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Young porcine endocrine pancreatic islets cultured in fibrin show improved resistance toward hydrogen peroxide

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Keywords: endocrine pancreatic islet culture, encapsulation, extracellular matrix, fibrin, hydrogen peroxide, porcine pancreatic islets

Abbreviations: Br-dUTP, 5-bromo-2'-deoxyuridine 5'-triphosphate; BSA, bovine serum albumin; DAB, diaminobenzidine; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; GSIS, glucose-stimulated insulin secretion; H₂O₂, hydrogen peroxide; HBSS, Hank's balanced salt solution; IBMX, 3-isobutyl-1-methylxanthine; IEQ, islet equivalents; PBS, phosphate buffered saline; RT, room temperature; T1DM, type 1 diabetes mellitus; TCPS, tissue culture polystyrene; TdT, terminal deoxynucleotidyl transferase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; UW, University of Wisconsin

Aim: To study the protective effect of a fibrin scaffold toward embedded young porcine endocrine pancreatic islets from hydrogen peroxide within the context of islet encapsulation in transplantation. **Methods:** After isolation and in vitro maturation, groups of 200 young porcine islet equivalents (IEQ) were embedded in a 200 μ L fibrin gel and exposed to 2 concentrations (10 and 100 μ M) of hydrogen peroxide (H₂O₂) to investigate the ability of fibrin to protect islets against apoptotic stimuli. As a control, young porcine islets were seeded in tissue culture polystyrene (TCPS) well plates and exposed to the same H₂O₂ concentrations. Islet integrity, viability and function were then investigated. **Results:** Morphologically, the integrity of islets embedded in fibrin gels was better preserved compared with that of islets cultured in TCPS plates, when exposed to H₂O₂. Immunofluorescence staining showed that insulin and glucagon expression was higher in islets cultured in fibrin. Overall, H₂O₂ incubation led to decreased insulin and glucagon expression. A TUNEL assay revealed elevated numbers of apoptotic cells for islets cultured in TCPS plates when compared with those embedded in fibrin. Islets cultured in TCPS plates and exposed to H₂O₂ had diminished ability to secrete insulin in response to glucose stimulation, whereas islets embedded in fibrin maintained their glucose responsiveness. Insulin trapped in fibrin was extracted and quantified, revealing insulin in the extract. **Conclusions/Interpretation:** Fibrin has a protective effect on young porcine endocrine pancreatic islets exposed to hydrogen peroxide.

Introduction

This study follows the hypothesis that cell-extracellular matrix interactions improve islet integrity, viability and function after exposure to immune system-derived apoptotic stimuli, such as hydrogen peroxide (H₂O₂). Apoptotic stimuli such as H₂O₂ are present during type 1 diabetes mellitus (T1DM) onset, autoimmune β -cell destruction, and after islet transplantation. Islet transplantation could offer a cure for T1DM as the islet graft could restore adequate endogenous insulin secretion for the patient. Even after the introduction of the Edmonton protocol, islet transplantation is still attempting to overcome the low success rates associated with cellular or functional loss of the

graft.¹ Two reasons for loss of such a graft include damage to the native extracellular matrix (ECM) after isolation and transplantation, and later, rejection of the donor graft by the host's immune system.² One of the macrophage-secreted mediators of the immune system response is H₂O₂, a small and uncharged molecule with the capacity to be microbicidal, cytotoxic, and tissue damaging, which can easily pass through the cell membrane.³ Physiological H₂O₂ concentrations during inflammation can range between 5 and 15 μ M.⁴ Recent studies have shown that the islet ECM, and more specifically, cell-integrin interactions, mediate apoptosis resistance and support viability and function of pancreatic β cells.^{5,6} Fibrin, a transient in vivo ECM, has been used to improve culture conditions for mouse

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Table 1. Number of cells stained positively for glucagon, insulin, and both glucagon and insulin, counted on immunofluorescence images of porcine pancreatic islets at 'Time Zero', in fibrin with no (F0), 10 (F10), and 100 (F100) μM H_2O_2 , and on TCPS with no (T0), 10 (T10), and 100 (T100) μM H_2O_2 . Percentages of glucagon- and insulin-positive cells are shown in brackets.

| Condition | Total number of cells counted* (100%) | Glucagon-positive cells | Insulin-positive cells | Positive cells |
|-----------|---------------------------------------|-------------------------|------------------------|----------------|
| Time Zero | 1925 | 443 (23.0%) | 548 (28.5%) | 991 (51.5%) |
| F0 | 2332 | 403 (17.3%) | 482 (20.7%) | 885 (38.0%) |
| F10 | 2348 | 319 (13.6%) | 433 (18.4%) | 752 (32.0%) |
| F100 | 2746 | 164 (6.0%) | 301 (11.0%) | 465 (17.0%) |
| T0 | 4174 | 735 (17.6%) | 368 (8.8%) | 1103 (26.4%) |
| T10 | 3725 | 314 (8.4%) | 194 (5.2%) | 508 (13.6%) |
| T100 | 2908 | 144 (5.0%) | 104 (3.6%) | 248 (8.5%) |

*Variations in the cell numbers counted depend on sample sizes, which vary from one 4- μm -thick paraffin section to another.

