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Research Article

Designing and Validating a New Method the TUNEL Microwave (TUNEL-MW) for Rapid Quantification of Apoptosis in Islets Following Isolation and Post-Thaw

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ABSTRACT

Background: Among the current quality control assays used in islet transplantation, there is an urgent need for more appropriate assays that measure cell damage via apoptosis that are accurate and rapid. Although the Terminal Uridine Nucleotide End Labeling (TUNEL) is a popular marker for apoptosis, the protocol takes 4 hours to complete. In this regard, microwave assisted histoprocessing, which shortens the time taken for processing, holds promise. Keeping this in mind, a new TUNEL Microwave (TUNEL-MW) method, for rapid quantification of apoptosis, was designed, developed and validated.

Method: Two lots of post-thaw isolated human islets cultured for 24 hours, 3 days, 5 days and 7 days i.e. 8 samples, were used for the study. Dewaxed and rehydrated tissues were processed for routine histology, stained with haematoxylin and eosin (H&E) and the conventional TUNEL was carried out as per manufacturer's instructions. For the TUNEL-MW, kit instructions were modified and microwave-assisted histoprocessing was done. The assessment of apoptotic index (AI%) by light microscopy (LM) was carried out by a pathologist who was completely blinded to the study.

Results: The new TUNEL-Microwave (TUNEL-MW) developed by us reduced processing time from 4 hours to 30 minutes (saving 3½ hours). Results were validated by univariate linear regression ($r^2 > 0.990$), coefficient of variation (<5% between all three methods) and the Bland Altman plot comparing AI% determined by the new TUNEL-MW with the conventional TUNEL and with LM (gold standard).

Conclusion: TUNEL Microwave appears to be an ideal method. It is simple and takes just 30 minutes to perform and can therefore be used along with existing quality control measures to rule out or measure apoptosis prior to islet release for islet transplantation.

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Introduction

The Terminal Uridine Nucleotide End Labeling (TUNEL) assay is a popular method for the detection of apoptosis [1-18]. Apoptosis first described by Kerr *et al.* is the endpoint of an energy-dependent cascade

of molecular events initiated by numerous stimuli [19-24]. It is a vital process and is a natural form of programmed cell death designed to eliminate unwanted cells. It occurs during development, during situations where tissue homeostasis must be restored, as a defense mechanism and following cell injury [19-24]. The relevance of this phenomenon to islet transplantation is that both cold and warm

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ischaemia of the post-mortem pancreas and islets on cryopreservation and indeed the very process of islet isolation itself are known to make islets vulnerable to apoptosis [25-28]. Ischaemia and hypoxia seen in post-mortem pancreata, isolated islets and in post-thaw isolated islets activate intracellular signals and cause the leakage of cytochrome c into the cytosol which results in a cascade of events that trigger apoptosis within the islets.

Similar molecular changes occur during the disruption of islet cell-matrix interactions with acinar tissue during islet isolation [29-39]. The loss of interstitial matrix and the basement membrane following poorly controlled digestion compromises islet cell viability and function, regulating both cell differentiation and survival leading to induction of apoptosis [29-39]. Given these conditions, the inference is that all islets for transplant, whether freshly isolated or post-thaw, are potentially vulnerable to apoptosis. Therefore, it is prudent that standard quality control measures prior to islet release for transplantation incorporate assays that measure apoptosis, thus ensuring the administration of a safe, pure, potent and viable product, namely the islets. Among the many assays specifically directed at measuring cell damage via apoptosis, current quality control assays used in islet transplantation are restricted to membrane integrity assays [40, 41]. However, loss of membrane integrity is more a measure of necrosis while the hallmark of apoptosis is loss of nuclear integrity [19-24, 40-54]. Another drawback of membrane integrity assay is that apoptotic bodies are known to exclude dyes and therefore underestimation of the percentage of damaged cells is a strong possibility [19-24, 40-54].

One of the reasons for the continued preference of membrane integrity as a measure of cell damage is that, while there are several more appropriate assays that measure cell damage via apoptosis and necrosis, they do not meet the criteria for their selection as quality control assays used in islet transplantation. Specifically, while a quality control assay must be sensitive, appropriate, repeatable and valid, it must also be rapid and simple to perform [23-36]. The other obstacle when evaluating cell damage in isolated or native islets is that not all assays are appropriate for cell clusters [40, 41]. Among the markers of apoptosis, cell morphology still serves as the gold standard for the identification of apoptosis and its distinction from necrosis [11, 22]. The light microscopic morphology of apoptosis includes shrinkage of cells, condensation of nuclear chromatin peripherally under the nuclear membrane and formation of apoptotic bodies by fragmentation of the cells and the nuclei [11, 22].

