Report for The Renal Association:

Nerve Growth Factor Gene Therapy for Diabetes-associated Cardio-renal Complications

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Date of Submission : 21st September 2013

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Background

Diabetes mellitus has emerged as a major threat to human health in the current century. Recent epidemiological data by the International Diabetes Federation (IDF) have reported 371 million suffering from diabetes as of 2012 (1).

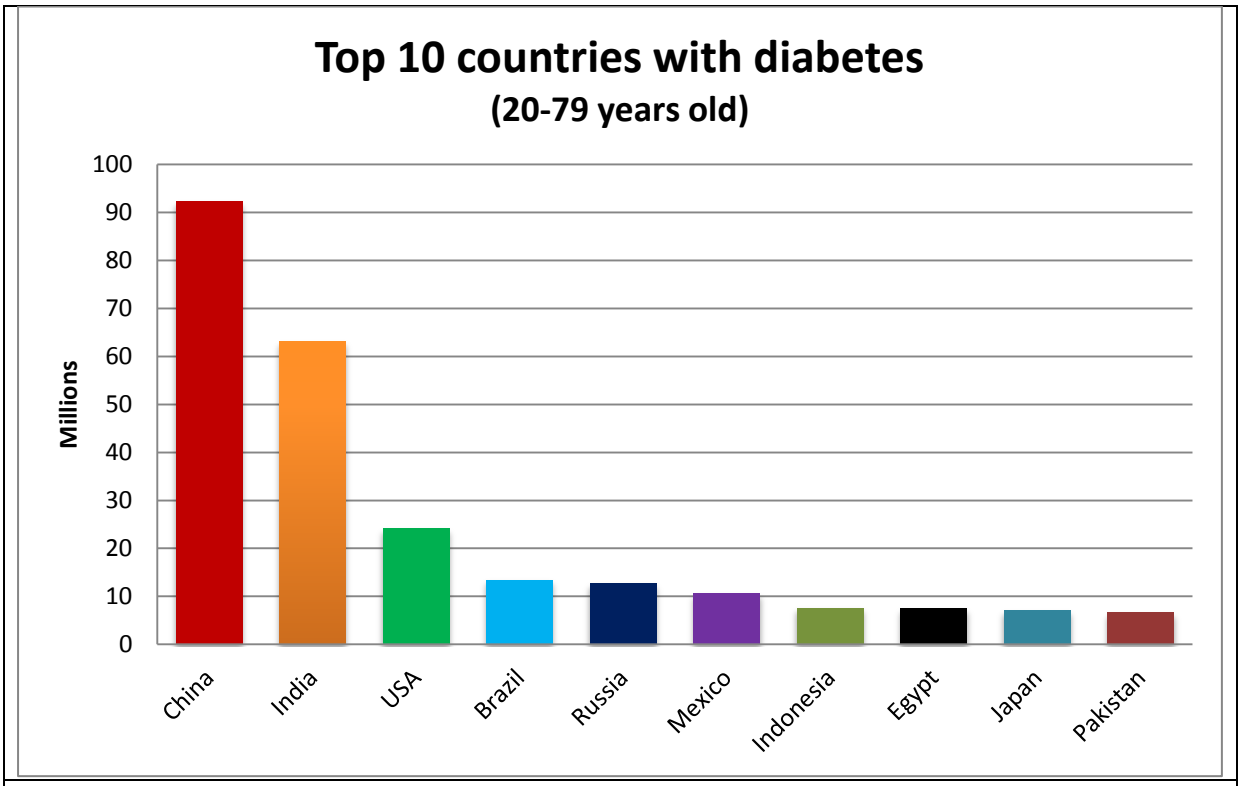

Figure 1. Bar chart showing the top 10 countries with diabetics, aged between 20-79 years old. Adapted from *Guariguata, 2012* (1, 2)

Diabetes is associated with severe cardiovascular and renal complications, and the discovery of drugs to treat diabetes has become stagnant over the past few years. Nerve growth factor (NGF) belongs to a family of proteins called neurotrophins, which are a class of growth factors that regulates axonal growth, survival, differentiation and myelination (3, 4). Recent studies from the group in Bristol have shown that the effects of NGF are not confined to the nervous system and NGF has been shown to be implicated in the survival of cardiomyocytes (5).

NGF expression has been shown to be altered in diabetes (6). The group in Bristol investigated the effects of NGF gene therapy using an adeno-associated virus (AAV) vector to prevent diabetic cardiomyopathy in mice (5). The results showed that mice infected with AAV carrying the recombinant NGF gene were spared from progressive decline in cardiac function at 12 weeks post NGF transfer compared to non-NGF treated mice. Since the relationship between NGF and diabetic cardiomyopathy has been investigated, it is only fitting that we investigate the relationship between NGF and diabetic nephropathy. Thus, this study tested the hypothesis that, by treating cardiovascular complication with NGF overexpression, this same treatment could simultaneously exert a beneficial effect on the diabetic kidney.