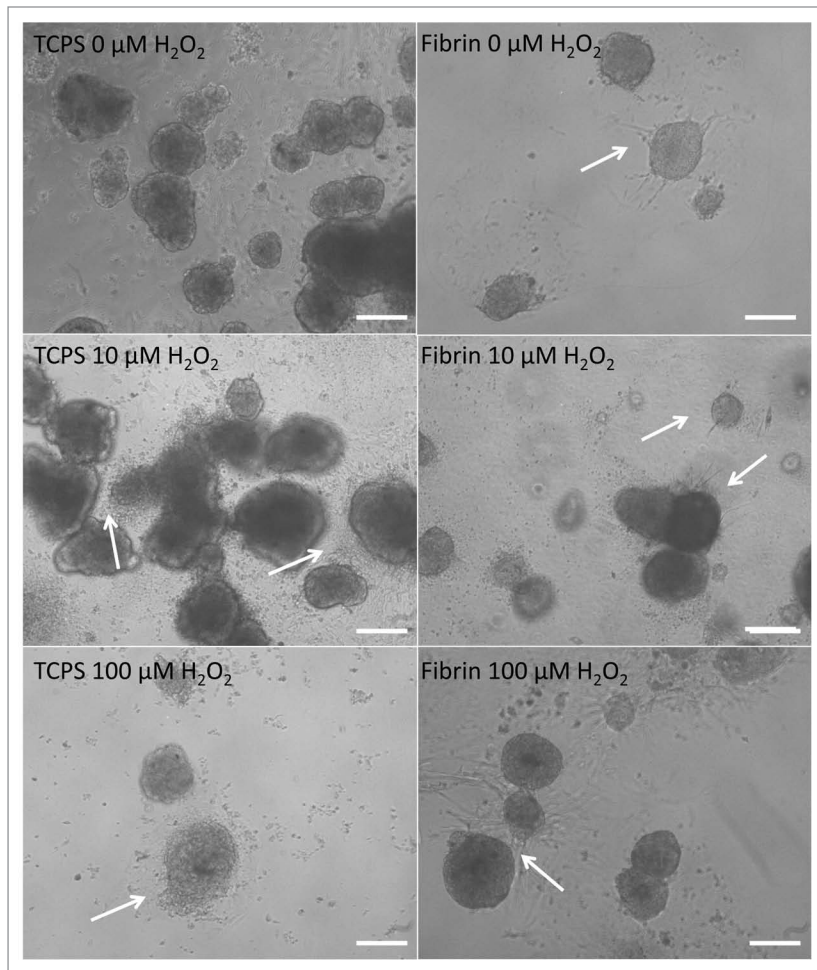


Figure 1. Morphology of young porcine endocrine pancreatic islets cultured for 12 h in TCPS plates with no H_2O_2 , 10 μM H_2O_2 and 100 μM H_2O_2 . Morphology of islets cultured for 12 h in fibrin with no H_2O_2 , 10 μM H_2O_2 and 100 μM H_2O_2 . 10x magnification. Bars represent 50 μm .

and human pancreatic islets before transplantation.^{7,8} Mouse islets pre-cultured in fibrin and implanted in mice have shown improved survival, increased insulin content and yielded a larger population of functional islets.⁷ Human islets embedded in

fibrin gels enriched with perfluorodecalin showed less apoptosis and maintained their insulin secretion in response to glucose.⁸

It is hypothesized that fibrin protects young porcine endocrine pancreatic islets from hydrogen peroxide. In accordance, culturing young porcine endocrine pancreatic islets embedded in fibrin could decrease pre- and post-transplantation-related complications, thus improving islet integrity, viability and insulin secretion. This study is a first step toward improved xeno-islet grafts for human transplantation.

Results

Islets in fibrin maintained their typical morphology whereas islets cultured in TCPS plates dispersed

After the overnight culture and 12 h incubation with 0, 10, and 100 μM H_2O_2 , islets cultured in fibrin showed the first signs of sprouting, forming capillary-like structures (Fig. 1D–E). The occurrence of sprouts decreased with increasing H_2O_2 concentrations. Islets in fibrin were viable with little or no effect from H_2O_2 . Almost no single cells could be observed in proximity of the islets, indicating that islets in fibrin did not start to disintegrate. Islets cultured in TCPS plates were dispersed after incubation with 100 μM H_2O_2 . The grade of dispersion increased with the H_2O_2 concentration (Fig. 1A–C).

Islets cultured in fibrin maintained their responsiveness to glucose concentrations and secreted more insulin than islets in TCPS plates

The results of the glucose-stimulated insulin secretion (GSIS) assay performed after the 12 h H_2O_2 incubation indicated that islets in fibrin not only maintained their responsiveness to different glucose concentrations but also secreted relatively more insulin when compared with their respective controls in TCPS (Fig. 2A). As shown in

Table 2. Number of cells stained positively for integrin α_5 , counted on immunofluorescence images of porcine pancreatic islets at 'Time Zero', in fibrin with no (F0), 10 (F10), and 100 (F100) μM H_2O_2 , and on TCPS with no (T0), 10 (T10), and 100 (T100) μM H_2O_2 .

| Condition | Total number of cells counted* (100%) | Integrin α_5 -positive cells | Percentage (positive) |
|-----------|---------------------------------------|-------------------------------------|-----------------------|
| Time Zero | 2644 | 779 | 29.5% |
| F0 | 2541 | 1790 | 70.4% |
| F10 | 2253 | 1206 | 53.5% |
| F100 | 2300 | 1116 | 48.5% |
| T0 | 2798 | 836 | 29.9% |
| T10 | 2236 | 398 | 17.8% |
| T100 | 2591 | 332 | 12.8% |

*Variations in the cell numbers counted depend on sample sizes, which vary from one 4- μm -thick paraffin section to another.