The TUNEL assay used to detect apoptosis is based on the concept that following the leakage of cytochrome c into the cytosol, caspases are activated which on hydrolysis cause cleavage of the cytoskeletal and nuclear matrix proteins. The cytoskeletal proteins are converted into shrunken cells (apoptotic bodies) and the nuclear DNA is broken down into 180 to 200 base pairs by Ca⁺⁺ and Mg⁺⁺ dependent endonucleases [1-18]. The double-stranded, low molecular weight DNA fragments as well as single strand breaks ('nicks') in high molecular weight DNA can be identified by labeling free 3'-OH termini with modified nucleotides in an enzymatic reaction [1-18]. The existing TUNEL assay that measures apoptosis in terms of loss of nuclear integrity is a simple assay to perform. The drawback is that it takes approximately 4 hours to complete. It was hypothesized that if incubation times can be reduced via the use of the microwave then the possibility of the incorporation of

the TUNEL as a quality control parameter checking for apoptosis, in isolated or native islets will enhance the scope of existing quality control tests.

Why the Microwave?

The microwave oven consists of metal walls and a magnetron which emits microwaves at a frequency of 2.45 GHz. This frequency is ideal for both scientific heating and cooking purposes. Whenever rapid diagnosis is required, microwaves are utilized as they provide quick fixation, rapid processing and support the performance of immunohistochemical stains. The work of several pioneers like; Mayers, Kok and Boon and Anthony Leong showed that the microwave helped reduce the processing and paraffin sectioning time for cell blocks and fresh tissues to as low as 32 minutes [55-60]. "Microwave transparent materials" such as glass and plastic, allow total transmission of these waves to biological tissue. Biological materials allow penetration and absorption of microwaves to varying degrees following which depolarization of molecules like water, cell membrane proteins etc. occur.

This biophysical change causes breakage of protein bonds, alteration of membrane permeability and the unmasking of hidden surface receptors. These physiological alterations form the basis for rapid fixation, histological processing, antigen retrieval, staining and labelling. However, the penetrative capacity for the medium, the type and size of the tissue, the size, shape and type of container used, the procedure being carried out and the positioning of the load, determine the duration and output required for processing. By controlling the input of the current using a range of power settings, the level of emitted waves can be controlled and rapid methods for processing standardised [55-60].

Methodology

Two lots of post-thaw isolated human islets (Case-I and Case-II) cultured for 24 hours, 3 days, 5 days and 7 days i.e. 8 samples of post-thaw islets were used for the study. The kit used for the quantification of apoptosis was the In-Situ Cell Death Detection Kit, (Fluorescein, Cat. No. 1 684 795, Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, USA). The post-thaw, culture, islet fixation, agar and paraffin embedding of islets and the TUNEL protocols used have been described in detail by Priya *et al.* 2013 [61]. Pancreatic tumor tissue following therapy was used as the control as it has a naturally high apoptotic index. For the negative control the TUNEL reaction mixture contained only label (fluorescein) and for the positive control, the reaction mixture contained both label and enzyme.

As the combined staining of the samples with fluorescein (label-TUNEL) and the nuclei using haematoxylin (H&E) stain reduced the intensity of fluorescence the same section could not be used to evaluate apoptosis by light microscopy and the TUNEL. This drawback was circumvented by staining the "reference section" with H&E stain and the sections on either side of the reference section were used for the two TUNEL procedures namely, the conventional TUNEL and the newly developed TUNEL-Microwave (TUNEL-MW). Thus, the distance between a TUNEL section and the reference section was 5µm and that between the two TUNEL sections was 10µm. Thus, the possibility of variations in results due to sampling variations was avoided. The

Olympus Compound Microscope was used for light microscopic (LM) assessment of apoptosis in post-thaw cultured islets and the Confocal Microscope LSM 510, Zeiss was used for the TUNEL and TUNEL-MW. For both the TUNELs and LM; sections were sampled in 20 random fields at a total magnification of x400. Thus, the possibility of bias due to geographical variation in apoptosis in the section examined was avoided.