Methods

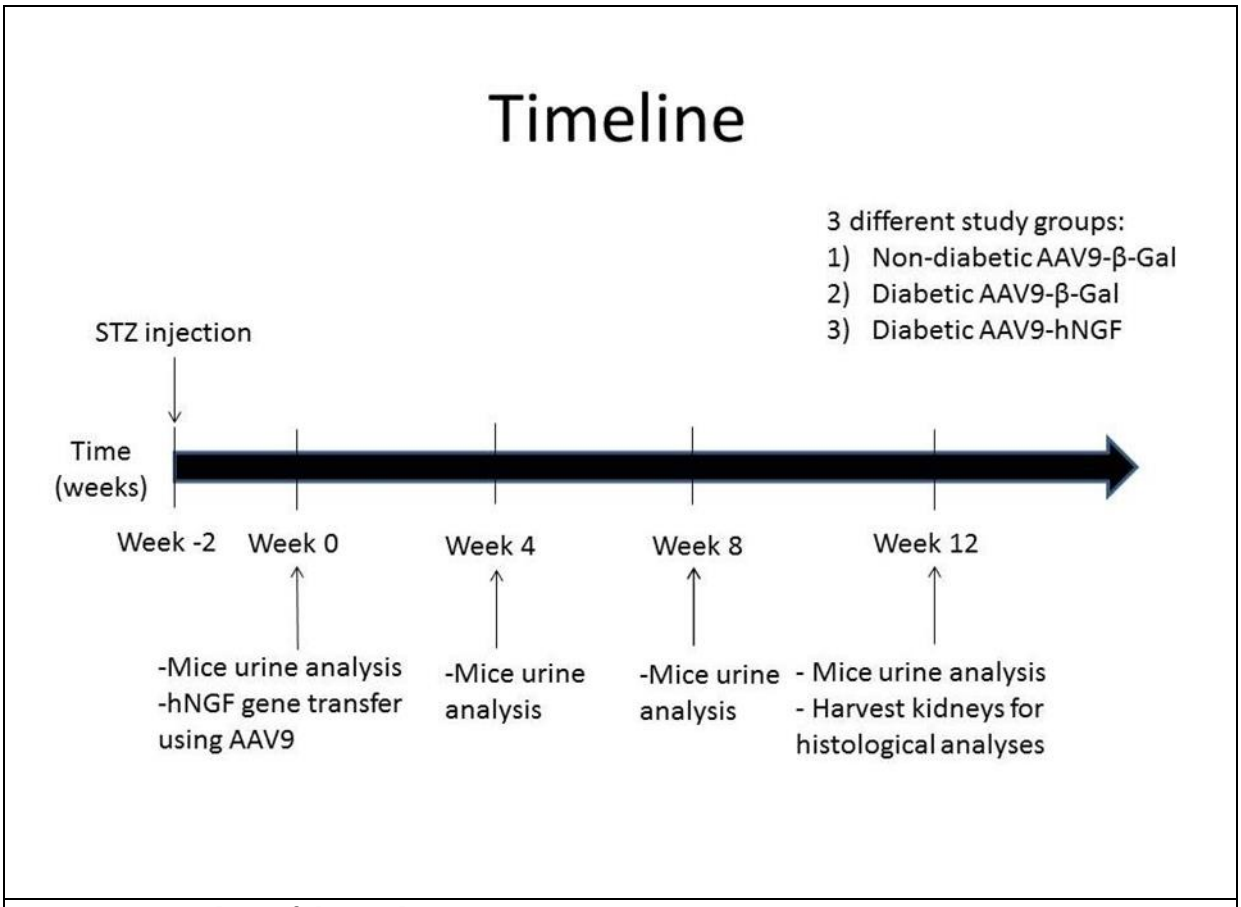

Figure 2. Timeline of the study
STZ= Streptozotocin; β-Gal= β-Galactosidase; hNGF= human nerve growth factor; AAV9= Adeno-associated virus 9

We studied three groups of mice: 1) **diabetic AAV9-hNGF**, 2) **diabetic AAV9-β-Gal** and 3) **non-diabetic AAV9-β-Gal** accordingly**.**

Kidneys of mice were harvested at week 12 post gene transfer and were then analysed for three histopathological features:

1. Vascular density of the renal cortex (capillaries and arterioles). This was investigated through staining of vascular endothelial cells with isolectin-B4 and staining of vascular smooth muscle cells with α-smooth muscle actin (α-SMA).

2. Amount of interstitial fibrosis. Fibrotic tissues were stained with Picrosirius Red dye and revealed using a polarised light filter under microscopy. Fibrotic tissues appeared bright red.

3. Apoptotic cells within the renal cortex. Apoptotic cells were recognised by TdT-mediated dUTP Nick End Labeling (TUNEL staining) and In Situ Apoptosis Fluorescein Detection Kit (ApopTag staining). Apoptotic cells appeared purple, while non-apoptotic cells appeared blue when viewed using fluorescence microscopy.

Results:

Immunohistochemistry for Vascular Density

Isolectin-B4 staining for Endothelial Cells

NGF gene therapy using AAV9 had no effect on the capillary density of the diabetic kidney at 12 weeks post NGF gene transfer.

The mean capillary density for non-diabetic AAV9- β-Gal was of 348.8 ± 51.2 capillaries/mm2 (n=5). Mean for diabetic AAV9 β-Gal was 402.5 ± 38.4 capillaries/mm2 (n=5). Mean for the treated group, diabetic AAV9- hNGF, was 393.0 ± 97.5 capillaries/mm2 (n=5). Results are shown in Figure 3. Analysis of Variance (ANOVA) comparing the different groups showed that there was no significant difference between the groups.

Student's T- test comparing the means between non-diabetic AAV9 β-Gal vs diabetic AAV-9 β-Gal showed that p= 0.126. When comparing diabetic AAV9 β-Gal vs diabetic AAV9-hNGF, p= 0.861. When comparing non-diabetic AAV9 β-Gal vs diabetic AAV9-hNGF, p= 0.395.

