Influence of heme oxygenase-1 gene transfer on the viability and function of rat islets in *in vitro* culture

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Abstract

AIM: To investigate the influence of heme oxygenase-1 (HO-1) gene transfer on the viability and function of cultured rat islets *in vitro*.

METHODS: Islets were isolated from the pancreata of Sprague-Dawley rats by intraductal collagenase digestion, and purified by discontinuous Ficoll density gradient centrifugation. Purified rat islets were transfected with adenoviral vectors containing human HO-1 gene (Ad-HO-1) or enhanced green fluorescent protein gene (Ad-EGFP), and then cultured for seven days. Transfection was confirmed by fluorescence microscopy and Western blot. Islet viability was evaluated by acridine orange/propidium iodide fluorescent staining. Glucose-stimulated insulin release was detected using insulin radioimmunoassay kits and was used to assess the function of islets. Stimulation index (SI) was calculated by dividing the insulin release upon high glucose stimulation by the insulin release upon low glucose stimulation.

RESULTS: After seven days culture, the viability of cultured rat islets decreased significantly ($92\% \pm 6\%$ vs $52\% \pm 13\%$, $P < 0.05$), and glucose-stimulated insulin release also decreased significantly (6.47 ± 0.55 mIU/L/30IEQ vs 4.57 ± 0.40 mIU/L/30IEQ, 14.93 ± 1.17 mIU/L/30IEQ vs 9.63 ± 0.71 mIU/L/30IEQ, $P < 0.05$). Transfection of rat islets with adenoviral vectors at an MOI of 20 was efficient, and did not impair islet function. At 7 d post-transfection, the viability of Ad-HO-1 transfected islets was higher than that of control islets

($71\% \pm 15\%$ vs $52\% \pm 13\%$, $P < 0.05$). There was no significant difference in insulin release upon low glucose stimulation (2.8 mmol/L) among Ad-HO-1 transfected group, Ad-EGFP transfected group, and control group ($P > 0.05$), while when stimulated by high glucose (16.7 mmol/L) solution, insulin release in Ad-HO-1 transfected group was significantly higher than that in Ad-EGFP transfected group and control group, respectively (12.50 ± 2.17 mIU/L/30IEQ vs 8.87 ± 0.65 mIU/L/30IEQ; 12.50 ± 2.17 mIU/L/30IEQ vs 9.63 ± 0.71 mIU/L/30IEQ, $P < 0.05$). The SI of Ad-HO-1 transfected group was also significantly higher than that of Ad-EGFP transfected group and control group, respectively (2.21 ± 0.02 vs 2.08 ± 0.05 ; 2.21 ± 0.02 vs 2.11 ± 0.03 , $P < 0.05$).

CONCLUSION: The viability and function of rat islets decrease over time in *in vitro* culture, and heme oxygenase-1 gene transfer could improve the viability and function of cultured rat islets.

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Key words: Islet viability; Islet function; Heme oxygenase-1; Gene transfer; Adenoviral vectors

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INTRODUCTION

Diabetes affects more than 200 million people worldwide^[1]. The mainstay treatment for type I diabetic patients is chronic insulin injection. While exogenous insulin therapy has dramatically reduced mortality in diabetes, patients often succumb to the long-term sequelae of diabetic angiopathy, either in the form of nephropathy, neuropathy or retinopathy. Vascularized pancreas transplantation reliably restores normoglycemia and maintains long-term glucose homeostasis, but it has significant surgical morbidity and mortality^[2]. In 2000, with the success of the 'Edmonton protocol', which had produced insulin independence in 85% of type I diabetic patients one year after allogeneic islets transplantation combined with a non-steroid immunosuppressive regimen, islet transplantation has progressed from research to clinical reality^[3].

The technique to maintain isolated islet preparations in tissue culture has been adopted by most islet transplant centers. Islet culture for a brief period (24 to 72 h) has emerged as the current standard procedure prior to clinical transplantation^[4,5]. It offers advantages over immediate infusion post-isolation, enabling assessment of islet quality and safety, and reducing islet immunogenicity as well as recipient travel to the transplantation site and immunosuppression before transplantation^[6]. But *in vitro* culture of islets has been shown to result in loss of viable tissue over time, and a decrease in glucose responsiveness has been observed in those islets which survive^[7,8].