$$\text{Apoptotic Index (\%)} = \frac{\text{Cells in Islet Positive for Apoptosis}}{\text{Total Cells counted}} \times 100$$

I New TUNEL Microwave (TUNEL-MW)

i Microwave Oven Used

The oven should have a 1 to 10 range preferably. The power output of the microwave oven should be low (between 800 to 900W); the reason being, that domestic microwave ovens have a cycle time, each lasting one minute and consisting of three phases. The “preheat time” of the magnetron followed by “full emission” of 800W or 900W for a short time and then a short duration during which the magnetron is completely

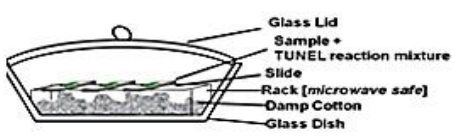
“switched off”. The various ranges are obtained by altering the time of the switching ‘on’ and ‘off’ of the magnetron and not by controlling the amount of emission. Thus, our calculation of 350W output required for tissue processing which is approximately at the range of power 3 or power 4 ensures that the tissue is exposed to 800W for a shorter time within each cycle time [55-60]. Thus, a microwave oven with very high output like 1250W could harm the tissue and cause false positives.

Dewaxed and rehydrated tissue was permeabilized and antigen retrieval was carried out by microwave irradiation using 200ml 0.1 M Citrate buffer, pH 6.0 at 350 W for 5 min (like the manufacturer’s protocol). Following this, three rinses in cold PBS were carried out in the microwave at Power 4 (350W) for 2 minutes each. The TUNEL mixture was applied and incubation was carried out at Power 4 for 2 minutes in a humidified chamber as shown in (Figure 1a, processing time for TUNEL procedure) and (Figure 1b, humidified chamber). The reaction was stopped by three rinses in cold PBS at Power 4 (350W) for 2 minutes each and the slides were then mounted using anti-fade. Samples were directly viewed using the confocal microscope LSM 510 (Figure 1e), using the same excitation wavelength as mentioned in the manufacturer’s protocol.

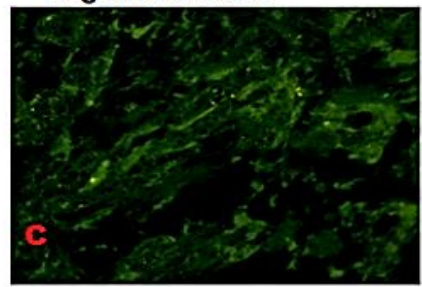
a)

Procedures	Duration of each Procedure	
	TUNEL	New TUNEL-MW (developed by us)
Deparaffinization and rehydration	Routine	Routine
Antigen retrieval	5 min	5 min
3 washes in PBS; 15 minutes each	45 min	6 min
Incubation with TUNEL reaction mixture	2 hours	2 min
3 washes in PBS; 15 minutes each	45 min	6 min
Dehydration and Mounting	Routine	Routine
Total Time taken (Approximate)	4 hours	30 minutes
Total Time Saved :	3 ¼ Hours	

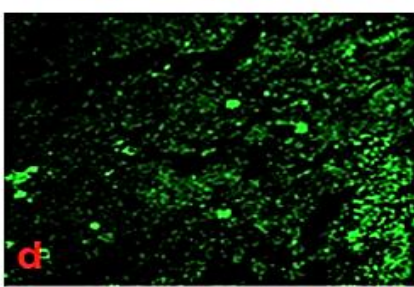
b) Humidified Chamber



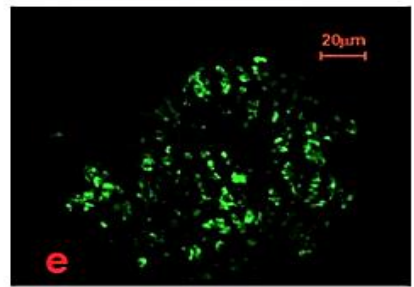
Negative Control



Positive Control



Post-thaw Isolated



c) Negative Control *
d) Positive Control *
e) A cluster of post-thaw isolated human islets [TUNEL-MW] following 24 hrs culture embedded first in agar and then in paraffin.
Note *: Sections were taken from a pancreatic tumour following treatment.