Figure 2, the insulin secretion of islets in TCPS exposed to 10 and 100 μM H_2O_2 were normalized to the insulin secretion of islets in TCPS cultured without H_2O_2 in response to the initial low glucose concentration (LG) conditions. Similarly, the insulin secretion of islets embedded in fibrin exposed to 10 and 100 μM H_2O_2 were normalized to the insulin secretion of islets in fibrin cultured without H_2O_2 in response to the initial low glucose concentration (LG) conditions. This allows better comparing the effect of culture conditions on insulin secretion. Absolute insulin secretion of 3 islet batches in 3 repeats is shown in **Figure 2B**.

Analysis of the extract revealed that some insulin remained trapped within the fibrin gels (**Fig. 3**). The mass of insulin secreted by 200 IEQ in fibrin with no exposition to H_2O_2 and by those in fibrin exposed to 100 μM H_2O_2 was 0.26 and 0.20 μg , respectively. Therefore, approximately 5–6% of the total mass of insulin remained trapped in the fibrin mesh. Further development is needed to develop an extraction protocol to quantify the insulin trapped in the fibrin.

Insulin and glucagon expression is diminished in islets cultured in TCPS plates

Immunofluorescence staining for insulin and glucagon was performed (**Fig. 4**). Cells staining positively for insulin were manually counted. At least 10 random fields per condition were examined, containing an average of 3 islets per field. Islets cultured in fibrin and on TCPS plates showed decreasing insulin and glucagon expression with increasing H_2O_2 concentration when compared with the 'Time Zero' control (**Table 1**). On TCPS, islets had a lower number of insulin-positive cells than glucagon-positive cells and overall lower levels of expression of both molecules as opposed to fibrin. Islet cells cultured in fibrin had higher numbers of insulin-positive cells than glucagon-positive cells but overall lower expression of both molecules when compared with islets at 'Time Zero'.

Integrin α_5 is strongly expressed in fibrin-embedded islets

Islets were stained for the $\alpha_v\beta_3$ and the α_5 integrins. The staining for $\alpha_v\beta_3$ was overall very weak (data not shown) and showed no differences between the culture conditions. In contrast, the integrin α_5 was expressed more strongly in islets cultured in fibrin compared with islets on TCPS (**Fig. 5**). A decrease in integrin α_5 could be noted with increasing H_2O_2 concentration. **Table 2** lists a manual count of at least 5 fields

with an average of 3 islets per field. A higher percentage of cells expressed α_5 integrin in the fibrin set when compared with islets on TCPS.

Fibrin embedding protects islets against apoptosis

Paraffin sections of islets in each condition were treated with a TUNEL assay and then imaged and counted. At least 10 random fields of each condition performed in triplicate were counted manually (**Fig. 6A and B**). All brownish and black spots within the cell nuclei were counted. Fibrin-embedded islet cells were significantly less apoptotic than the islet cells in TCPS plates. The incidence of apoptosis increased with increasing H_2O_2 concentrations (**Fig. 6C**).

Discussion

This study confirms fibrin has a protective effect when used to embed young porcine endocrine pancreatic islets after exposure to H_2O_2 (10 and 100 μM). It is known that pancreatic islets are separated from their ECM after isolation and consequently lose their typical spherical morphology and disperse if cultured in suspension.⁷ Islet cells may suffer from anoikis, a form of apoptosis induced by cell detachment from its ECM.⁹ Additionally, in this study, elevated islet dispersion in suspension was observed with increasing concentrations of H_2O_2 whereas islets cultured in fibrin exhibited sprouting formation. It has been shown that fibrin gels promote microvessel formation and re-vascularisation of pancreatic porcine islet grafts in mice when transplanted subcutaneously.¹⁰

Recent studies showed that interactions between pancreatic islets and their ECM regulate insulin secretion and cell proliferation, and prevent apoptosis.^{11,12} In this study, we showed that islets cultured on TCPS lost their glucose responsiveness when exposed to H_2O_2 , whereas islets embedded in fibrin maintained it and even secreted relatively more insulin in response to glucose (**Fig. 2**). Without the provision of a three-dimensional environment, isolated pancreatic islets undergo more apoptosis than islets supported by fibrin, agarose, collagen and/or other ECM-like networks during a given culture period before being transplanted. When recovered from those matrices and transplanted, islets showed improved resistance toward apoptosis and improved survival when exposed to the immune system of the