Several phenomena, including activation of free radicals, apoptosis and necrosis, may be responsible for these effects. Therefore, approaches towards enhancing islets resistance to these insults would facilitate both clinical and investigative trials. As a cellular graft, islets are especially suited for gene therapy. An attractive strategy for protecting islets in *in vitro* culture is to use gene therapy to transduce islets with cytoprotective genes that can make islets more resistant to injury.

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in the heme degradative pathway that catalyzes the oxidation of heme into biliverdin, carbon monoxide (CO), and free iron^[9,10], and it has been described as a ubiquitous inducible stress protein capable of cytoprotection *via* radical scavenging and apoptosis prevention. Overexpression of HO-1 by chemical induction or gene therapy has been used to reduce the deleterious effects of oxidative stress and apoptosis in various cell types and animal models^[11-14].

Compared with chemical induction, gene transfer can provide effective, targeted, and relatively persistent expression of HO-1. The aim of the present study was to investigate the influence of HO-1 gene transfer on the viability and function of cultured rat islets, and to explore the potential value of HO-1 gene transfer in islet transplantation.

MATERIALS AND METHODS

Animals

Twenty male Sprague-Dawley rats weighing 250 to 300 g were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences.

Rat islet isolation and culture

Rat islets were isolated from the pancreata of the outbred male Sprague-Dawley rats by a collagenase digestion technique and discontinuous Ficoll density gradient centrifugation^[15]. The main bile duct was located and clamped at both ends. Ten milliliters of collagenase P (Roche Applied Science, Indianapolis, Ind, USA) solution (1 g/L, pH 7.8) was injected into the duct and then the distended pancreas was surgically resected, and incubated at 38°C for 15 min. The digested gland was vigorously shaken for 10 s and the digestion was stopped by Hank's solution (4°C) with 100 mL/L fetal calf serum (Gibco, BRL, USA). The tissue was filtered through a 600 µm screen, and then washed by Hank's solution twice. Islets were purified by centrifugation at 3000 r/min for 20 min on discontinuous Ficoll (Pharmacia Fine Chemicals,

Uppsala, Sweden) gradients. After several washes with Hank's solution, islets were suspended in RPMI-1640 medium (Gibco, BRL, USA) containing 100 mL/L fetal calf serum (Gibco, BRL, USA), 20 mmol/L HEPES (Sigma-Aldrich Chemicals, Louis Mo, USA), 100 kU/L of penicillin and 100 g/L of streptomycin at 37°C in a humidified atmosphere of 50 mL/L CO₂. Islets purity was assessed by dithizone (Sigma-Aldrich Chemicals, Louis Mo, USA) staining, and islets were counted and scored in size. An algorithm was used for the calculation of 150 µm-diameter islet equivalent number (IEQ).

Adenoviral vectors

Adenoviral vectors were prepared by use of the AdEasy system (Stratagene, Baltimore, USA). Human HO-1 cDNA was cloned into pAdTrack-CMV. Once constructed, the shuttle vector was linearized with *Pme* I and co-transformed into *E. coli* BJ5183 together with pAdEasy-1, the supercoiled viral DNA plasmid. Transformants were selected on kanamycin and the recombinants were subsequently identified by restriction digestion. Once a recombinant was identified, it was produced in bulk using the recombination-deficient XL10-Gold[®] strain. Purified recombinant adenoviral plasmid DNA was then linearized by *Pac* I to expose its inverted terminal repeats (ITR) and transfected into HEK293 cells where deleted viral genes necessary for virus assembly were complemented *in vivo*. The pAdEasy-1 vector will not replicate in cells other than complementing cells (293 cells). This vector has been developed to infect but not replicate in non-permissive target cells. Ad-EGFP was generated using the same system and supplied by the Institute of Genetics of Fudan University. Viral titers were determined by plaque assay and expressed as plaque forming units per mL (pfu/mL). Viral titers of Ad-HO-1 and Ad-EGFP were 1.96×10^9 and 1.99×10^9 pfu/mL, respectively.

