Gene Delivery using Adenoviral Vector into the Organotypic Culture System of the Chick Retinae

Mohamed A. Al-Griw *
Abdulhakem S. Elnfati *

Abstract:

Damage to retina during development correlates with many ophthalmological pathologies, such as retinal dysplasia and degeneration. Virus-mediated gene transfer into identified retinal cells may offer a great potential for therapeutic interventions. This study describes a three-dimensional culture system of the embryonic chick retina. Samples from 17 and 19 days old retinae were isolated from eyeball and were cultured for up to 10 days. Selection of embryonic stage and specimen processing were optimized for culturing. The ability of a replication-deficient adenovirus vector (AVV) carrying an early cytomegalovirus (CMV) gene promoter to efficiently transfer a green fluorescent protein (GFP) gene into the cultured embryonic chick retinae was analysed. The results indicated that retinal tissue slices did not show any change in their cytoarchitecture. The results also showed that the GFP expression was maximal about 24 hours after infection of retinal slices and were maintained for several days. They also showed that GFP expression was widespread in the retinal slices. Moreover, the results also showed that injecting of AVV did not show any detectable cytopathic effects - these were only observed for very high titres of infection. The approach developed here is a valuable tool that can be used to assess the effect of pharmacological manipulations on retinal cells and to transfer of effective therapeutic genes into the retina, which might also facilitate the search for understanding the mechanisms

* Department of Zoology, Faculty of Science, University of Tripoli, Tripoli-Libya

responsible to vision-related diseases and to identify potential therapeutic interventions.

Keywords: embryonic chick retina; organotypic culture system; genetic transformation; toxicity.

Introduction:

Injury to retinae is present in many ophthalmological pathologies, such as central retinal artery obstruction, central retinal vein obstruction, carotid obstruction and glaucoma [1]. Certain ocular diseases are quite rare, whereas others, such as cataract, age related macular degeneration and glaucoma are very common, especially in the ageing population [1-3]. The prevalence of many of these diseases will continue to increase as the worldwide population continues to age. In this respect, age-related vision impairment is expected to double in many countries [1].

Of particular importance in the understanding of any biological system or injury induces cell death is the model that is used to study that system. The central nervous system (CNS), especially retinal tissues from rat [4], mouse [5], and chicken [2-3] were highly used to study embryonic development, cellular differentiation, pharmacology, transplantation and other medical aspects [6]. Many studies reported that complete retino-architecture can be obtained and maintained in retinal cultures for long culture periods [2-6]. The need for well-organized *in-vitro* culture systems, which can mimic an *in-vivo* situation, is still a high priority, in spite of remarkable advances made since the last century. This reflects the need to study the mechanism of injury in the context of the complex cellular matrix that exists in the retina where the injury to one cell type has an effect on other cell types [7]. Perhaps most importantly in the normal tissue environment the close proximity of the different cells may well result in a more significant increase in local glutamate concentrations than would be seen within a perfused single cell type culture system. An organotypic culture, which combines the ease of access found in monolayer cultures with the morphological structures and cellular

populations found *in-vivo* [8], can be a useful midpoint in this process. Successful preservation of the cytoarchitecture over a period of several weeks was observed in slice culture techniques from rodent CNS [3-4, 9].

The investigation of transfer protocols not only provides the ability to test candidate therapeutic agents, but also allows the refining of potential *in-vivo* delivery systems. The latter is valid only if the transfer approach faces the same environmental issues found in the *in-vivo* tissue. Development of gene therapies for various neurological disorders is an active area of research. Therefore, a particular priority was to develop a functioning model system that enables us to deliver a gene and measure its cytotoxicity on the retinal cytoarchitecture effects whilst maintaining precise control of experimental conditions throughout the course of a ten day experiment. The fluorescence procedure, using fluorescence microscopy was employed in the experiments permitted the analysis of the viability of cells/slices infected with adenoviral vector (AVV) expressing green fluorescence protein (GFP) under the control of cytomegalovirus promoter. Using this system, organotypic cultures of chick retinal slices reached a near *in-vivo*-like tissue organization, illustrating that this model is a reasonable approximation of the conditions faced by viral gene transfer methods *in-vivo.* This is based on the observation that the optimal conditions for AVV infection is the same as those previously found with live animal models [10]. The data presented here support the idea that organotypic culture system offers not only a bench to work on therapeutic constructs, but also optimization of delivery, and may allow retinal genetic researchers to choose a simplified appropriate transfection method, which produces the maximum number of positive intact cells, for testing the efficacy of potentially therapeutic constructs.