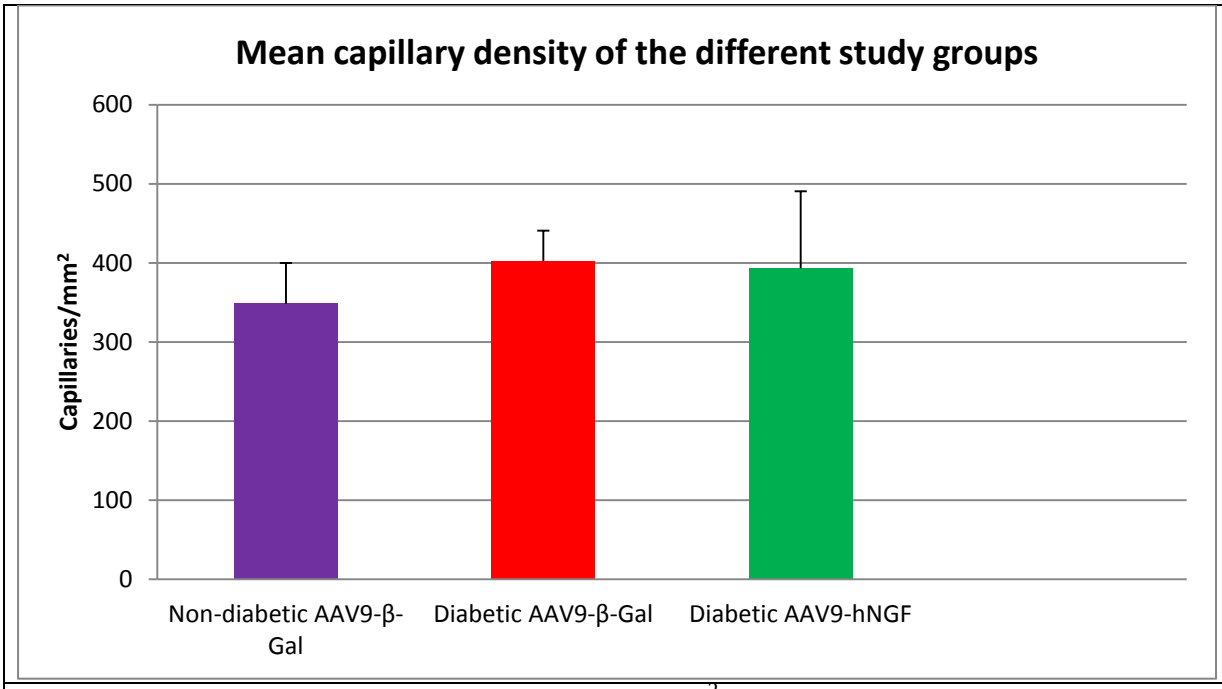

Figure 3. Bar graphs showing the mean capillary/mm^2 of the different study groups. All data are expressed as mean ± standard deviation (SD).
AAV9= Adeno-associated Virus 9; β-Gal= β-Galactosidase; hNGF= human nerve growth factor.

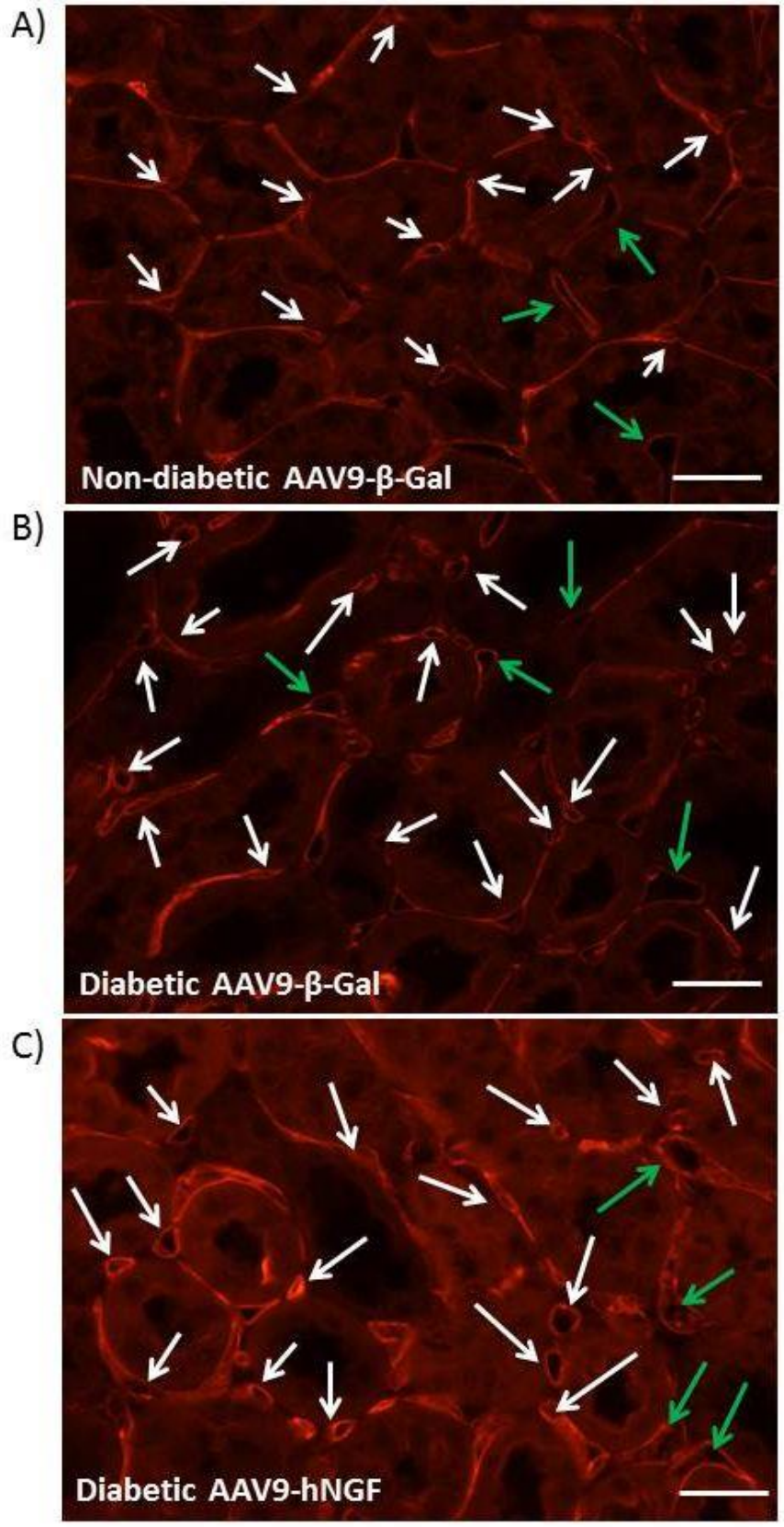

Figure 4. Effect of NGF gene transfer using AAV9 on the microvasculature within the kidney at 12 weeks post gene transfer. Images represent microphotographs of the renal cortex at 40X magnification. Endothelial cells were stained with Isolectin-B4 and appeared bright red under fluorescence microscopy. Capillaries are indicated with white arrows. Larger vessels are indicated with green arrows. Scale bar= 20µm.