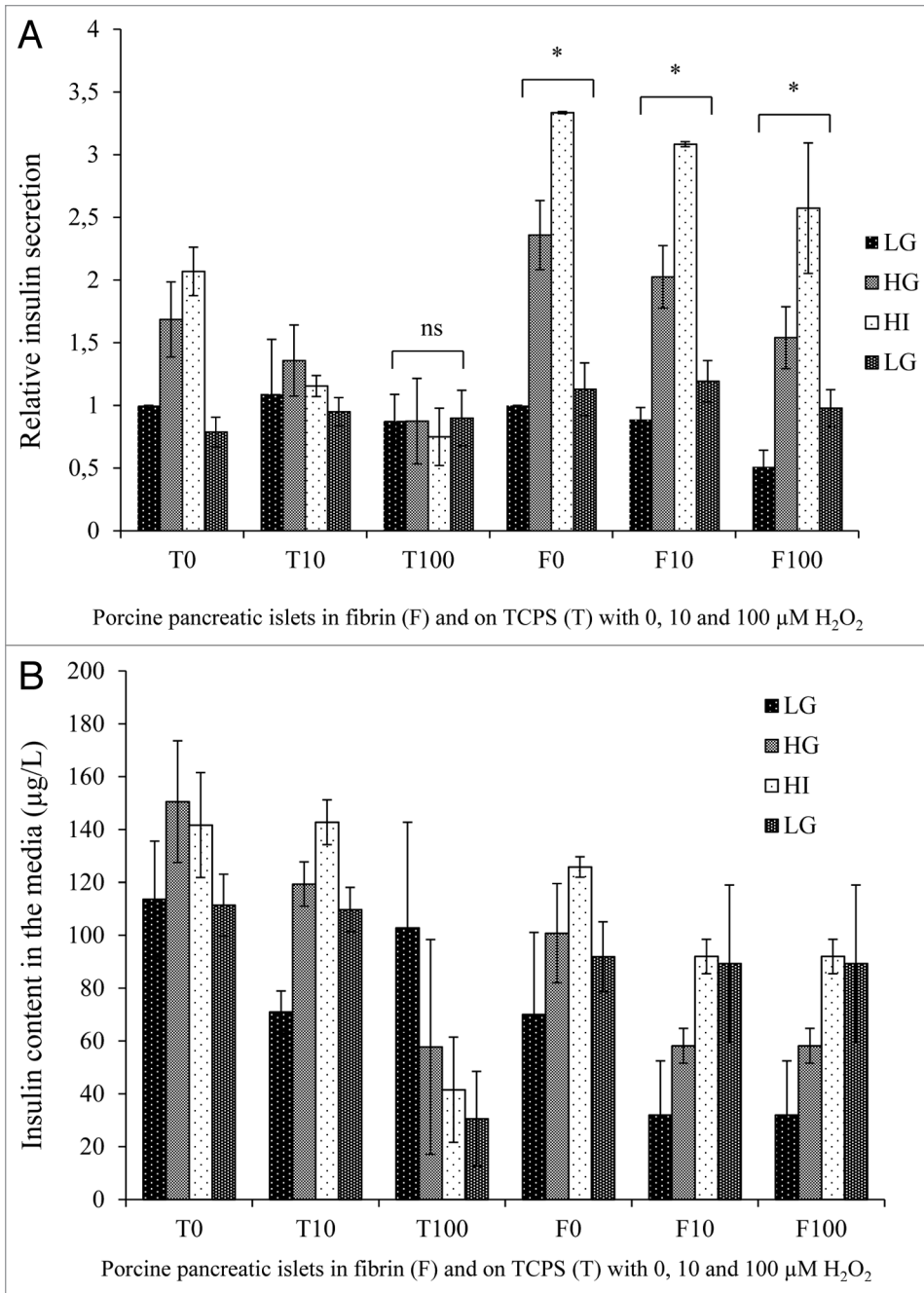


Figure 2. (A) Relative insulin secretion of young porcine endocrine pancreatic islets after 1 h stimulation with low glucose concentration (LG, 2.8 mM), high glucose concentration (HG, 28 mM), high glucose concentration + 3-isobutyl-1-methylxanthine (IBMX) (HI, 28 mM + IBMX) and a second low glucose concentration (LG2, 2.8 mM). Insulin secretion of islets in TCPS/fibrin was normalized to the insulin secretion of islets in TCPS/fibrin with no H₂O₂ in response to low glucose concentration. (B) Three repeats of a glucose-stimulated insulin secretion assay with LG, HG, HI and a final LG. Shown are the insulin contents in the media (microgram per liter) of porcine pancreatic islets (200 IEQ) cultured on TCPS (T) or in fibrin gels (F) with 0, 10 or 100 μM H₂O₂. * represents a value of *P* < 0.05, 'ns' means no statistical significance.

host. A higher insulin secretion in response to glucose was also observed in those islets.^{6-8,11-13} In our cultures, relative insulin secretion was also higher in the fibrin set with islets maintaining their glucose responsiveness whereas non-embedded TCPS

cultures did secrete insulin but were not able to respond to higher glucose concentrations (Fig. 2A). Our results confirm that fibrin-embedded islets are significantly less apoptotic even after exposure to 10 or 100 μM H₂O₂ compared with islets cultured in TCPS plates. The 2 different H₂O₂ concentrations were chosen to represent a physiological concentration i.e., 10 μM, and a higher concentration i.e., 100 μM, shown to induce elevated levels of apoptosis.^{4,14}

Insulin and glucagon expression was better preserved in islets cultured in fibrin (Table 1) but diminished with increasing H₂O₂ concentrations. Overall, numbers of glucagon- and insulin-positive cells appeared to be low, with just 52.5% of all cells at 'Time Zero' staining positively for both. After islet culture, decreasing percentages were observed. These relatively low numbers could be due to the time passed between isolation and culture, the culture time itself, the varying cell composition of pancreatic islets, and/or even the region of origin of the islets within the pancreas.¹⁵

One possible explanation for the beneficial effects of fibrin on the survival of pancreatic islets after H₂O₂ exposure could be the cell-integrin-fibrin interactions.¹¹ It has been shown that integrins are essential for islet survival, resistance to apoptosis, and islet cell maintenance.^{5,6,16} Porcine pancreatic islets express several integrins e.g., α_v and α₅.¹⁷ We found evidence that islets in fibrin and on TCPS expressed integrins, α_vβ₃ and α₅, with a much weaker overall α_vβ₃ expression. Integrin α₅ was expressed more strongly in islets cultured in fibrin than in islets on TCPS and expression decreased with increasing H₂O₂, indicating a possible integrin-dependent protective effect of fibrin toward pancreatic islet cultures (Table 2). After a 12 h culture in fibrin, integrin α₅ expression