Materials and methods:

Organotypic culture system of chicken retina Preparation:

The 42 White Leghorn Retinae were organotypically cultured as described previously [8, 11-12]. The chick eyeballs

were removed under aseptic conditions and immediately placed into a Petri dish containing ice-cold MEM (minimum essential medium supplemented with 2.3 mM (v/v) penicillin/streptomycin, Invitrogen). The retinae were dissected away from the eyeball, embedded in a plastic mold containing cool, molten 4 % agar (37 ºC, Melford). Intact retinal slices were cultivated on cell culture inserts (Millipore, 1.0 µm, Falcon) placed in 6-well culture plates (Falcon) containing 1 ml culture medium, containing 50 % MEM with earls salts (Sigma), 25 % HBSS (Hanks balanced salt solution 1X) (Invitrogen), 20 % normal horse serum (Invitrogen), 4.6 mM L-glutamine (Sigma), 21 mM D-glucose (Fisher Scientific), penicillin/streptomycin solution stabilized (Invitrogen), 4.2 µM (v/v) L-ascorbic acid (Aldrich-Sigma) and 11 mM NaHCO3 at pH 7.2-7.4. The cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2. Culture medium was changed to serum free and supplemented with 0.3 % B27 growth supplement (Invitrogen) and thereafter the medium was refreshed every 72 hours with cultures maintained for up to 10 days. Each treatment, together with the appropriate control, was replicated 3 or 4 times.

Histology:

The tissues from different culture days beginning from day *in-vitro* 0 till 10 were washed three times with PBS (phosphate buffered saline) and were fixed in 4% paraformaldehyde (PFA; Sigma) for an hour. The fixed sections were stained with hematoxilin-eosin to evaluate retinal morphology under different conditions.

Immunohistology:

Retinal slices were fixed in buffered 4% PFA and were embedded in paraffin wax (Sigma). 4 µM chicken retina cross-sections were incubated with 100 µl of primary antibody [polyclonal rabbit anti-chicken mpdz (Santa Cruz Biotech)] for 90 minutes at room temperature or overnight in dark at 4 ºC. The slides were washed three times with PBS and then subjected to 100 µl of fluorescent conjugated secondary antibody (Alexa Fluor®,

Invitrogen, Molecular Probes) for overnight in dark at room temperature. The slides were washed three times with PBS. To examine the specificity of immunolabelling with the antibodies, control tissues were processed without primary antibodies, which resulted in no immunostaining.

Quantification of cell survival/mortality:

To stain all cell nuclei, the retinal slices were placed on glass slides and were fixed in 4% PFA in PBS for 1 hour. After three PBS washes, the sections were treated with nuclear dye 4`,6-dimidino-2-phenylindole (DAPI; 0.5 µg) (Vector Laboratories) for 5 minutes. Due to prior fixation this step allows permanent documentation of all cell nuclei present in the tissue independent of whether the cells were intact or dead at the time of fixation. After three final PBS washes, the tissues were dried on the heating plate and cover slipped. The slices were then inspected microscopically for cell integrity. Since the blue staining of cell nuclei by DAPI is dependent on disintegrated cell membranes, the number of stained cells reflects the degree of cell mortality.

For labeling of viable and dead cells, calcein-AM (4 mol/L) and ethidium homodimer-1 (6 mol/L) were used, according to the Molecular Probes (Invitrogen). Viable calcein-AM labeled cells appear green, whereas dead ethidium homodimer-1 stained cells appear red. The Retinal slides were washed once with PBS and were inspected microscopically for cell mortality.

Adenoviral vector preparation and infection:

Recombinant adenoviral vector (AVV) expressing green fluorescent protein (GFP) under the control of the cytomegalovirus (CMV) promoter has been described previously [13-14]. High titre virus was prepared by four rounds of infection of human embryonic kidney (HEK) cells and subsequent CsCl gradient density centrifugation [15]. For adenoviral delivery, retinal slices were cultured with culture media containing approximately 10^9 pfu/ml of adenoviral particles. For each retinal slice, several visual fields were analyzed and the numbers of dead cells present

determined and normalized in terms of the dead cells per field exhibited by a control slice non-treated with AVV-GFP.

Microscopy and imaging:

To assess the cellular damage on the retinal cytoarchitecture, the sections were subjected to phase contrast microscopy or fluorescence microscope (Eclipse E1000; Nikon), as appropriate. Imaging was done for each fluorescent channel separately and processed through extended focus imager, and all the pictures from different channels were combined as overlays and stored in tiff image format. To quantify immunoreaction (pixel) intensity, flattened image stacks were opened in imageJ (NIH analysis software), thresholded with constant pixel intensity level settings to a white background and then analyzed with the analyze particles function. Five visual fields from each retina were analyzed, giving a total of 20 measurements per condition. Quan titication was performed independently by an observer blinded to the experimental conditions.