α-Smooth Muscle Actin Staining for Smooth Muscles

NGF gene therapy using AAV9 had no effect on the arteriole density of the diabetic kidney when stained using α-smooth muscle actin (α-SMA).

Our results showed that, as expected, the non-diabetic group had the highest mean number of arterioles/microphotograph compared to both diabetic groups. However, overexpression of NGF did not seem to have any effect on the arterioles when compared to diabetic control (diabetic AAV9-β-Gal) (Figure 6). ANOVA comparing the different groups showed there was no significant difference between the groups.

Student's T- test comparing the means for arterioles between non-diabetic AAV9 β-Gal vs diabetic AAV-9 β-Gal showed that p= 0.078. When comparing diabetic AAV9 β-Gal vs diabetic AAV9-hNGF, p= 0.757. When comparing non-diabetic AAV9 β-Gal vs diabetic AAV9-hNGF, p= 0.063.

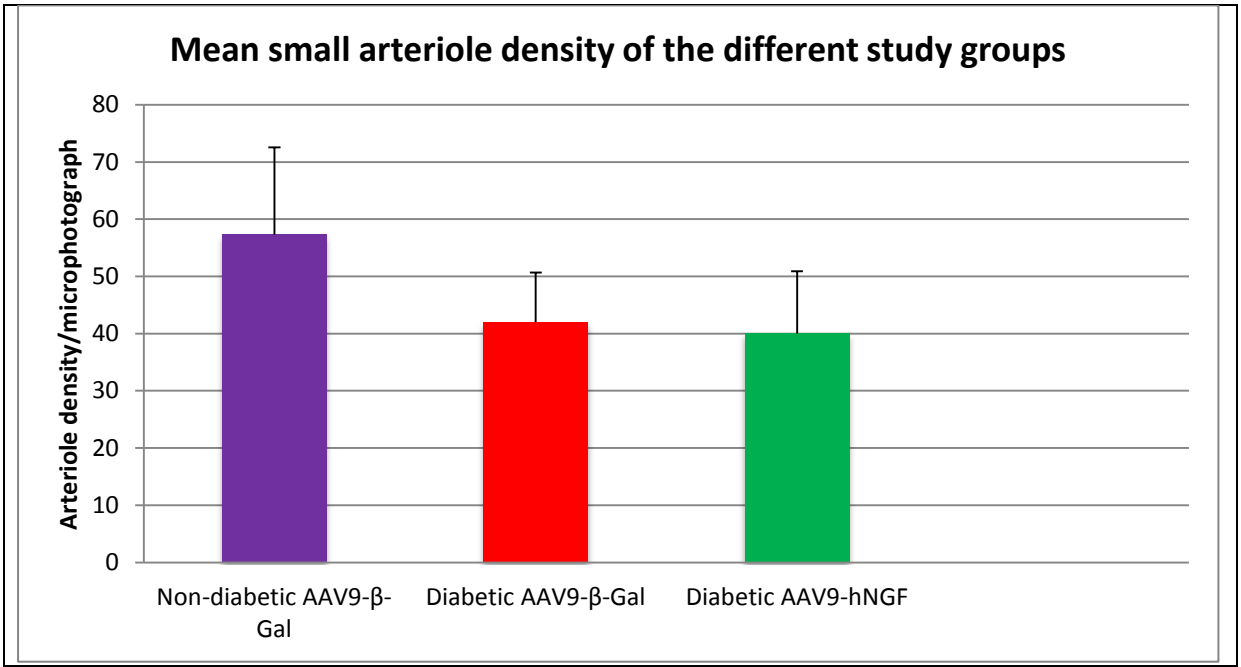

Figure 5. Bar graphs showing the mean small arteriole density of the different study groups. Data for small arterioles are shown separately as these vessels are particularly targeted in diabetes. All data shown are expressed as mean ± standard deviation (SD).
AAV9= Adeno-associated virus 9; β-Gal= β-Galactosidase; hNGF= human nerve growth factor.