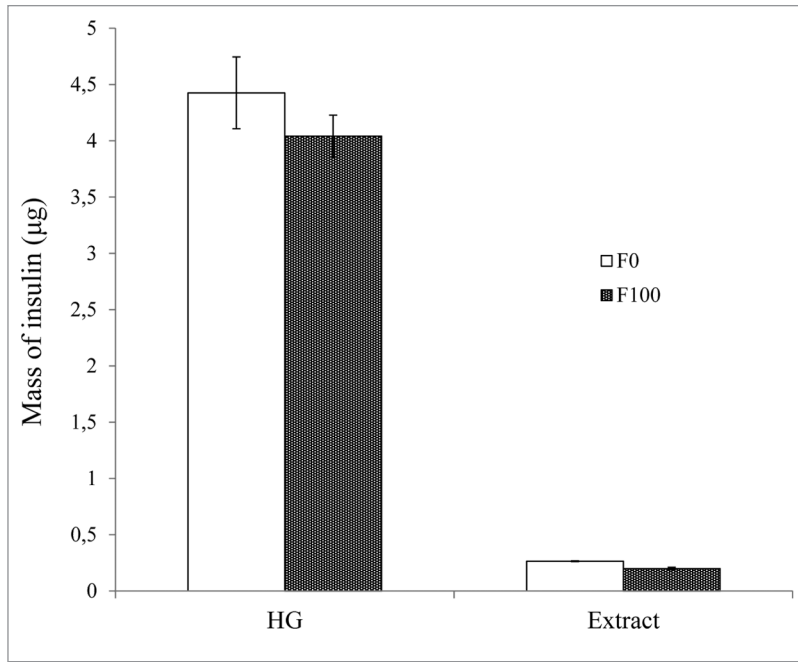


Figure 3. Mass of insulin (in micrograms) secreted into the media by 200 IEQ embedded in fibrin and exposed to 0 (F0) and 100 μM H_2O_2 (F100) in a high glucose concentration condition (HG) and the mass of insulin (in micrograms) in the extract of fibrin gels exposed to the same conditions.

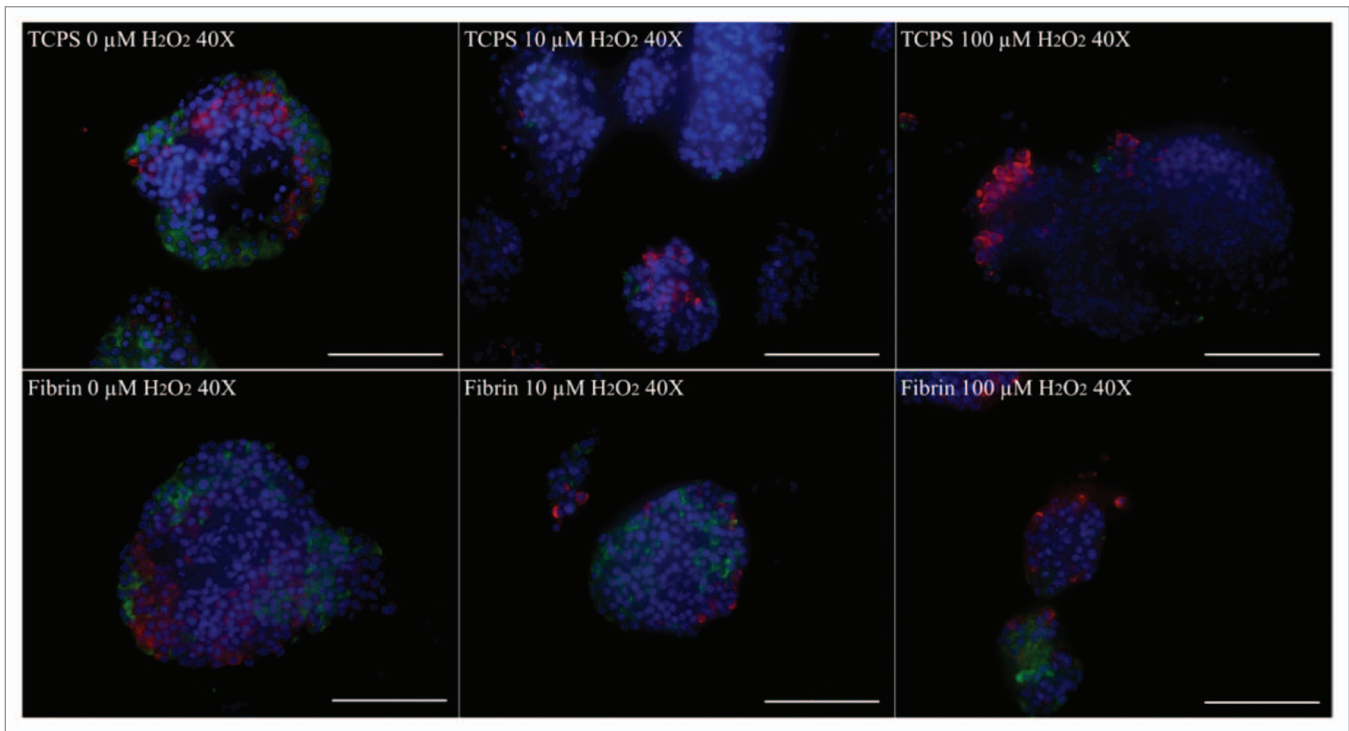


Figure 4. Immunofluorescence staining for insulin (green) and glucagon (red) of young porcine endocrine pancreatic islets. Nuclei are DAPI stained in blue. 40x magnification. Bars represent 100 μm .

related to the available binding sites in the fibrin mesh for the islet cell integrins.^{5,9,11} Immunofluorescence techniques are one of the few methods currently available to investigate integrin expression of islets in fibrin gels. Other traditional methods such

as RT-PCR and Western blotting pose several problems when working with tissues or cells embedded in gels, as the extraction of the cell materials from the tissues (here, the islets) embedded in fibrin, without affecting the cells, is challenging.