Statistics:

Statistical analysis was performed using SPSS software (version 20). To quantify cells in control and other conditioned retinal slice cultures, three cover slips with at least six visual fields per cover slips were taken for each data point. At least 4 independent biological replicates were performed and statistical data were then calculated. Data within each time-point were tested for normality using the Kolmogorov-Smirnov test (with Lilliefors correlation). All data were represened as mean ± SEM. Statistical differences were assessed by Student's t test or One-way ANOVA followed by Post-Tukey test, as appropriate. Results were considered to be statistically significant at $P \leq 0.05$.

Results:

Preservation of cytoarchitecture of the embryonic chicken retina:

To determine the optimum period for selection of embryonic stage, retinae from different developmental stages of

chick embryos were cultured and then cell mortality assay was performed (Figure 1A). Compared to co-cultures, we found that organotypic cultures more closely mimic the environment *in-vivo* behavior, which allowed us for straight-forward identification of retinal cells. The results obtained here showed that retinal tissues were able to form regular and higher order organotypic structures. The results also showed that retinal cells from 17 day old embryo were able to survive the culture conditions even until 10 days, while retinae from 19 day old embryo did not keep their structures after one week as judged by cell mortality assay (Figure 1B). Therefore, in all subsequent experiments, retinal tissues from 17 day old embryo were used.

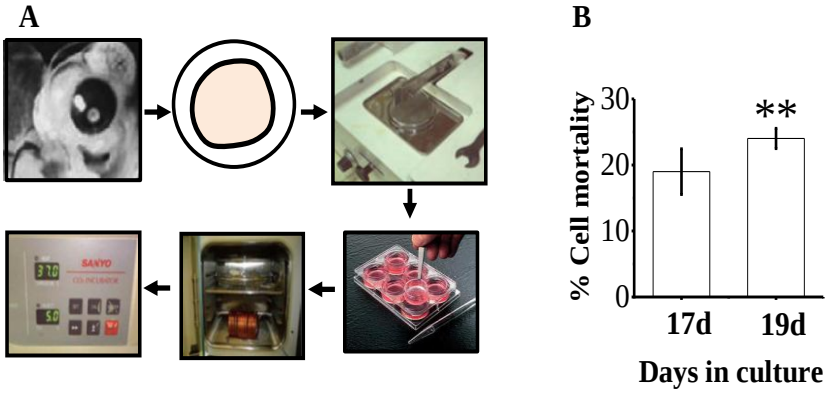

Figure 1. Organotypic culture system of chick retinae. (A) Schematic representation of the method of preparing organotypic cultures of chick retinae. Eyes from embryonic (E) 17 and 19 embryos were removed and whole retinal cups were isolated. The tissues were cultured for 10 days. (B) Quantification of the % of cell mortality. Data are expressed as mean ± SEM for at least 4 independent biological replicates. $^{**}P \leq 0.01$. unpaired student *t*-test.

To determine the optimum period for culturing, 17 day old retinae were cultured and were then fixed at serial time points (Figure 2A). It is important to mention that a few pyknotic cells were seen throughout the cultured tissues which might have been the result of apoptosis (Figure 2B). In comparison with 7 or 10 day old cultures, 0 day old cultures (acute cultures) displayed a

significant (P = 0.005) reduction in the extent of viability from 130.49 ± 4.55% to 83.56 ± 3.77% (Figure 2C). This reduction in the cell survival may be due to long-term effects of retinal slices to dissection procedures. Further analysis of cell survival showed that there were no significant differences in cell survival between retinal slices cultured for 7 days and those cultured for 10 days (Figure 2C, P = 0.116). Culturing of chick retinas for 20 days showed lower viable cells compared with that chick retinas cultured for 10 days (Figure 2C). Therefore, in all subsequent experiments, 7 and 10 day old cultures were used as the culture start point to induce injury and 10 DIV as culture endpoint.