Figure 1: New TUNEL Microwave (TUNEL-MW) method.

ii Precautions Taken to Prevent False Positives

- i. Artificial nicks and uncoiling of the DNA occurs at temperatures above 90°C. To circumvent this, the temperature was maintained below 90°C using both short cycles and rapid cooling (chilled buffers and ice).
- ii. A positive and negative control of pancreatic tumor tissue following therapy was used with each run.
- iii. As islets are composed of a cluster of cells, the resulting combined fluorescence interferes with the accuracy of the counting. By using the confocal microscope LSM 510 “point focusing” was made possible.

II Statistical Analysis

Validation was by univariate linear regression, coefficient of variation and the Bland Altman plot [62-64].

Results

Figures 1 a-e presents the images of results of the newly developed TUNEL-MW method taken via the confocal microscope LSM 510. The negative control (Figure 1c) was strongly negative for the TUNEL while the positive control (Figure 1d) was strongly positive for the same,

indicative of the specificity of the TUNEL. The table (Figure 1a) illustrates the ability of the new method to cut down processing time from approximately 4 hours to 30 minutes.

Validation of the AI% obtained by the New TUNEL-MW against the AI% obtained by the conventional TUNEL and via LM (gold standard). Figure 2a presents the univariate linear regression as a scatter plot of the results of the AI% by LM (gold standard) versus AI% by TUNEL-MW, $r^2=0.993$ ($p < 0.001$) and AI% determined by conventional TUNEL versus AI% by TUNEL-MW $r^2=0.999$ ($p < 0.001$) (result not shown). The coefficient of variation was $< 5\%$ for all three methods. To confirm that AI% by the new TUNEL-MW did not exceed $\pm 2SD$, which is the statistically allowed limit, the Bland & Altman plot (217-219) was used (Figures 2bi & 2bii). The mean difference for the AI% by LM (gold standard) versus the AI% by new TUNEL-MW (Figure 2bi) was -0.0148 and the $1.96SD$ ranged from $+0.0695$ to -0.0843 . The mean difference for the AI% by the classic TUNEL versus the AI% by new TUNEL-MW (Figure 2bii) was -0.0217 and the $1.96SD$ ranged from $+0.0052$ to -0.0485 . To determine the measure of consistency or agreement of values within cases for all three methods, the intra class coefficient of correlation was determined. The intra class “coefficient of correlation” between the AI determined by LM and the TUNEL-MW was $r=0.997$ and that between the conventional TUNEL and the TUNEL-MW was $r=0.999$.

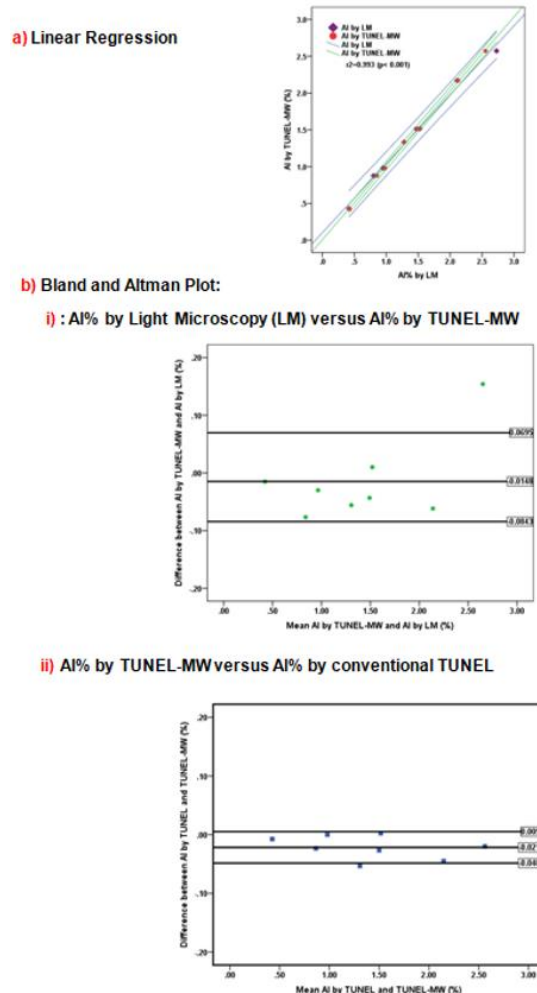


Figure 2: Validation of the New TUNEL Microwave (MW).