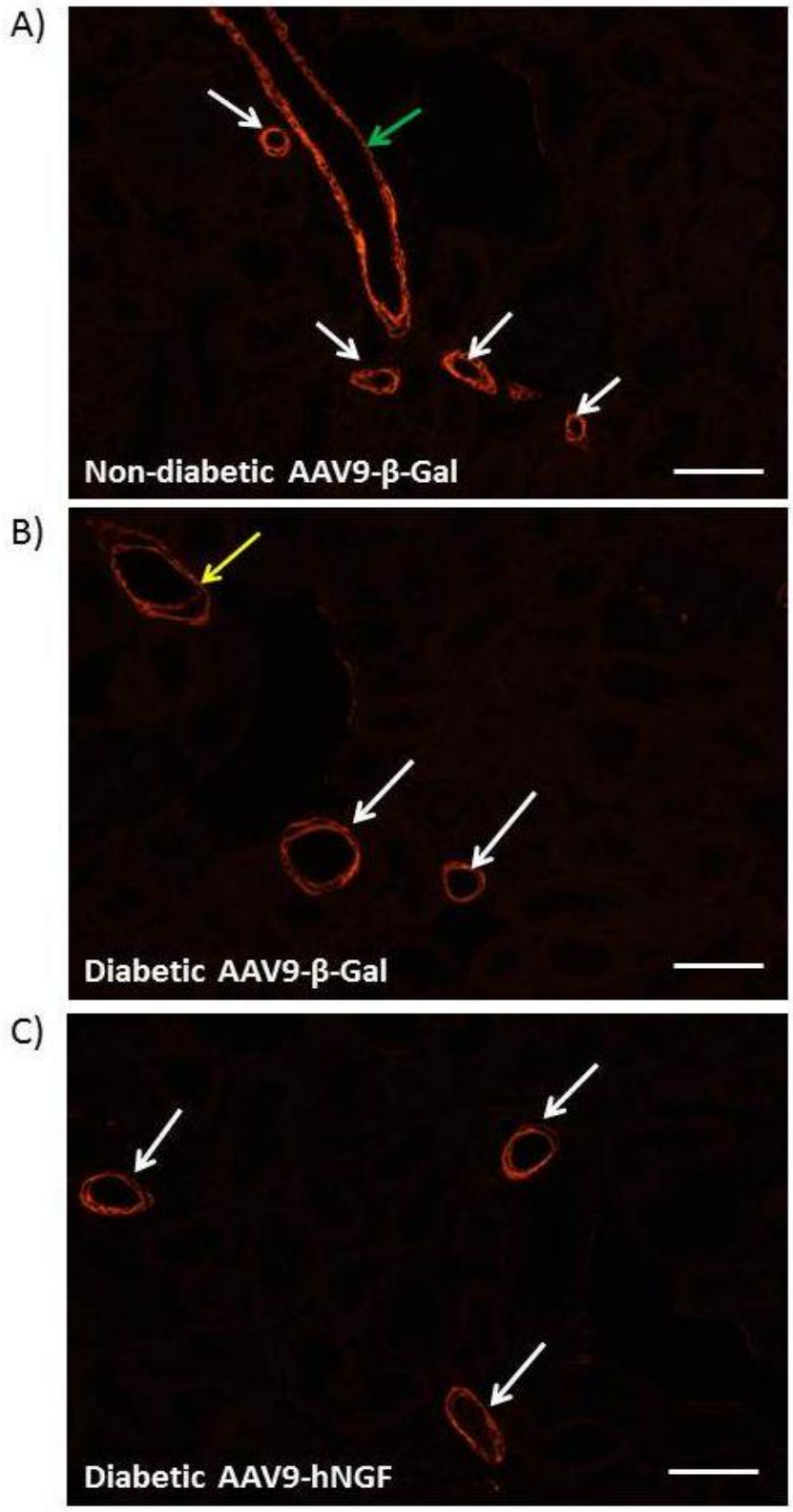

Figure 6. Effect of NGF gene transfer using AAV9 on the microvasculature within the kidney at 12 weeks post gene transfer. Images represent microphotographs of the renal cortex at 40X magnification. Smooth muscles were stained with α-Smooth Muscle Actin (α-SMA) and appeared red. Small arterioles are indicated with white arrows. Medium arterioles and arteries are indicated with yellow and green arrows respectively. Only small arterioles were selected for analysis. Scale bar= 50µm.

4.2 Picrosirius Red staining for fibrotic tissue

NGF gene therapy using AAV9 had no effect on the amount of fibrotic tissue within the renal interstitium when stained using Picrosirius Red solution.

Although the p value comparing the different groups were >0.05, our data showed that the non-diabetic group (non-diabetic AAV9-β-Gal) and the group with NGF overexpression (diabetic AAV9-hNGF) had a reduction in interstitial fibrosis within the renal cortex when compared to the diabetic control group (diabetic AAV9-β-Gal). Overall, the group treated with NGF had the least amount of fibrosis present within the kidney. The mean percentage fibrosis (% of total area) for non-diabetic AAV9-β-Gal was 0.065 ± 0.068%. Mean for diabetic AAV9-β-Gal was 0.127 ± 0.100%. Mean for diabetic AAV9-hNGF was 0.036 ± 0.014%. ANOVA comparing the different groups showed that there was no significant difference between the groups.

Student's T- test comparing the means between non-diabetic AAV9-β-Gal vs diabetic AAV9-β-Gal showed that p= 0.273. When comparing diabetic AAV9-β-Gal vs diabetic AAV9-hNGF, p= 0.081. When comparing non-diabetic AAV9-β-Gal vs diabetic AAV9-hNGF, p= 0.383.