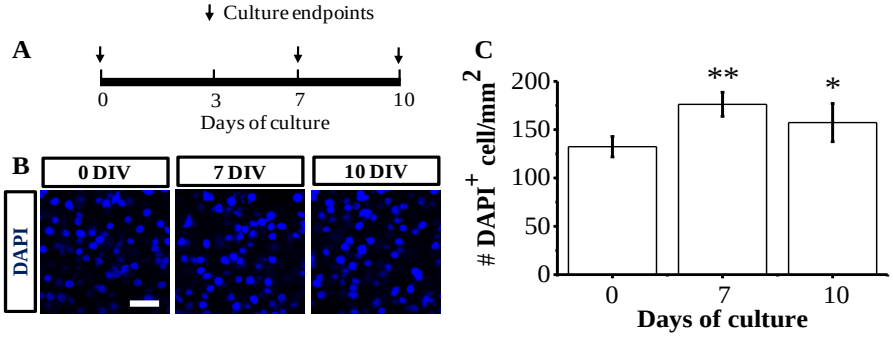

Figure 2. Quantitative analysis of cell survival in organotypic cultures of retinal slices. (A) Schematic of experimental paradigm. Retinae were organotypically cultured and stained, and were inspected at serial time points. (B) Blue DAPI labeling identifies cell nuclei . Sale bar: 50 µm. 40X magnification. (C) Quantification of the % $DAPI^+$ cells in chick retinae cultured. Data are expressed as mean ± SEM for at least 5 independent biological replicates. *$P \leq 0.05$, **$P \leq 0.01$. One-way ANOVA followed by Post-Tukey test.

Expression of mpdz protein in organotypic cultures of the chick retinae:

MPDZ (multiple PDZ domain protein 1) is an essential protein for normal development of the retina and may have a role in many vision-related diseases [16]. Therefore, we performed immunohistologic analysis on White Leghorn retinas with anti-chick mpdz (Figure 3) to investigate MPDZ expression in our

culture system as an indicator of normal retinal cytoarchitecture. After 10 days in culture, we found that there was a minimal retinal disorganization in some cultures leading up to this developmental stage. MPDZ immunoreactivity was observed as intense brown staining at the retinal pigment epithelium (OLM).

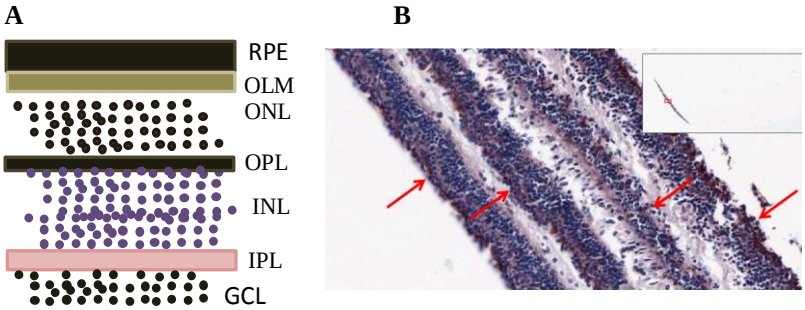

Figure 3. Localization of mpdz in 10 day old culture of the wild-type chick retina. (A) Schematic representation of the retinal cytoarchitecture. (B) Fluorescence images of retinal slices. Arrowheads point to mpdz protein (purple). nuclear staining dye (DAPI; blue). X20. Scale bars, 50 µm. RPE (retinal pigment epithelium), OLM, ONL (outer nuclear layer), OPL (outer plexiform layer), INL (inner nuclear layer), IPL (inner plexiform layer), and GCL (ganglion cell layer).

Genetic transformation of the organotypic cultures of chick retinae:

In the first instance, we sought to develop an experimental protocol that mimics more of an *in-vivo*-like environment under favourable conditions. Such a model would allow us to develop effective therapeutic strategies to treat vision-related diseases. Next we aimed to test the feasibility of transform retinal cells using an AVV-GFP that would help us in transferring of therapeutic genes into the retina cells. A microapplication gene delivery system was tested on organotypic cultures of retinal slices. 3 µl (10^9 pfu) of the adenoviral stock solution was added to the culture medium and left in contact with the slices for 24 hours prior to each fixation (Figure 4A). Our results showed that GFP^+ cells were found throughout the slice without any apparent specific cellular or regional pattern

(Figure 4B). At the edges of the culture slices, transfected cells were more dense. Expression of the transgene was stable over time and did not evolve in both intensity and localization, until at least 10 days post-infection. There was no difference in the mean number of GFP^+ cells between slices cultured for 3 days and those cultured for 7 days. There was no difference in the mean number of GFP^+ cells at most time points (Figure 4C).