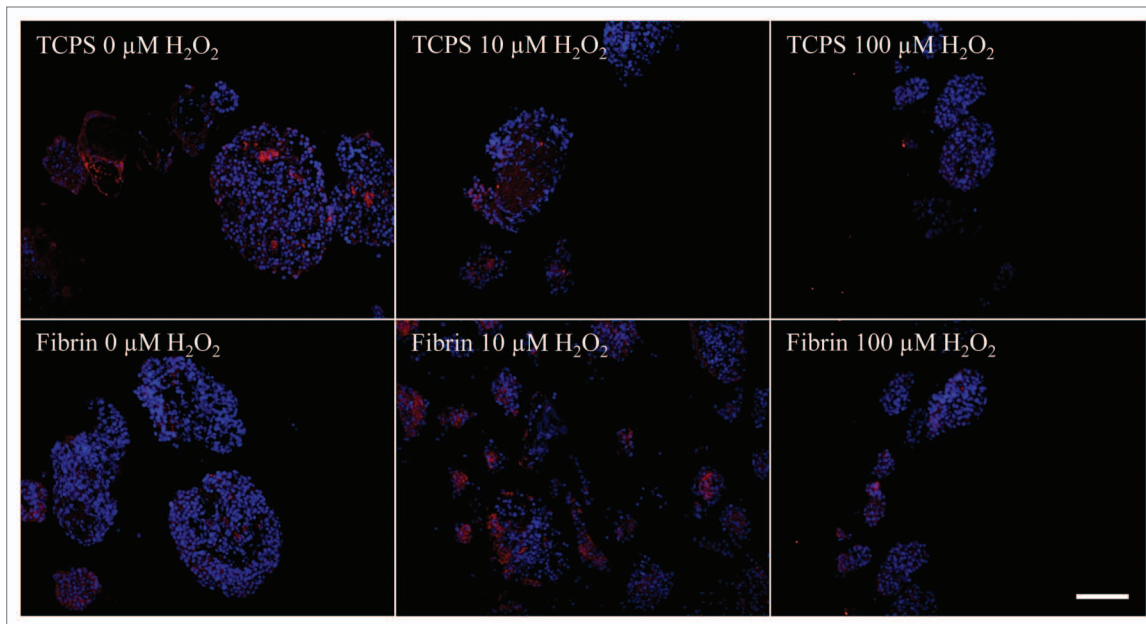


Figure 5. Immunofluorescence staining for integrin $\alpha 5$ (red) of young porcine endocrine pancreatic islets. Nuclei are DAPI stained in blue. 20 \times magnification. Bars represent 50 μm .

Finally, it can be argued that fibrin could simply constitute a mechanical barrier protecting the islets by delaying the H_2O_2 diffusion. It has previously been shown that rhodamine B, a fluorescent dye with a molar mass of $479.02 \text{ g}\cdot\text{mol}^{-1}$, diffuses quite rapidly in fibrin gels (2 mg fibrinogen per mL)¹⁸ considering the time scale (12 h) of the H_2O_2 experiments. Therefore, it can be assumed that the diffusion of H_2O_2 , with a molar mass of $34 \text{ g}\cdot\text{mol}^{-1}$, is not hindered through the fibrin matrix. Interestingly, fibrin was found to retain small amounts of insulin. Theoretically, more insulin could be trapped inside the gels because the extraction method used to extract insulin does not guarantee that all insulin was recovered and/or remained stable during the extraction process. Not measuring all insulin trapped in the gels could yield a lower apparent insulin secretion in 3D cultures.

This study revealed that fibrin, when used to embed young porcine endocrine pancreatic islets, protects those islets from the destructive effects of H_2O_2 . In the context of islet transplantation, these results suggest that fibrin could provide protection for such a graft. Further studies with endocrine pancreatic islets embedded in fibrin will include immune cells and immune system-derived effectors to further investigate the potential protective effects of fibrin.

Materials and Methods

Isolation of young porcine endocrine pancreatic islets

Young porcine endocrine pancreatic islets were provided by the islet isolation facility at the Department of Surgery, University California Irvine, under the ethical protocol IACUC #2008-2823. Pancreata from young Yorkshire pigs (14–22 d) were removed using rapid surgical procurement and placed in University of Wisconsin (UW) solution for organ preservation.

Pancreata were minced and digested in a low dose collagenase MA/BP protease enzyme mixture (001-2020, Vitacyte) for 15 ± 2 min in a 37°C water bath and mixing at 60 rpm. Cold (4°C) HBSS supplemented with HEPES and 10% BSA was added to the tissue-enzyme mixture to stop the enzymatic digestion and the partially digested tissue was force-filtered through a 500 μm metal mesh.

