CREATOR(S):	IWUAGWU, B.U., CRUICKSHANK, S.F., ROWE, I. and KNOTT, R.M.
TITLE:	Use of a three dimensional porcine retinal explant model to detect HIF1 alpha for understanding diabetic retinopathy.
YEAR:	2017

Use of a three dimensional porcine retinal explant model to detect HIF1α and targets for understanding diabetic retinopathy

B.U. Iwuagwu, S. F. Cruickshank., I. Rowe & R.M. Knott.
School of Pharmacy & Life Sciences, Robert Gordon University,
Aberdeen AB10 7GJ. Scotland, United Kingdom

ROBERT GORDON UNIVERSITY•ABERDEEN

Abstract

Diabetic retinopathy (DR) is a major microvascular complication associated with diabetes mellitus and is a leading cause of blindness [1]. Hypoxia and hyperglycemia are suggested to play crucial pathophysiological roles [2,3], but the exact mechanisms are complex and the precise events are still debated. Models of DR, including organotypic cultures (retinal explants) contribute significantly to studies in DR [4].

Here, a retinal explant model which comprised of whole porcine retina embedded within an agarose-collagen three-dimension (3D) matrix (explants, see fig.1) is utilised. Explants were cultured in 5.5mM (low) or 25mM (high) glucose for up to 24 h.

The aim of this work is to investigate the expression and localization of the hypoxia inducible factor -1 alpha (HIF1α), a critical transcription factor in DR [2,3], and other targets: endothelial proliferation marker (CD31), glial fibrillary acidic protein (GFAP) and cellular proliferation marker (Ki67) in explants.

Materials & Method

Porcine ocular globes were dissected and whole retina embedded within a 0.5% agarose - collagen (0.25mg/ml) 3D matrix in low (5.5mM) or high (25mM) glucose DMEM media.

Fig.1 Explant

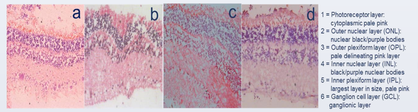
Explants were maintained at 37ºC, in a 95% air humidified incubator. Mean oxygen concentration within matrix was 10%. Explant vessel viability was confirmed with contractile response to potassium chloride and angiotensin II. Explants were harvested at 0.5 h, 4 h and 24 h post culture, fixed overnight in 10% neutral buffered formalin (NBF) and incubated in 30% sucrose. Serial sections (20µm) were cut using a cryostat. Tissue integrity was ascertained with Mayer's hematoxylin and Eosin stain, and imaged with a light microscope.

To detect the expression and location of targets, sections were washed and permeabilised by washing 3X in 50mM Tris buffered saline (TBS) plus 0.1% Triton X-100 (TBST) for 15mins, followed with incubation with 10% normal goat serum/1% bovine serum albumin (BSA) (Santa Cruz) for 2 hours at room temperature (RT) with final incubation with 1:20 primary mouse anti: hif1α, cd31, gfap and ki67 (Thermofischer) overnight at 4ºC.

Specimens were washed gently 3X with TBST. Non-specific endogenous peroxidase reaction was blocked with 0.3% H_2O_2 in 0.3% normal serum (goat) in TBS for 15 mins at RT, and incubated with 1:200 secondary goat anti-mouse IgG (H+L) antibody, DyLight 488 (Thermofischer scientific) for 1 h at RT, washed 3X with TBST and counterstained with DAPI. All antibodies were tested on fresh retinal tissues and activity demonstrated before use on explants. Images were taken with a Leica fluorescent microscope. All assays were repeated n ≥ 3 for each time point and representative micrographs presented.

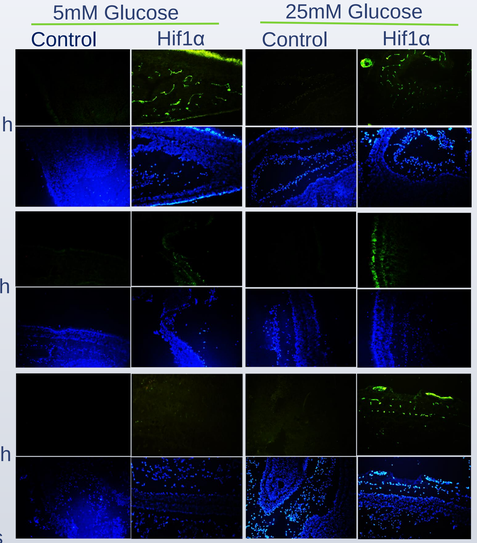
Results

1. Tissue integrity of explants confirmed over a 24 h period with H&E (fig.2)



Explants at 0.5 h (b), 4h (c) and 24 h (d) post culture stained with haematoxylin and eosin (H&E) showed tissue preservation over 24 h. Explants were comparable to the uncultured sample (a), with retinal layers identified; photoreceptors, outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL) (200X)

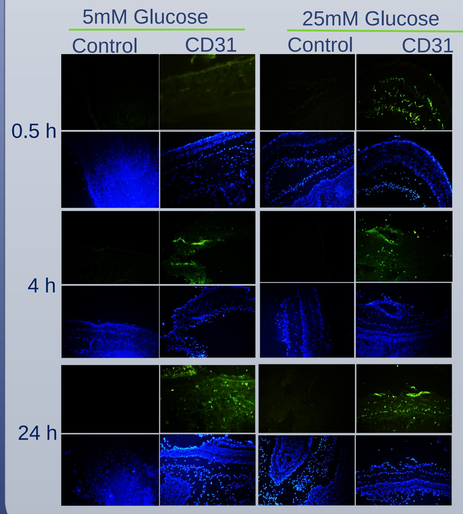
2. Effect of glucose concentration on detection of Hif1α (fig. 3)