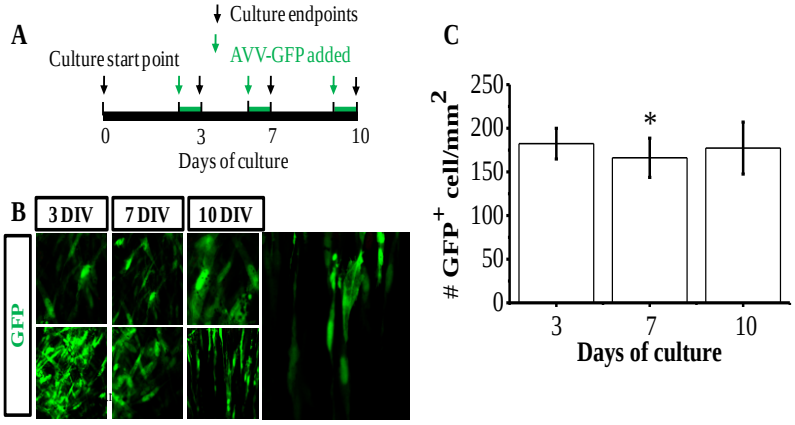

Figure 4. GFP expression after gene transfer by AVV in organotypic cultures of chick retinae. (A) Schematic of experimental paradigm. Retinal slices were organotypically cultured and were infected with AVV-GFP. (B) Fluorescence images of GFP^+ cells (green). Cell processes can be seen to course in and out of the plane of focus of the slice. Scale bar: 40 µm. (C) Quantitative analysis of the course of GFP^+ cells. Data are represented as mean ± SEM for at least 4 independent biological replicates. * versus 0 days old cultures. *$P \leq 0.05$. One-way ANOVA followed by Post-Tukey test.

Efficient transfer of GFP gene into the organotypic cultures of chick retinae:

To test the suitability of this AVV for gene transfer, we examined the possible cytotoxic effects. The infected retinal slices were incubated with ethidium homodimer-1, which is used to stain dead cells. As expected, some dead cells were stained in the retinal cultures by ethidium homodimer-1 (Figure 5A). This suggested that AVV had very little, if any, cytotoxic effect on the infected

cells. There was no difference between non- and infected cultures with regard to cell mortality (Figure 5B).

Next we determined the efficiency of AVV-GFP to transform retinal cells. The infected cultures were fixed and were then stained with DAPI stained nuclei (Figure 5C). After 24 hours of AVV infection, the mean density of fluorescent cells was 673 cells per μm^2 in 10 days old of culture (Figure 5D). This was equivalent to ~ 69 % of cells in this region, as detected by DAPI nuclear stain.

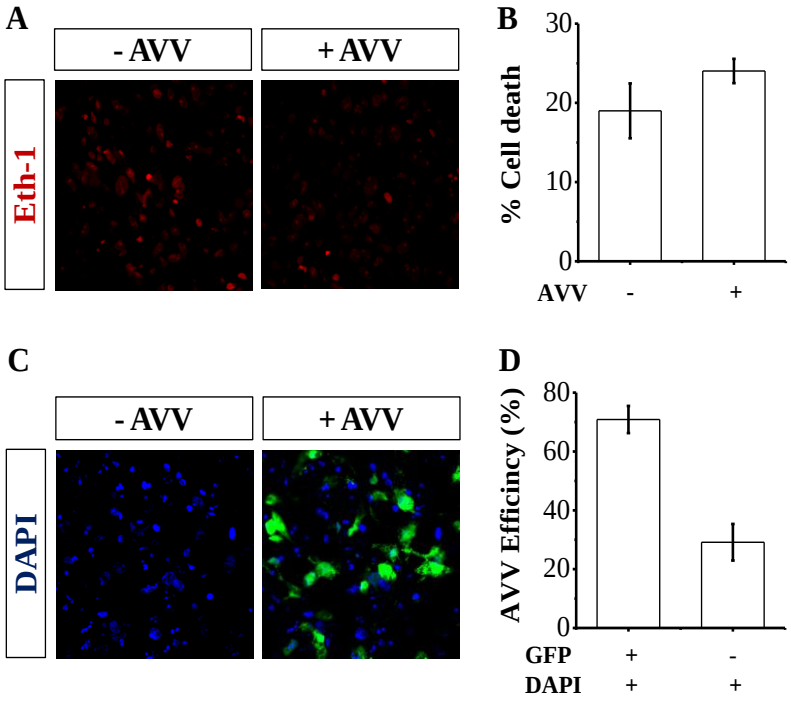

Figure 5. Cell survival in organotypic cultures of retinal slices after AVV-GFP infection. (A) Immunofluorescent images for dead cells (ethidium homodimer-1, red). (B) Quantification of the % of cell death. (C) Immunofluorescent images for viable cell (GFP, green). Blue color shows DAPI-stained nuclei. (D) Quantification of the AVV efficiency to deliver GFP gene into cultured retinal slices. Data are represented as mean ± SEM for at least 4 independent biological replicates. *$P \leq 0.05$. Unpaired student's *t* test.