Tissue clusters were then cultured at 37°C , 5% CO_2 in T-175 suspension flasks in media. The medium used was a novel culture medium supplemented with proteins and enzymes designed to improve β -cell function (GLP-1 antagonists, insulin, etc.) and exclusion of exocrine tissue, and containing 10% porcine serum.¹⁹ The islets were allowed to mature for 8 d with media changed every 48 h, which has been shown to further decrease exocrine tissue.²⁰ After which, samples for islet counts as well as for islet viability and function were taken and aliquots were collected, packaged in a 50 mL conical tube with culture medium and shipped via overnight express shipment at room temperature. The total number of IEQ has been calculated as described elsewhere.²¹

Young endocrine porcine islet culture

Upon arrival, porcine endocrine pancreatic islets were inspected, washed and placed in fresh media made of CMRL-1066 medium (11530, Invitrogen) containing 10% fetal bovine serum (FBS, 12483, Invitrogen), 25 mM HEPES (H3375, Sigma-Aldrich), 1% penicillin-streptomycin (15140-122, Invitrogen) and 2 mM L-glutamine (25030-149, Invitrogen) at pH 7.4. Islets were either embedded and cultured in fibrin gels or cultured in tissue culture polystyrene (TCPS) plates. Fibrin gels with a total volume of 200 μL (2 $\text{mg}\cdot\text{mL}^{-1}$ bovine fibrinogen, F8630, Sigma-Aldrich) containing 200 IEQ were prepared in a 48-well TCPS plate as described previously.²² After jellification with 1 $\text{U}\cdot\text{mL}^{-1}$

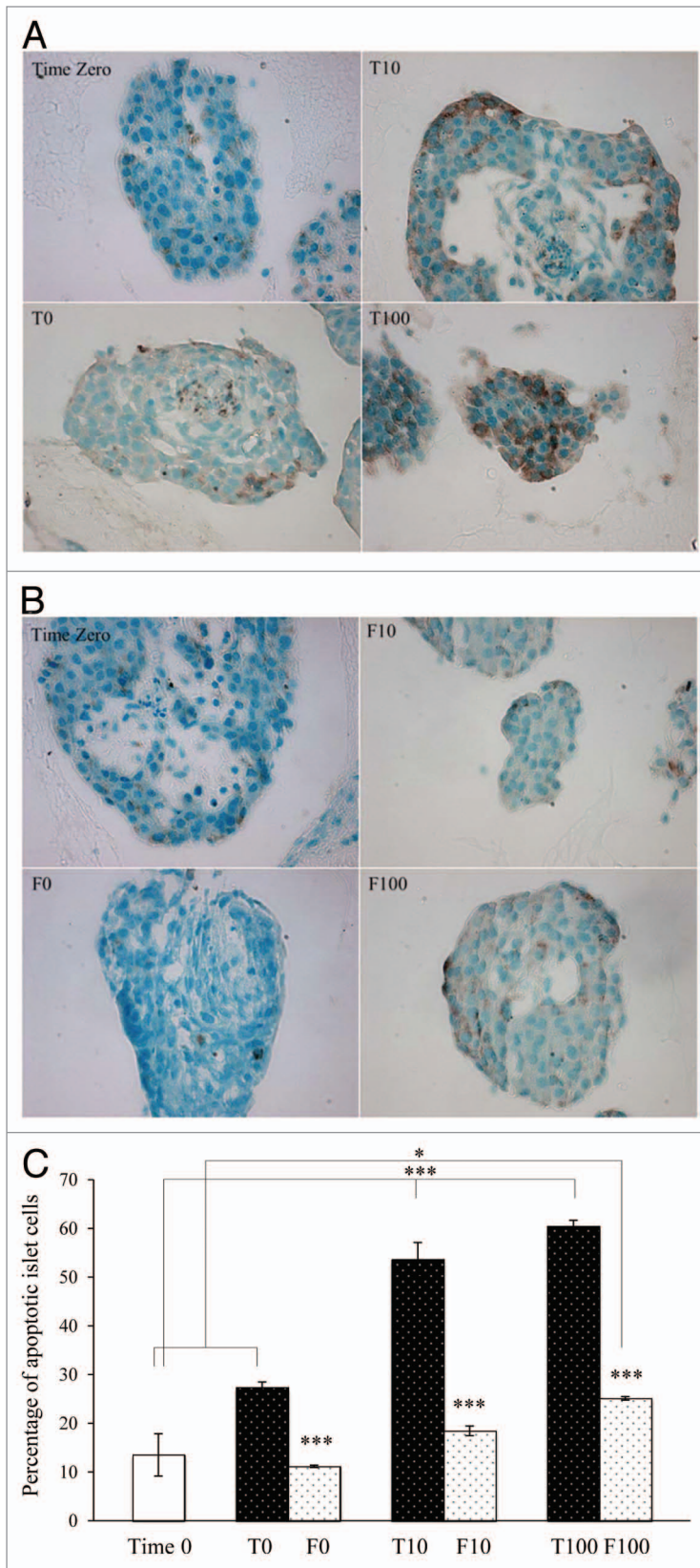


Figure 6. Light microscopy images of young porcine endocrine pancreatic islets treated with the APO-BrdU IHC staining kit. 40× magnification. Islets cultured in TCPS plates with 0, 10 or 100 μM H_2O_2 compared with Time Zero (A) and islets embedded in fibrin with 0, 10, or 100 μM H_2O_2 compared with Time Zero (B) are shown. The percentage of apoptotic cells (brown) in the total cell population (blue and brown) is shown in **Figure 6C**. ** represents a value of $P < 0.05$, **** represents a value of $P < 0.001$. *** compared Time Zero with T10 and Time Zero with T100 and each T-set compared with the corresponding F-set.