Hif1α was detected in all explants at 0.5 h post culture. Detection for hif1α was diminished by 4 h and was completely absent in low glucose explants within 24 h, which may suggest a resolution of hypoxic signals. High glucose appeared to drive hif1α expression because hif1α signal was sustained at all time points in high glucose explants. Activation of hif1α may signify oxygen dependent and/or glucose-mediated oxygen-independent hif1α activation. Hif1α expression was associated with vessel-like structures, photoreceptor and also within retinal layers. (200X)

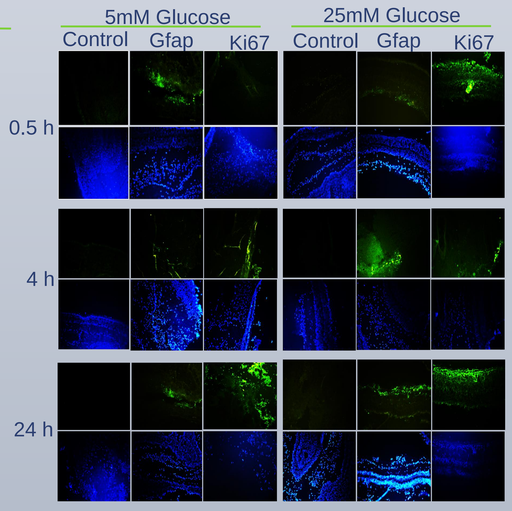
3. Effect of glucose concentration on detection of CD31 (fig. 4)

CD31, an endothelial cell proliferation marker was detected in high glucose explants at all time points. CD31 expression at 0.5 h may suggest an early response of the endothelial cells to effects accruing from high glucose. In low glucose explants, CD31 was not detected until after 4 h. CD31 expression was associated with vessel-like structures and retinal layers (200X)



3. Effect of glucose concentration on detection of GFAP and Ki67 (fig. 5)

The expression of gfap, a stress marker, was seen at all time points in both explants. Early response may relate to response from Mùller cells in the ganglion cell layers, becoming more invasive and seen on vessel-like structures (200X)



Ki67, a cellular marker for proliferation was detected in high glucose explants as early as 0.5 h which may suggest an early cellular proliferation signal in high glucose explants. This observation seems to correlate with early detection of CD31, an endothelial proliferation marker at 0.5 h in high glucose explants. In low glucose explants, Ki67 was not detected until after 4 h.(200X).

Summary & Conclusion

A three-dimensional (3D) retinal explant model was used to culture whole adult porcine retina (explants) (fig.1) in low/high glucose for up to 24 h and tissue integrity was confirmed with H&E (fig. 2).

Hif1α was detected in all explants at 0.5 h post culture (fig.3). Hif1α expression in low glucose explants was diminished at 4 h, and was completely absent within 24 h. However, hif1α expression was sustained in the presence of high glucose up to 24 h. Early detection of hif1α at 0.5 h in all explants may relate to an oxygen-dependent hif1α activation arising from a state of acute systemic hypoxia post sacrifice [5]. Whilst, sustained activation in the high glucose explants may relate to a glucose-mediated oxygen-independent hif1α activation [6]. Hif1α was detected on vessel-like structures, within inner retinal layers and photoreceptor, which can be characteristic of acute systemic hypoxia, a state which may have been precipitated by circulation cessation post sacrifice [5].

CD31 & Ki67, both markers of proliferation were detected in high glucose explants at all time points (0.5 – 24 h) but signals were detected in low glucose explants after 4 h. Thus, high glucose may be responsible for early proliferation signals in high glucose explants at 0.5 h. Endothelial cell (CD31) and cellular proliferation (Ki67) was detected in vessel-like structures and within retinal layers (fig. 4).

GFAP, a glial cell stress marker [7], was detected in all explants at all time points. Gfap was localised inward from the ganglion cell layer, and was also associated with vessel-like structures.

In conclusion, explants were responsive to culture conditions and targets were detected. For application, explants can be exposed to different external stimuli such as; high pressure, hypoxia and/or high glucose and changes in the expression and/or localisation of target(s) of interest examined, as demonstrated here. Verification of study results with Western blot is needed, with confocal microscopy and cell-specific staining. More mechanistic studies are needed to understand cellular/signalling pathways that are activated/downregulated in relation to the targets studied.

References

1. Lai A. K. and Lo A. C. (2013) Journal of Diabetes Research, 2013, 1-29.
2. Catrina et al (2004) Diabetes, 53:3226-3232.
3. Thangarajah et al (2009) PNAS, 106, 13505-13510
4. Matteucci et al (2015) BioMed Res Int; 2015, 1-16
5. Mowat et al (2010) PLoS One; 5 (6) e11103
6. Déry et al (2004) Int Journal of Biochem & Cell Biol ; 37, 535–540
7. Lewis & Fisher (2003) Int Rev Cytol;230:263-90

Acknowledgement

Special thanks to the American Society of Biochemistry and Molecular Biology (ASBMB) for Graduate/Postdoc Travel Award (2017), and to Robert Gordon University, Scotland for full scholarship for doctoral thesis. Many thanks to QPL, Brechin, Scotland for the kind donation of porcine ocular globes