Discussion:

In this study, we highlighted a simple but most effective an *ex-vivo* model system to culture embryonic chick retinae for extended periods. *In-vitro*, retinal tissues could be cultured as monolayers, dissociated cells, slices, reaggregates or explants [3, 5, 17-21]. Monolayer retinal cultures are quite useful in studying the expression of proteins by individual cells which lack interactions with other cell types [17-21]. However, dissociated cell cultures are useful in conducting cell proliferation or apoptotic assays [18]. Reaggregation cultures of embryonic chick retina are of great importance in three dimensional tissue reconstruction [19] and tissue engineering [20-21]. Though the above-mentioned culture systems have their own significance and advantages, tissues could be considered to mimic more of an *in-vivo*-like environment under favourable conditions because of their tendency to preserve the retinal cytoarchitecture *in-vivo*. Because chicken eyes are relatively large, organotypic culture model of chick retinae could be used to test therapies for human blindness [22]. Therefore, in this study we used an organotypic cultures of chick retinae, which provides a more natural environment to the cultured retinal slices. Compared to co-cultures, organotypic cultures more closely mimic the environment *in-vivo* that allowed us for straight-forward identification of retinal cells. We used retinas from 17 chick embryos which are immature at this stage. We used whole retinal slices instead of retinal tissue parts as explants. During the course of several culture days, the retinal tissues gradually reached a cytoarchitecture that is highly comparable to *in-vivo* retina. Adding to the above-mentioned advantages, such whole retinal slices serve as excellent model system in following the developmental and differentiation pattern of all retinal layers [3, 5]. In particular, the formation of the inner plexiform layer, and differentiation of specific cell types, contributie to inner plexiform layer (IPL) [3, 5, 19, 23]. Retinas from developmental stages at E19 were used to study the efficiency of these slices in forming higher order structures. We found that retinae from E19 days were not able to

survive in culture for more than a week (not shown). A simple explanation could be due to the large size of retinal tissue that had to grow in relatively smaller space [19, 23].

In many instances it will be particularly desirable to observe and quantitate the effect of the culture setting on specific cell types. In this way the progress of the different cell types and the benefits or otherwise of a particular intervention can be demonstrated for these cell types. MPDZ, an essential protein in retinal development is found in the central nervous system [16] retina [24] and many other tissues, where it has been shown to localize to tight, gap, neuromuscular, synaptic, and adherens junctions [16, 24]. In the eye, MPDZ localizes to the outer limiting membrane (OLM) [24]. Recent studies have highlighted the importance of MPDZ function in normal eye development [16]. Therefore, in this work antibody to MPDZ protein was used as an indicator to normal retinal development.

Previously, GFP transgenic animals were used for such imaging with CNS elements [25-27] and these systems give excellent opportunities to follow the progress of injury during a persistent insult. Although these methods may well work within our protocol we decided in the first instance to genetically transform all retinal cells with an AVV carrying GFP gene driven by a CMV promoter. In a previous study, an AVV carrying GFP and lacZ genes driven by CMV was used to transform cells in organotypic cultures of brain slices [28]. In this work, it was demonstrated that this strategy can be applied successfully to retinal slices held in culture and that staining is sufficient to track the majority of retinal cells within a tissue sample when subject to culture conditions, whilst having no cytopathic changes were observed at light and fluorescence microscopic levels (Figure 3-4). Using AVV transformation would make it possible to assess global injury to the slices during the entire culture period and given the correct protocols AVV transformation of slices with GFP would certainly be useful for assessment of differing treatment conditions to alleviate injury. Within the conditions of our culture we found

that the staining of cells remained active for in excess of the 10 days required for our protocol (Figure 5).

Conclusion:

In conclusion, authors have developed a methodology that can be adapted to a variety of treatment strategies for the analysis of developmental plasticity and toxicological effects of foreign agents for regenerative studies. The method is versatile and has the advantage of enabling studies to be carried out in discretely controlled conditions and when the method is used in conjunction with the analysis techniques presented it is possible to monitor a large variety of cellular processes that are relevant in the study of many vision related diseases. It can be also used to test the actions of various transgenes in organotypically prepared retinal slices and provide evidence that the concentration of transgene may be sufficient to achieve biologically significant actions.