Discussion

The battery of quality control assays in current use in islet transplantation include functionality assays (insulin vs. glucose response), sterility assays (mycoplasma and endotoxin levels), islet quantification (via islet equivalents) and membrane integrity assays (fluorescent dyes exclusion assays) [40, 41]. The protocols followed in islet isolation and cryopreservation increase the vulnerability of islets towards apoptosis, the lack of definite quality control measures to evaluate apoptosis prior to islet release for transplantation presents a serious drawback [40, 41]. One of the reasons for the current lack of suitable measures of apoptosis is the lack of assays that are reliable, appropriate and quick. Early markers of apoptosis like Bcl-2, caspases etc. might yield high false positives due to a transient decline (as in Bcl-2) or elevation (as in caspases) during islet isolation and thaw [1-18]. Their levels might be restored to normal physiological limits when islets are transplanted into suitably primed recipients. Therefore, markers directed at the hallmark of apoptosis namely of nuclear integrity might be more appropriate in indicating the percentage of cells that are irreversibly damaged and whose loss will affect the volume of the functional islet mass post-transplant [1-18].

The conventional TUNEL, an established assay to quantify apoptosis was therefore selected for this study [1-18]. Its advantage is that the simplicity of the protocol and the assessment of results allow it to be carried out by a trained technician. Its drawback is that it takes approximately 4 hours to perform. In order to cut the processing time for the TUNEL, we designed a new method which incorporated the use of the microwave which we named as TUNEL-Microwave (TUNEL-MW) to serve as a candidate quality control measure to be used in routine islet transplantation. Figure 1a summarizes the processing time for the new TUNEL-MW assay developed; the new TUNEL-MW method cuts down processing time by approximately 3½ hours. By successfully decreasing incubation time for the TUNEL by the new TUNEL-MW together with confirmation of the reliability of the TUNEL as a measure for apoptosis, we propose that the TUNEL-MW could be used as a quality control measure in routine islet transplantation.

The gold standard for the evaluation and quantification of apoptosis is histomorphology via electron microscopy [11, 22]. The drawback with electron microscopy is that it is time consuming, expensive and the area of tissue that can be sampled/examined is extremely small. Further, when the apoptotic index of a cell population is relatively low it is impractical to use electron microscopy as a method of measuring apoptosis [65]. Light microscopy however affords a wider area of tissue sampling, is inexpensive and when strict criteria is adhered to by skilled pathologists, is a very reliable technique [11, 22]. To validate the new TUNEL-MW, the apoptotic index measured by this method was compared to that measured by the classic TUNEL and the reference method chosen for this study namely light microscopy (H&E stain). The three methods were found to be highly correlated ($r > 0.90$, $p < 0.001$) and the measure of consistency or agreement measured by the intra class coefficient of correlation for all three methods was $r > 0.990$ ($p < 0.001$).

The following are the advantages of the New TUNEL Microwave Method:

- i. With the use of a humidified chamber this method prevents drying of the sample and the fluorescein label used during the microwaving process.
- ii. It allows for diagnosis of apoptosis in both native islets, and fresh and post-thaw isolated islets.
- iii. It significantly reduced incubation times, thus reducing the timing of the entire procedure considerably.
- iv. Does not require highly specialized equipment; a simple domestic microwave may be used, thus keeping the costs down.
- v. As no special experience is essential for the adoption of the microwave TUNEL method, and the TUNEL kit being a simple one, it provides a new convenient assay for the rapid detection of apoptosis.

One of the limitations is that the procedure requires standardization based on the microwave oven's power output, type and thickness of tissue sample and size of the load used. The TUNEL method is also sensitive to the duration of fixation that a tissue is exposed to. In conclusion, the new TUNEL-MW to detect and measure apoptosis has reliability and reproducibility that matches both the apoptotic index as measured by light microscopy (LM) and the classic TUNEL. Simple, and requiring 30 minutes to perform, it can be used to complement existing quality control measures to rule out or measure apoptosis prior to release of islets for transplantation.

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Author Contributions

Dr Priya Miranda, Dr Lakey JRT, and Dr Mohan V conceived the study. Dr Lakey JRT, and Dr Mohan V, supervised the study and revised the manuscript. Dr Priya Miranda conceived, coordinated, performed the study, performed the statistical analysis and wrote the first draft of the article. Dr Meera Govindarajan supervised the microwave assisted histoprocessing technique. Cell turnover was assessed by Dr Ganthimathy S and she also revised the manuscript. Dr Anjana RM gave valuable input and revised the manuscript. Dr Thiagarajan V and Dr S Gunasekaran revised the manuscript.

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