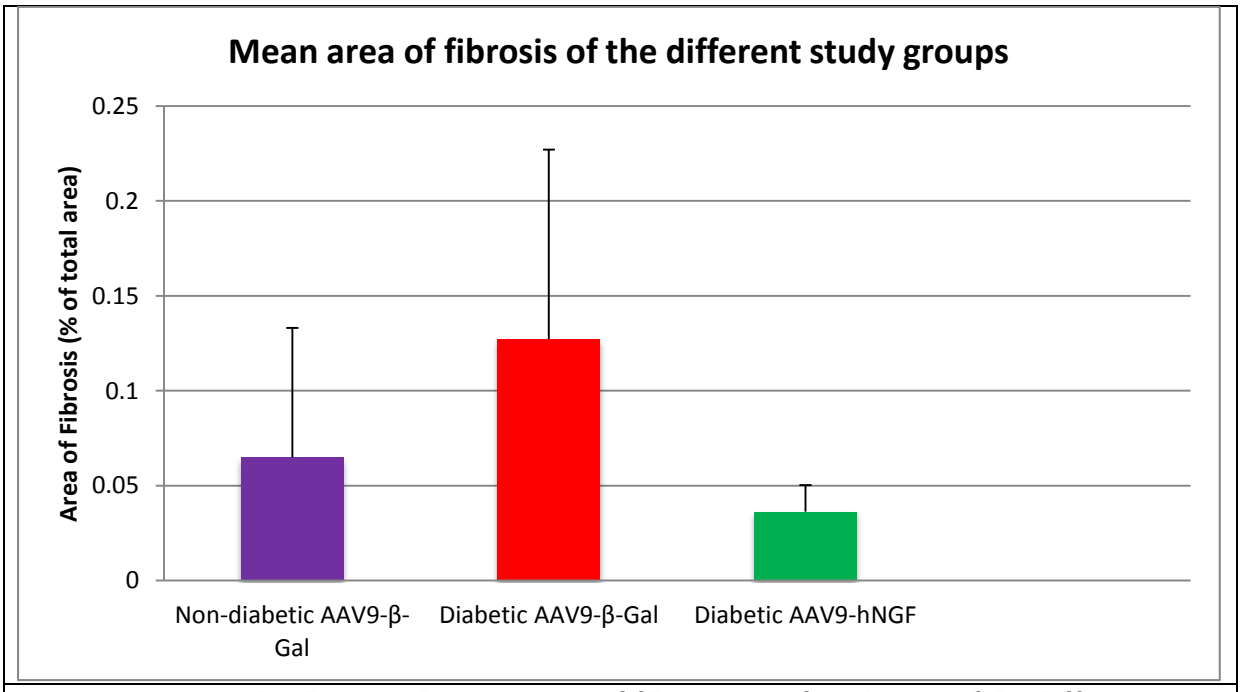

Figure 7. Bar graphs showing the mean area of fibrosis (% of total area) of the different study groups. All data shown are expressed as mean ± standard deviation (SD). AAV9= Adeno-associated virus 9; β-Gal= β-Galactosidase; hNGF= human nerve growth factor.

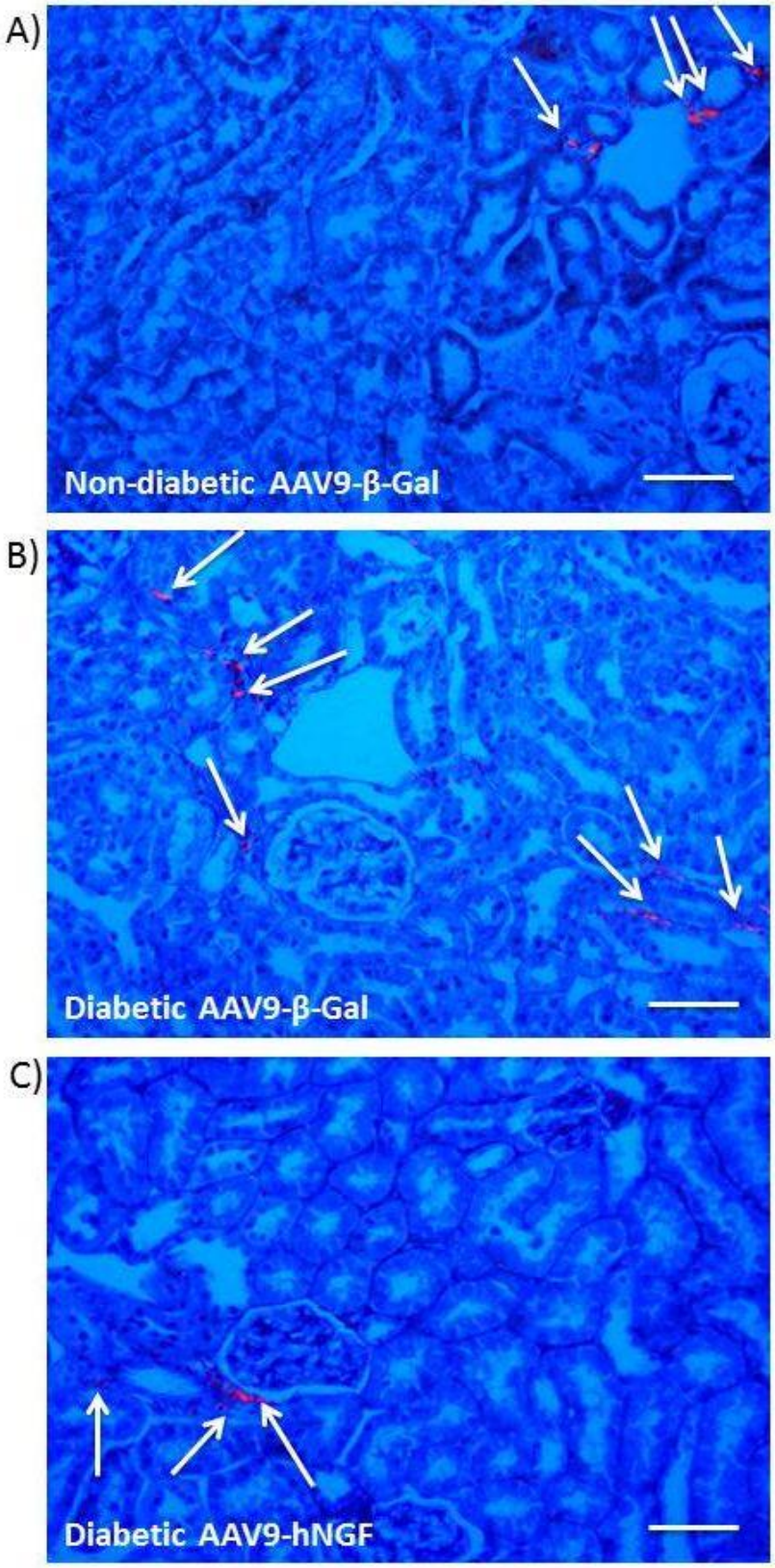

Figure 8. Effect of NGF gene transfer using AAV9 on the amount of fibrotic tissue within the renal interstitium at 12 weeks post gene transfer. Images represent microphotographs of the renal cortex at 20X magnification. Fibrotic tissues were stained with Picrosirius Red solution and appeared bright red when viewed using a polarised light filter, and are indicated with white arrows. Scale bar= 50µm.