إدخال جين بروتين الفلورسنت الأخضر باستخدام الناقل الفيروسي الأدينوفيروس في خلايا شبكية الدجاج في نظام زراعة عضوي نمطي

محمد عبدالسلام القريو*
عبدالحكيم شعبان النفاتي*

المستخلص:

الضرر الذي قد يحدث للشبكية أثناء مرحلة النمو الجنيني قد يكون مرتبطاً بعدة أمراض بصرية مثل خلل تكون أو تحلل نسيج الشبكية. استخدام تقنية نقل جين محمل على فيروس ناقل إلى داخل خلايا شبكية العين قد يوفر إمكانية لاكتشاف بعض العلاجات لبعض هذه الأمراض. تصف هذه الدراسة استعمال نظام زراعة ثنائي الأبعاد لدراسة تأثير وفاعلية تقنية النقل الجيني على خلايا شبكية العين في جنين الدجاج.

جمعت عينات خلايا الشبكية المستخدمة في هذه الدراسة من أجنة عمرها 17 و19 يوماً، وزرعت في نظام زراعة خاص ومضبوط لمدة عشرة أيام، ولقد تم دراسة قدرة التضاعف للادينوفيروس الناقل – المحتوي على مبادر لجين سيتوميقالوفيروس – على إدخال جين بروتين الفلورسنت الأخضر إلى خلايا شبكية عين جنين الدجاج المزروعة.

أوضحت النتائج أن قطاعات نسيج الشبكية لم تظهر أية تغيرات في تركيبها السيتوبلازمي أثناء عملية الزراعة، بالإضافة إلى أن التعبير الجيني لجين بروتين الفلورسنت الأخضر كان في أعلى مستوياته بعد 24 ساعة من عملية إدخال الفيروس، وحافظ على نفس المستوى لعدة أيام. أظهرت أيضا الدراسة أن عملية حقن الأدينوفيروس لم تسبب أية تأثيرات مرضية لسيتوبلازم الخلايا تم دراسة هذه النقطة فقط عند المعدلات العالية من عملية نقل الفيروس.

النهج الذي تم تطويره هنا هو طريقة عملية تقنية قيمة يمكن أن تستخدم لتقييم الضرر الذي قد ينشأ عن الاستخدامات الدوائية على خلايا الشبكية، بالإضافة إلى آلية لنقل جينات علاجية فعالة إلى داخل شبكية العين، والتي قد تساعد البحث العلمي في فهم الآليات المسؤولة عن الأمراض المرتبطة بالنظر وابتكار طرق علاجية ممكنة لهذه الحالات.

* قسم علم الحيوان- كلية العلوم - جامعة طرابلس.

References:

1. Clark, A.F. and T. Yorio, *Ophthalmic drug discovery*. Nature Review 2003. **2**: p. 448–459.
2. Kato, S., Negishi, K., Hayashi, Y., Miki, N., *Enhancement of neurite outgrowth and aspartate-glutamate uptake systems in retinal explants cultured with chick gizzard extract.* Journal of Neurochemistry, 1983. **40**: p. 929-938.
3. Hoff, A., Hämmerle, H., Schlosshauer, B., *Organotypic culture system of chicken retina.* Brain Research Brain Research, 1999. **4**: p. 237-248.
4. Rzeczinski, S., Victorov, I.V., Lyjin, A.A., Aleksandrova, O.P., Harms, C., Kronenberg, G., Freyer, D., Scheibe, F., Priller, J., Endres, M., Dirnagl, U., *Roller culture of free-floating retinal slices: a new system of organotypic cultures of adult rat retina.* Ophthalamic Research, 2006. **38**: p. 263-269.
5. Caffé, A.R., Viser, H., Jansen, H.G., Sanyal, S., *Histotypic differentiation of neonatal mouse retina in organ culture.* Current Eye Research, 1989. **8**: p. 1083-1092.
6. Johnson, T.V., Martin, K.R., *Development and characterisation of an adult retinal explant organotypic tissue culture system as an in vitro intraocular stem cell transplantation model.* Invest. Ophthalmol. Vis. Sci. , 2008. **49**: p. 3503-3512.
7. Melani, A., et al., *Selective adenosine A2a receptor antagonism reduces JNK activation in oligodendrocytes after cerebral ischaemia.* Brain, 2009. **132**: p. 1480-1495.
8. Stoppini, L., P.-A. Buchs, and D. Muller, *A simple method for organotypic cultures of nervous tissue.* Journal of Neuroscience Methods, 1991. **37**: p. 173-182.
9. Gähwiler, B.H., et al., *Organotypic slice cultures: a technique has come of age.* Trends Neuroscience, 1997. **20**: p. 471-477.
10. Stokes, C.E.L., et al., *Dynamics of a transgene expression in acute rat brain slices transfected with adenoviral vectors.* Experimental Physiology, 2003. **88**: p. 459-466.
11. Kluge, A., et al., *Tracing of the entorhinal-hippocampal pathway in vitro.* Hippocampus, 1998. **8**: p. 57-68.