Islet incubation with hydrogen peroxide (H_2O_2)

After the overnight culture, media were removed and replaced with 200 μL of either fresh medium with no H_2O_2 , 200 μL of fresh medium containing 10 μM H_2O_2 (H3410, Sigma-Aldrich), or 200 μL of fresh medium containing 100 μM H_2O_2 . The islets were then incubated for 12 h.

Glucose-stimulated insulin secretion (GSIS) and insulin entrapment in fibrin gels

As described in detail elsewhere, a GSIS test²³ was performed after the 12 h H_2O_2 incubation. 2.8 mM glucose solutions served as the low glucose (LG and LG2) steps and 28 mM glucose solutions were used to represent high glucose (HG) concentrations. An additional incubation step with 28 mM glucose + 50 μM 3-isobutyl-1-methylxanthine (IBMX; I5879, Sigma-Aldrich) was performed. Islets were incubated with the different glucose solutions for 1 h each in the sequence LG, HG, HI, and LG2. Insulin contents were measured using either an Insulin Porcine High Range ELISA kit (0.313 $\mu\text{g}\cdot\text{L}^{-1}$ sensitivity; 10-1223-01, Mercodia Inc.) or an Insulin Porcine ELISA kit (0.01 $\mu\text{g}\cdot\text{L}^{-1}$ sensitivity; 10-1200-01, Mercodia Inc.). Insulin content of the media was normalized to the volume of medium for each condition.

Insulin was extracted from fibrin gels containing the islets by rinsing the gels with 500 μL PBS and centrifuging them for 20 s (12,800 rpm). The insulin content of the fibrin extract was expressed as μg per 200 IEQ. Absorbance was measured using a Safire2 microplate reader (Tecan Group Ltd.).

Immunofluorescence

Islets in TCPS plates and in fibrin gels were collected into centrifuge tubes, fixed in 4% paraformaldehyde and processed for paraffin embedding. Immunofluorescence staining was performed according to standard protocols with paraffin sections consecutively cut at a thickness of 4 μm . Primary antibodies to insulin (ab7842), glucagon (ab18461) and $\alpha_v\beta_3$ integrin (ab7166) were purchased from Abcam. The primary antibody for the α_5 integrin (sc-10729) was purchased from Santa Cruz. As secondary antibodies a goat anti-mouse IgG Alexa Fluor® 488 (A11001, Invitrogen) and a goat anti-rabbit IgG Alexa Fluor® 555 (A21428, Invitrogen) were used. All sections were blocked in 10% goat serum and counterstained with DAPI (D1306, Invitrogen). Manufacturer recommendations were used for pre-treatment, dilutions, incubation time and temperature.

bovine thrombin (T9549, Sigma-Aldrich), the gels were covered with 200 μL of medium. An equal number of islets was re-suspended in 200 μL of medium and seeded in a 48-well TCPS plate.

Samples were imaged using an epifluorescence microscope (Nikon Eclipse).

Insulin and integrin α_5 -positive cells were counted manually. For each condition, at least 5 fields with an average of 3 islets on each were counted. The total cell population was represented by DAPI-positive cells. The percentage of cells staining positively for insulin or integrin α_5 over the total cell population was assessed.

TUNEL apoptosis assay

Paraffin sections of pancreatic islets cultured in fibrin or in TCPS plates were treated with an APO-BRDU-IHCTM kit (AH1001, Phoenix Flow Systems) to measure apoptosis by dual color immunohistochemistry. Since apoptosis is leading to a multitude of 3'-hydroxyl ends in the fragmented DNA, those ends can be labeled with Br-dUTP. A combination of biotin-labeled anti-BrdU antibody, horseradish peroxidase streptavidin conjugate, DAB and H₂O₂/Urea results in a brownish-black color generation in apoptotic nuclei whereas a methyl green solution for counter staining results in a light blue color of all cells within the population. After deparaffinization, samples were rehydrated and permeabilized with Proteinase K (1:100 in 10 mM Tris pH 8) at room temperature (RT) for 20 min. Following a PBS rinsing step, endogenous peroxidases were inactivated using 3% H₂O₂ in methanol (at RT for 5 min). After rinsing in PBS, samples were equilibrated with a reaction buffer provided with the kit, diluted in distilled water, at RT for 30 min. Immediately after equilibration, the DNA labeling solution containing the reaction buffer, TdT enzyme, Br-dUTP and distilled water, was applied for 90 min at 37 °C with samples placed in a humid chamber. All samples were then rinsed with PBS. This was followed by 10 min incubation with a blocking buffer at RT. After, antibody solution containing anti-BrdU-biotin and blocking buffer was applied to

each sample for 90 min at RT in a humid chamber. Samples were covered with blocking buffer after rinsing with PBS. A conjugate solution made in blocking buffer was then applied for 30 min at RT, followed by a PBS rinse. DAB solution (DAB/H₂O₂/Urea in tap water) was added and incubated for 5 min at RT. Samples were rinsed with tap water and immediately after, covered with a methyl green counterstain solution at RT for 5 min. Slides were dipped in 100% ethanol and later, xylene, before being mounted with a Permount solution (SP15100, Fisher Scientific). Samples were imaged with a Leica DMR HCS microscope system.

Statistical analysis

Data are presented as means \pm SD for 3 independent experiments. Differences in results were assessed by a single factor ANOVA. A probability (P) value < 0.05 was considered statistically significant, $P < 0.001$ was considered highly statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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