4.3 Immunofluorescence for Apoptotic Cells

We were unable to obtain any data regarding the amount of apoptotic cells within the renal cortex. Staining for apoptotic cells were performed using two different apoptosis kit:

1. TdT-mediated dUTP Nick End Labeling (TUNEL) and
2. In Situ Apoptosis Fluorescein Detection Kit (ApopTag)

Under fluorescence microscopy, apoptotic cells appeared purple while non-apoptotic cells appeared blue. The results from the TUNEL staining (Figure 9- left column) showed that >50% of cells per microphotograph were apoptotic (purple). This was deemed to be extremely unlikely, so the TUNEL procedure was repeated a second time, but it yielded the same result. The staining was then repeated using the ApopTag kit, and mice kidney specimens belonging to the same study groups which were stained using the TUNEL kit were used as comparison. The results from the ApopTag procedure were in stark contrast to that of the TUNEL staining. No apoptotic cell could be identified upon fluorescence microscopy and all cells appeared blue (Figure 9- right column). The ApopTag procedure was repeated twice, yielding the same results with no apoptotic cells to be identified.

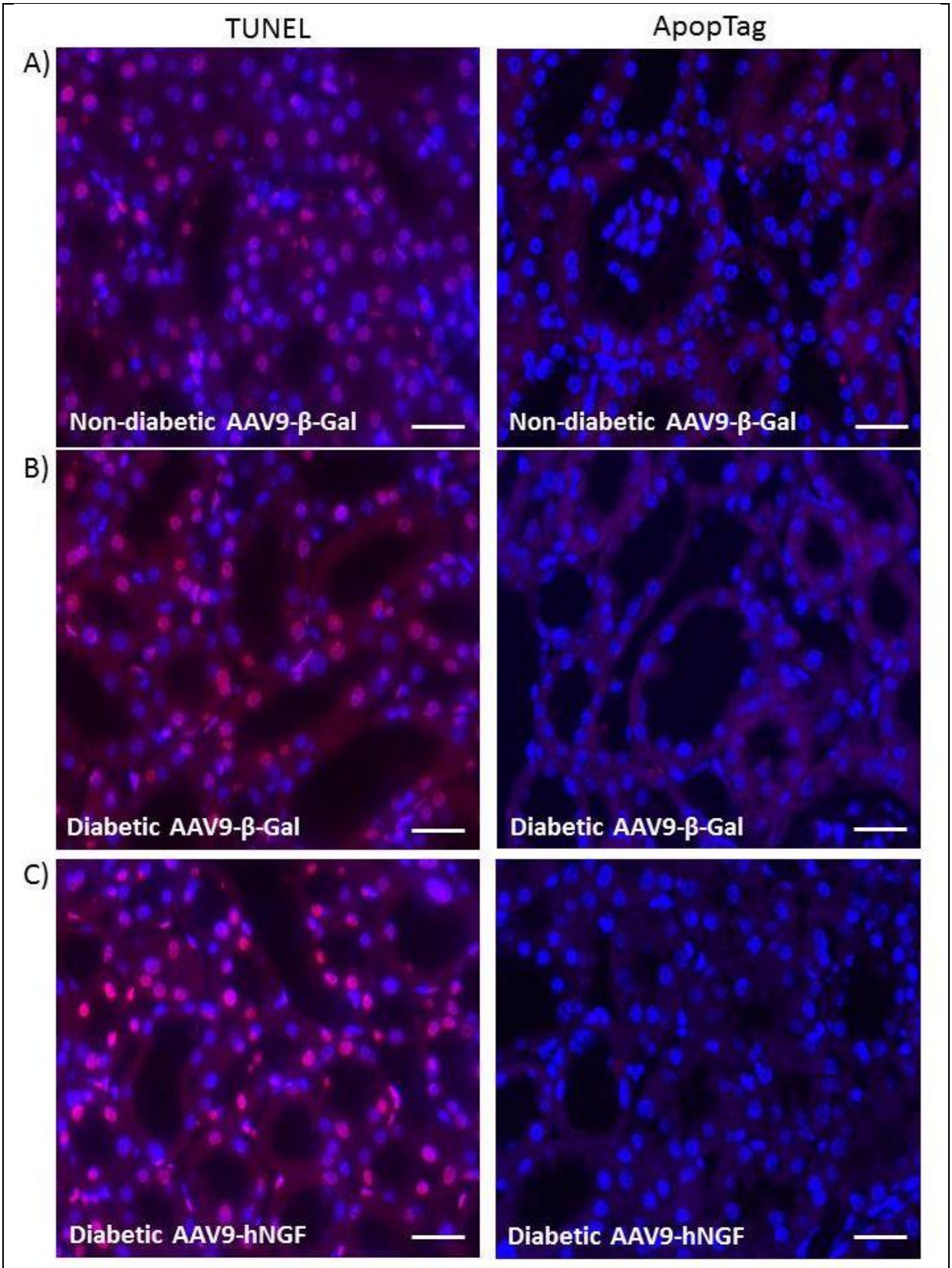

Figure 9. Impact of NGF gene transfer using AAV9 on the amount of apoptotic cells within the kidney at 12 weeks post gene transfer. Images represent microphotographs of the renal cortex at 40X magnification. Apoptotic cells appeared purple, while non-apoptotic cells appeared blue. Scale bar= 20µm

Discussion

Vascular density (capillaries and arterioles)

Our results showed that there were no significant difference between the groups ($p>0.05$), and at three months post gene transfer, no therapeutic benefit could be noted with NGF overexpression. We believe that since diabetes is a chronic condition, and complications associated with diabetes as seen in humans tend to manifest only after a prolonged duration of having the disease, the three months duration in our study sample may be insufficient for features of diabetic nephropathy to develop.