12. Ghoumari, A.M., E.E. Baulieu, and M. Schumacher, *Progesterone increases oligodendroglial cell proliferation in rat cerebellar slice cultures.* Neuroscience, 2005. **135**: p. 47–58.
13. Bingham, A.J., et al., *The repressor element 1-silencing transcription factor regulates heart-specific gene expression using multiple chromatin-modifying complexes.* Mol Cell Biol, 2007. **27**(11): p. 4082-92.
14. Wood, I.C., et al., *Interaction of the repressor element 1-silencing transcription factor (REST) with target genes.* J Mol Biol, 2003. **334**(5): p. 863-74.
15. He, T.C., et al., *A simplified system for generating recombinant adenoviruses.* Proc Natl Acad Sci U S A, 1998. **95**(5): p. 2509-14.
16. Ali M, H.P., McKibbin M, Finnegan S, Shires M, Poulter JA, Prescott K, Booth A, Raashid Y, Jafri H, Ruddle JB, Mackey DA, Jacobson SG, Toomes C, Lester DH, Burt DW, Curry WJ, Inglehearn CF, *Mpdz null allele in an avian model of retinal degeneration and mutations in human leber congenital amaurosis and retinitis pigmentosa*. Invest Ophthalmol Vis Science 2011. **52**: p. 7432-7440.
17. Dutt, K., Reif-Lehrer, L., *Glutamine synthetase induction in chick embryo retina monolayers.* Cell Biophys, 1980: p. 3, 1e17.
18. Gustmann, S., Dünker, N., *In vivo-like organotyoic murine retinal wholemount culture*. Journal of Vis. Exp., 2010. **35**: p. 1634.
19. Layer, P.G., Willbold, E., *Regeneration of the avian retina by retinospheroid technology*. Prog. Retin. Eye Res. , 1994. **13**: p. 197e230.
20. Layer, P.G., Robitzki, A., Rothermel, A., Willbold, E., *Of layers and spheres: the reaggregate approach in tissue engineering.* Trends Neuroscience, 2002. **25**: p. 131e134.
21. Rieke, M., Gottwald, E., Weibezahn, K.F., Layer, P.G., *Tissue reconstruction in 3D-spheroids from rodent retina in a motion-free, bioreactor-based microstructure.* Lab Chip, 2008. **8**: p. 2006e2213.
22. Pearson, R., A. Barber, and E.e.a. West, *Targeted disruption of outer limiting membrane junctional proteins (Crb1 and ZO-1) increases integration of transplanted photoreceptor precursors into the adult wildtype and degenerating retina.* Cell Transplant, 2010. **19**: p. 487-503.

23. Thangaraj, G., A. Greif, and P.G. Layer, *Simple explant culture of the embryonic chicken retina with long-term preservation of photoreceptors.* Experimental Eye Research, 2011. **93**: p. 556-564.
24. Van de Pavert, S.A., A. Kantardzhieva, and A.e.a. Malysheva, *Crumbs homologue 1 is required for maintenance of photoreceptor cell polarization and adhesion during light exposure.* Journal of Cell Science, 2004. **117**: p. 4169-4177.
25. Forsayeth, J.R. and P.D. Garcia, *Adenovirus-mediated transfection of cultured cells. University of California at San Francisco.* BioTechniques, 1994. **354**: p. 354-6, 357-8.
26. Foecking, M.K. and H. Hofstetter, *Powerful and versatile enhancer-promoter unit for mammalian expression vectors.* Gene, 1986. **45**: p. 101-105.
27. Salter, M.G. and R. Fern, *NMDA receptors are expressed in developing oligodendrocyte processes and mediate injury.* Nature, 2005. **438**(7071): p. 1167-71.
28. Miyaguchi, K., et al., *Gene transfer into hippocampal slice cultures with an adenovirus vector driven by cytomegalovirus promoter: stable co-expression of green fluorescent protein and lacZ genes.* Brain Research Bulletin, 2000. **51**: p. 195-202.