Adenovirus infection

Aliquots of 30 IEQ were resuspended in 0.5 mL serum-free culture medium and placed in a 24-well culture plate and incubated with Ad-HO-1 and Ad-EGFP vectors at a multiplicity of infection (MOI) of 20 at 37°C for 4 h with agitation every 1 h. MOI was calculated using the assumption that islets contain on average 1000 cells. After infection, islets were washed twice with culture medium and incubated for at least 48 h before further analysis to allow for transgene expression. Control islets were mock infected. Mock infected islets underwent a similar procedure, but were not exposed to viruses during the incubation period and were not incubated with any vectors.

Western blot analysis

Islet cells (48 h post-transfection) were washed with cold phosphate buffered saline (PBS) and lysed in 2% SDS, Tris-HCl 60 mmol/L (pH 6.8) buffer, incubated at 95°C, sonicated in a water bath at 37°C and centrifuged at 12000 r/min for 15 min. Assessment of the total protein content was carried out with the BCA detection kit (Pierce Biotechnology, Rockford, IL, USA). Aliquots corresponding to 100 µg of protein were subjected to electrophoresis on a 15% SDS-PAGE pre-cast gel

Figure 1 Purity and viability of freshly isolated rat islets. **A:** Islets stained with DTZ (red staining indicates islets; islet purity was above 90%); **B:** Islets stained with AO/PI (green staining indicated live cells, red staining indicated dead cells; islet viability was above 90%). Bar = 150 μ m, $\times$ 200 magnification.

and transferred electrophoretically to a nitrocellulose membrane. The membranes were incubated with 50 g/L non-fat dry milk in TBS (20 mmol/L Tris, 500 mmol/L NaCl, pH 7.5) overnight at 4°C to block non-specific binding. The blots are then incubated with the murine antihuman HO-1 monoclonal antibodies (StressGen, Victoria, BC, Canada) at a dilution of 1:200 for 2 h at room temperature; this was followed by a 1 h incubation with the AP-conjugated rabbit antimouse polyclonal antibody (Promega, USA) at a dilution of 1:1000. The protein bands were visualized by the NBT + BCIP staining system (Haoyang Biological Manufacture Co, Ltd, Tianjin, China).

Islet viability

Islet viability was evaluated by acridine orange (AO)/propidium iodide (PI) (Sigma-Aldrich Chemicals, Louis Mo, USA) fluorescent staining. The fluorescent dye, containing 10 μ L AO (670 μ mol/L) and 1 mL PI (750 μ mol/L), was used at a 1:10 dilution. After the islets were washed twice with Hank's solution, the fluorescent dye was added to each well. Ten minutes later, islets were analyzed under a fluorescence microscope. Living cells were identified by green staining (AO), whereas dead cells showed a brown-red staining (PI).

Islet function

In vitro function of freshly isolated islets and cultured islets was assessed by glucose-stimulated insulin release. Islets of different groups were washed with PBS twice, and incubated first in low (2.8 mmol/L) and then in high (16.7 mmol/L) concentrations of glucose in culture medium. The static incubation assay was performed in a 24-well flat-bottomed culture plate with 30 IEQ/well and 3 duplicate wells for each islet group. Supernatant from each well was collected after each 1 h incubation, and the concentration was measured using an insulin radioimmunoassay kit (Jiuding Biotech Co, Ltd, Tianjin, China). Stimulation index (SI) was calculated by dividing the insulin release upon high glucose by the insulin release upon low glucose stimulation.

Statistical analysis

Data are expressed as mean $\pm$ SD. Statistical and graphical analysis was performed with software of SPSS10.0. Analyses were performed using the two-tailed Student's t-test where appropriate.

Figure 2 Effect of culture on glucose-stimulated insulin release. The insulin release upon either high or low glucose stimulation decreased conspicuously after seven days culture, $P < 0.05$

RESULTS

Purity and viability of freshly isolated rat islets

The purity of freshly isolated islets was above 90% calculated from the ratio of dithizone stained cells to dithizone nonstained cells as a percentage of the total cell number (Figure 1A). The viability of freshly isolated islets was above 90% calculated from the ratio of AO staining cells (living cells) to PI staining cells (dead cells) as a percentage of the total cell number (Figure 1B).

Effect of *in vitro* culture on insulin release

The glucose-stimulated insulin release was used to assess the function of cultured rat islets. As shown in Figure 2, after 7 d culture, the insulin release upon low and high glucose stimulation decreased from 6.47 ± 0.55 to 4.57 ± 0.40 mIU/L/30IEQ, and from 14.93 ± 1.17 to 9.63 ± 0.71 mIU/L/30IEQ, respectively. In other words, islet function decreased conspicuously over time in *in vitro* culture.