Interstitial Fibrosis

The deposition of fibrotic tissues can be seen in any pathological condition of sufficient duration affecting a particular organ, with the kidney being no exception. Therefore, being a chronic condition, untreated diabetes will inherently lead to kidney fibrosis with widespread disruption of normal kidney architecture and a concomitant deterioration in renal function. Thus, deposition of fibrotic tissues within the kidney would be a characteristic feature of diabetic nephropathy.

Our results showed that there were no significant difference between the groups ($p>0.05$). However in line with the effects of diabetes on the kidney, we noted a trend in our results, in which the mean area of fibrosis (% of total area) for mice treated with β-Gal (diabetic AAV9-β-Gal), was higher than the NGF treated mice (diabetic AAV9-hNGF). This indicated that NGF may confer a protective effect against the deposition of interstitial fibrosis within the renal cortex.

Apoptosis

Apoptosis is a characteristic feature of the complications related to diabetes, including diabetic nephropathy. We attempted to investigate the overexpression of NGF on the amount of apoptotic cells within the renal cortex by performing two different apoptotic staining procedures: 1) TdT-mediated dUTP Nick End Labeling (TUNEL) and 2) In Situ Apoptosis Fluorescein Detection Kit (ApopTag). However, we obtained a negative result for both staining. Due to time constrain, we were unable to identify the cause of this negative result. However, one of our colleagues in the lab who had proposed the usage of the ApopTag kit will be re-investigating the usage of this kit on our kidney specimens on our behalf.

Limitations of Study

One of the main limitations of this study was the relatively small number of kidney specimens that were used for analyses. Another limitation was, we did not investigate the effects of NGF overexpression on the renal function. Determining the renal function may be a better indicator of NGF overexpression compared to histopathological analysis.

Future Work

As we have encountered various shortcomings during our study, most notably, in the failure of apoptotic staining, we plan to further investigate this apoptotic staining procedure to try to identify the main cause of this shortfall. Besides, since diabetes mellitus is a chronic condition and effects of diabetes are only seen after a prolonged duration of having the disease, the study should be repeated with the mice being kept for a longer period. We have proposed to keep the mice for five months (20 weeks) instead of three (12 weeks).

In the next phase of the study we also plan to perform immunohistochemical staining on neurons to investigate the effects of diabetes on the nervous innervation within the kidney. This is important because neurons are frequently affected in diabetes. In the heart, diabetes is known to cause disruption of cardiac innervation and predisposes to silent myocardial ischaemia which increases the risk of sudden death (7). Also, the innervations within the kidney is important to coordinate the processes of glomerular filtration, tubular secretion and tubular reabsorption with the activity of the renin-angiotensin aldosterone system (RAAS) which is the key factor in determining renal function (8).

Conclusion

Our findings suggest that overexpression of NGF using AAV9 vector has no beneficial effect as a treatment of diabetic nephropathy at three months post gene transfer. Although NGF overexpression over three months has shown significant promise to treat diabetic cardiomyopathy, this same therapy does not appear to be beneficial in the diabetic kidney, thus rejecting our initial hypothesis.

Moreover, even if we manage to obtain a positive outcome during the next phase of the study, additional research into safety of NGF gene therapy still needs to be addressed. As NGF is known to be pro-angiogenic and anti-apoptotic, these features may contribute to tumour growth, ocular pathology and arthritis (9). Thus, the potential benefits and adverse events of NGF have to be thoroughly evaluated before any clinical application can be considered.

Reflection

This eight-week study allowed me to gain a deep insight into how translational research work is being conducted. I have learnt how a research idea or project is envisioned, planned, writing of a proposal and eventually carrying out the research. I also learned how to perform basic laboratory skills, most notably:

1) Microtome- Learned how to prepare own slides with usage of microtome

2) Staining- Learned different staining techniques on tissues prepared by microtome such as Picrosirius Red staining for Fibrotic tissue

3) Immunohistochemistry and Immunofluorescence- Performed immunohistochemistry and Immunofluorescence to visualise vascular endothelial cells to estimate the vascular density within the kidney

4) RNA and protein extraction on kidney specimen

One thing which I truly appreciate, which was particularly challenging throughout this elective was the difficulty in collecting accurate and good data from laboratory procedures. I realised that laboratory work is not as straight forward as it sounds, and repeated failures of laboratory procedures are part and parcel of basic sciences' research. This was clearly reflected in my staining of apoptotic cells, where, even though I repeated the staining procedure multiple times, as well as using a different staining kit, I still could not identify the fault within the procedure which resulted in the negative result. Furthermore, I also learned that laboratory work is a slow process and requires significant amount of patience. Between each step of the staining procedures, a waiting time between 1-2 hours is common. Fortunately, I managed to use this time to plough through journals regarding the topic which I was researching on. Furthermore, each full staining procedure may take up to 2 full days for completion.

After being through all these hard and tedious work, I found it all the more rewarding when I was able to visualise the positive staining of my kidney specimens under the microscope. Visualising the fluorescence of target cells under the microscope was truly gratifying, as it indicates that the staining was successful. However, I soon realised that, obtaining a positive staining is only the first part of the equation. Collecting the data by counting the number of cells per microscopic view, is perhaps as tedious as performing the staining procedure itself.

Ideally, if time was not the limiting factor, I would be willing to collect data from greater number of kidney specimens to be included in this study. The data collected from this study would form the basis for future research work. I am truly inspired to dwell further, and hopefully, I will be given the opportunity to continue this project in the future.

Finally, I would like to thank Dr Marco Meloni and Professor Costanza Emanueli for allowing me to be a part of this very interesting and pioneering project. I am especially indebted to Dr Marco Meloni for sharing his infinite wisdom and his exemplary supervision by guiding me throughout the duration of this project. I truly enjoyed working on this project and had a most memorable time.

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