

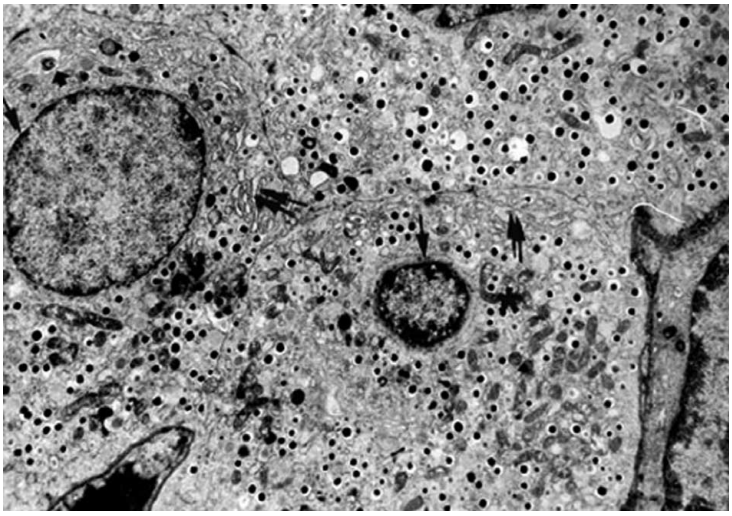

Fig. 5. Melatonin-ischemia-reperfusion group was shown better than ischemia and I/R groups. Dilated nuclear membrane and vesicles between them (Æ), dilated RER cisternae (double arrow), crystolysis in mitochondria (*) and myeline figures between dilated RER were seen (Uranyl acetate Lead citrate. Original magnification X 3000).

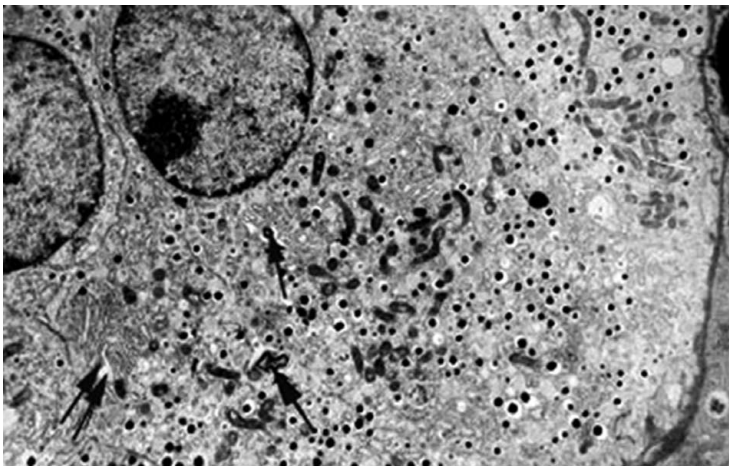

Fig. 6. Almost normal structural appearence of pancreatic tissue was seen in melatonin-ischemia group. Minimal dilatation in RER (double arrow), and small myeline figures in dilated RER cisternae (Æ) were seen (Uranyl acetate Lead citrate. Original magnification X 3000).

(Sanfey *et al*.; Gross *et al*., 1994; Rauen *et al*., 1994), liver (Perry & Wadhwa, 1988), stomach (Harris & Skalak,1993) and skeletal muscle (Schulz & Niederau, 1994) are important for tissue damage in the framework of postischemic reperfusion injury. The pivotal role of oxygen radicals and leucocytes for the deveplopment of acute pancreatitis is also a known fact. In vitro studies on isolated acinar cells have demonstrated that these cells are highly suspectible to oxidative stress and consecutive destruction of cell organelles. These changes can be prevented by antioxidants (Reiter *et al*., 2001a).

In the present study, we investigated the islet's b cells with electron microscopy on I/R and melatonin aplication before ischemia. Our results demonstrate that I/R application has damaging effects on this cells and melatonin appears to play a cytoprotective role.

The pineal hormone melatonin has recently been shown to have free radical scavenging ability (Tan *et al*.; Reiter *et al.,* 2001a). Pierrefiche *et al.* were the first to show that melatonin reduces lipid peroxidation which is devastating to the functional integrity of the cell mebranes. Lipid peroxidative stress is known to decrease membrane fluidity in microsomes and other cellular membranes. Alterations in membrane fluidity have major consequences in terms of cellular function and, thus, melatonin's ability to stabilize membranes may further contribute to cell protective actions of this molecule (Garcia *et al*., 1997).

In this study, we show that membrane structure especially nuclear membrane is very sensitive to I/R. On the other hand melatonin application has reversed the dilated nuclear envelope to its normal stuructural appearence.

The result of oxygen radical formation is damage to an array of biomolecules found in tissues, including nucleic acids, membrane lipids, enzymes, and receptors. Membrane-associated polyunsaturated fatty-acids are readily attacked by OH in a process that results in the peroxidation of lipids. Peroxidation of membrane lipids can disturb membrane fluidity and cell compartmentation, which can result in cell lysis. Thus oxygen radical-initiated lipid peroxidation and protein oxidation may contribute to the impaired cellular function and necrosis associated with reperfusion of ischemic tissues (Cuzzocrea & Reiter, 2002; Reiter *et al*., 2001b) Our study revealed that pancreatic I/R caused significant degenerative changes on pancreatic b cells especially on membranous organelles and this observation is in agreement with previous studies.

In conclusion, as administration of melatonin prevented pancreatic malfunction and inhibited the generation of free radicals in damaged pancreatic tissue, melatonin appears to play a cytoprotective role in the pancreas damaged by I/R. It seems likely that melatonin with its efficiency as a free radical scavenger and an antioxidant, merits consideration as a potential therapeutic agent in pancreatic I/R injury.

YILDIRIM, A.; TUNCER, M. C.; PAMUKÇU, O.; AKTAS¸, A. & AKKUS, M. Efectos de la isquemia/reperfusión sobre células ß del páncreas y efectos protectores del tratamiento de melatonina. ***Int. J. Morphol., 27(2)***:519-525, 2009

RESUMEN: Los radicales libres del oxígeno son considerados como uno de los componentes más importantes que participan en las alteraciones fisiopatológicas del tejido durante la isquemia-reperfusión (I/R). En este estudio, se investigó el supuesto efecto protector del tratamiento de melatonina sobre la lesión pancreática I/R. Ratas Sprague Dawley machos fueron sometidas a 30 minutos de oclusión del pedículo pancreático seguido de 90 minutos de reperfusión. La melatonina (10 mg/kg) fue administrada 30 minutos antes de la isquemia o de la aplicación I/R. Al finalizar los periodos de reperfusión, las ratas fueron decapitadas. Fueron tomadas muestras pancreáticas para el análisis en microscopía electrónica de transmisión. Los resultados indicaron que la isquemia ocasionó daño en las células ß demostrado por la dilatación entre el núcleo interior y la membrana exterior y la degeneración de los islotes de células pancreáticas, los que fueron revertidos por el tratamiento de melatonina. Como la administración de melatonina revirtió estos daños microscópicos, parece probable que ella proteja al tejido pancreático contra el daño oxidativo.

PALABRAS CLAVE: Páncreas; Células ß; Melatonina; Isquemia; Reperfusión.

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