Exogenous gene expression in adenovirus-transduced rat islets

Typical fluorescence micro-photographs of rat islets transfected with Ad-EGFP at an MOI of 20 were taken at 48 h post-transfection. As shown in Figure 3A and B, the fluorescence in Ad-EGFP transfected islets was intense. Western blot was used to detect the expression of human HO-1 protein in three groups of islets. Figure 4 shows that human HO-1 protein was detected in Ad-HO-1 transfected islets but not in uninfected or Ad-

Figure 3 Ad-EGFP transduced islets. Microphotographs were taken at 48 h post-transfection (A, B). Fluorescence was intense at an MOI of 20 (B). Bar = 200 μ m, $\times$ 100 magnification.

Figure 4 Western blot for hHO-1. Islets infected with Ad-HO-1, Ad-EGFP, or mock infected for 48 h were examined by Western blot. HO-1 expression was only detected in Ad-HO-1 group. A: control group; B: Ad-HO-1 group; C: Ad-EGFP group.

EGFP transfected islets. Therefore, adenovirus mediated exogenous gene transfer into rat islets was successful.

Effect of HO-1 gene transfer on the viability of cultured rat islets

The viability of islets was assessed by AO/PI fluorescent staining.

Representative pictures are shown in Figure 5. The viability of control islets was greatly reduced as shown by the large number of dead cells within the islets (Figure 5A). Nevertheless, the number of dead cells was reduced when islets were transfected with HO-1 gene (Figure 5B). Quantitative analysis of islet viability was performed by using fluorescence microscopy to determine the proportion of living cells within islets. As shown in Figure 5C, islet viability decreased greatly after 7 d culture ($92\% \pm 6\%$ *vs* $52\% \pm 13\%$, $P < 0.05$), but HO-1 gene transfer could reverse the viability reduction ($71\% \pm 15\%$ *vs* $52\% \pm 13\%$, $P < 0.05$).

Influence of adenoviral transfection on insulin release

Since adenoviral transfection at a high transfecting dose may damage islets and interfere with their biologic function^[16], glucose-stimulated insulin release was employed to evaluate the influence of adenoviral transfection at an MOI of 20 on insulin release. As shown in Figure 6, there was no significant difference in glucose-stimulated insulin release between Ad-EGFP transfected islets and control islets at 7 d post-transfection (4.30 ± 0.40 *vs* 4.57 ± 0.40 mIU/L/30IEQ, 8.87 ± 0.65 *vs* 9.63 ± 0.71 mIU/L/30IEQ, respectively). It demonstrated that adenoviral transfection at an MOI of 20 was safe for islets.

Effect of HO-1 gene transfer on the function of cultured rat islets

After 7 d culture post transfection, the insulin release upon low and high glucose of uninfected (control islets),

Ad-EGFP transfected, and Ad-HO-1 transfected islets were 4.57 ± 0.40 *vs* 9.63 ± 0.71 mIU/L/30IEQ, 4.30 ± 0.40 *vs* 8.87 ± 0.65 mIU/L/30IEQ, 5.67 ± 0.99 *vs* 12.50 ± 2.17 mIU/L/30IEQ, respectively. SI of control group, Ad-EGFP group, and Ad-HO-1 group were 2.11 ± 0.03 , 2.08 ± 0.05 , 2.21 ± 0.02 , respectively. As shown in Figures 6 and 7, the insulin release upon high level glucose stimulation and SI of Ad-HO-1 transfected islets were significantly higher than those of Ad-EGFP transfected islets and control islets ($P < 0.05$). Therefore, HO-1 gene transfer can improve the function of cultured islets.

DISCUSSION

With recent advances in techniques of islet isolation and the introduction of more potent and less diabetogenic immunosuppressive therapies, islet transplantation has progressed from research to clinical reality, and it has been regarded as a safe and viable route to achieve insulin independence in a population of patients with type I diabetes^[3]. Many transplant protocols incorporate a period of short-term (24 to 72 h) islet culture before transplantation for the recipient to be treated with immunodepleting agents^[4,5,17,18], and to provide time for *in vitro* assessment of islet quality. Short-term islet culture indeed has some benefits, such as purification of the islet preparation, immunomodulation^[6], and possibly improved allograft survival. However, cultured islets are known to degrade rapidly^[19,20], and lose viability and functional responsiveness to glucose stimulation with the extension of culturing time^[7,8]. Islet loss as high as 30% to 50% has been reported after 48 hours of culture^[21].

The islet, once removed from its natural surroundings within the pancreas and placed within the alien environment of the culture plate, becomes deprived of normal physiological organization and exposed to a number of hostile factors, such as hypoxia, activation of free radicals, apoptosis and necrosis that cause its premature demise. The quality of the transplanted islets is of the utmost importance to successfully achieve normal levels of glucose in transplant recipients, therefore, preservation of viability and function of islets post-isolation is a pre-requisite in islet transplantation. Pancreatic islets, as a cellular graft, are especially suited for gene therapy, as they can be infected efficiently *ex vivo* and then transplanted with minimal systemic exposure of the recipient to the vector.

Figure 5 Effect of HO-1 gene transfer on the viability of cultured rat islets. At 7 d post-transfection, the control group showed the presence of numerous dead cells (red) at the center of the islets (A), while the Ad-HO-1 group showed more living cells (B). Bar = 200 μ m, $\times$ 100 magnification. The islet viability was decreased greatly after 7 d culture ($P < 0.05$), whereas HO-1 gene transfer could reduce the loss of islet viability ($P < 0.05$).

Figure 6 Effect of HO-1 gene transfer on insulin release of cultured rat islets. At 7 d post-transfection, no significant difference in glucose-stimulated insulin release was detected between Ad-EGFP transfected islets and control islets, however, the insulin release upon high glucose stimulation of Ad-HO-1 transfected islets was significantly higher than that of Ad-EGFP transfected islets and control islets, $P < 0.05$.

Figure 7 Effect of HO-1 gene transfer on stimulation index of cultured rat islets. The SI of Ad-HO-1 transfected islets was significantly higher than that of Ad-EGFP transfected islets and control islets, $P < 0.05$.

HO-1 has been identified as a ubiquitous stress protein induced in many cell types by various stimulants, such as hemolysis, inflammatory cytokines, oxidative stress, heat shock, heavy metals, and endotoxin^[22]. HO-1 is the rate-limiting enzyme of heme degradation into its byproducts biliverdin, CO, and iron^[9,10]. Biliverdin is subsequently reduced into bilirubin, a powerful anti-oxidant, and it may inhibit the generation of reactive oxygen species^[23].

CO has a cytoprotective role in different systems^[24-26], including pancreatic β -cells^[27]. Heme catabolism by HO-1 also releases free iron, which has the potential to exacerbate the cytotoxic effects of reactive oxygen species^[28,29].

However, generation of intracellular free iron upregulates the expression of ferritin^[28,29], which has a high capacity to store free iron^[29]. Ferritin has been shown to protect endothelial cells against activated neutrophils as well as H₂O₂-mediated cytotoxicity^[29], suggesting that some of the effects of HO-1 may be mediated by ferritin^[28,29]. As HO-1 is not expressed constitutively, it has been demonstrated that overexpression of HO-1 by chemical induction can protect islet cells from apoptosis and improve *in vivo* function after transplantation^[22]. Compared with chemical induction, gene transfer can provide effective, targeted, and relatively persistent expression of HO-1.

Adenoviral vectors are useful for efficient gene delivery

to differentiated and non-proliferating cells, such as isolated pancreatic islets^[30]. In addition, adenoviral vectors can be produced in high titers and there is no risk of insertional mutagenesis as their genomes are not integrated into chromosomes. In the present study, recombinant adenoviral vectors were generated by use of the AdEasy system, and employed to transfect rat islets at an MOI of 20. According to fluorescence photographs taken at 48 h post-transfection, the expression of EGFP was intense. Furthermore, human HO-1 protein was detected by Western blot in Ad-HO-1 transfected islets at the same time. This demonstrated that recombinant adenoviral vectors were efficient to deliver exogenous genes into rat islets *in vitro*. Insulin release upon glucose stimulation was a measure of islet function, which was a prerequisite to any gene therapy program for diabetes treatment by islet transplantation^[31]. In our experiments, no significant difference in glucose-stimulated insulin release was detected between Ad-EGFP transfected islets and control islets at 7 d post-transfection. This is consistent with the results reported by others showing that adenoviral vectors at a low transfecting dose (MOI 20) provided effective transfer of a marker gene into islet cells without impairing cell function^[16].

Even though many clinical islet transplant protocols culture islets for only 24 to 72 h, islets were cultured for seven days in our study. This culture period was selected to minimize the effects of isolation factors on islet function, while maximizing the effect of culture. After

seven days culturing, the insulin release upon either low or high concentration glucose stimulation was significantly lower than that of freshly isolated islets ($P < 0.05$). This is consistent with previous reports showing that islet function degrades in *in vitro* culture over time. Nevertheless, the insulin release upon high concentration glucose stimulation and stimulation index (SI) of Ad-HO-1 transfected islets were significantly higher than those of Ad-EGFP transfected islets and control islets ($P < 0.05$). As glucose-stimulated insulin release was a favourable marker of the function of islets, our study demonstrated that HO-1 gene transfer conferred cytoprotection and improved islet function in *in vitro* culture. This was probably related to the effect of CO, which was not only a stimulator of insulin release but also a trigger of the transients of $[Ca^{2+}]_i$ assumed to coordinate the secretory activity of the β -cells^[32]. Furthermore, results of fluorescence microscopy studies with AO/PI staining indicated that HO-1 gene transfection significantly improved islet viability and survival during *in vitro* culture.

Despite the efficient transfection capacity of adenoviral vectors, the transgene cannot integrate into the host cell genome, leading to transient transgenic expression. In our study, we found that the EGFP expression time in cultured islets was more than 3 wk (data not shown). However, for some therapeutic strategies, such as islet cytoprotection in *in vitro* culture or early after transplantation, temporal expression of the cytoprotective transgene mediated by adenoviral vectors is still recommended because the factors related to early islet injury usually play a major role in the outcome of islet transplantation.

In summary, we demonstrated that adenoviral vectors could successfully transfer exogenous HO-1 gene into rat islet cells, and HO-1 gene transfer could improve rat islet viability and function in *in vitro* culture. Strategies using HO-1 gene transfer to islets might lead to better outcome in islet transplantation.

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COMMENTS

Background

With recent advances in methods of islet isolation and the introduction of more potent and less diabetogenic immunosuppressive therapies, islet transplantation has progressed from research to clinical reality. Islet culture for a brief period (24 to 72 h) has emerged as the current standard procedure prior to clinical transplantation. Short-term islet culture indeed have some benefits, such as purification of the islet preparation, immunomodulation, assessment of islet quality, and possibly improved allograft survival. But *in vitro* culture of islets has been shown to result in a loss of viability over time, and a decrease in glucose responsiveness has been observed for those islets which survive.

As a cellular graft, islets are especially suited for gene therapy applications. HO-1 has been described as a ubiquitous inducible stress protein capable of cytoprotection via radical scavenging and apoptosis prevention. Overexpression of HO-1 can be chemically induced, but compared with chemical induction, gene transfer can provide effective, targeted, and relatively persistent expression of HO-1. It's an attractive strategy to protect islets *in vitro* culture by using gene therapy to transduce islets with cytoprotective gene that can make islets more resistant to injury.

Research frontiers

Although advances in islet isolation and less diabetogenic immunosuppression have moved islet transplantation forward from research to clinical reality, many challenges have to be faced with, such as keeping islet viable, single donor grafts, limited donor supply, tolerance induction.

Innovations and breakthroughs

Overexpression of HO-1 by chemical induction can protect islet cells from apoptosis and improve islet function. We used gene technique to modify rat islets *in vitro*, and found that HO-1 gene transfer could protect islet viability and function after seven days culture. Compared with chemical induction, gene transfer can provide effective, targeted, and relatively persistent expression of HO-1.

Applications

HO-1 gene transfer can improve islet viability and function. It suggests a potential therapeutic application for HO-1 gene in improving islet survival/function in islet transplantation.

Terminology

Islet equivalent (IEQ): islets with an average diameter of 150 μ m.

Peer review

In this manuscript, Chen *et al.* have analyzed whether adenovirus-mediated gene transfer of heme oxygenase-1 into rat isolated pancreatic islets, affect the long term viability of the cells. This manuscript is well written, experiments and analyses of data are adequately performed.

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