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Research Article

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Central Axonal Response to Stretch Injury: An Immunohistochemical Study

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ABSTRACT

Diabetes mellitus is a chronic disorder characterized by impaired metabolism of glucose and it has many complications affecting the micro vascular level of the eye and kidneys and a variety of clinical neuropathy. It is associated with premature macro vascular diseases such as myocardial infarction and stroke, and peripheral vascular complications leading to ischemia and necrosis with putrefaction of the lower limbs.

The objective of this study is to describe the clinical, pathophysiological findings and surgical management of diabetic foot and how we can improve our outcomes and develop a guideline for the management of diabetic foot. It is a retrospective study of data on a total number of 33 patients admitted to Breast and Endocrine Department at Tripoli Medical Center over a one year period (from first of January 2014 to thirty one of December 2014).
We conclude that diabetic foot syndrome is a major complication of non-controlled diabetes mellitus which can be prevented by education and proper management of diabetes mellitus. Amputation can be minimized in diabetic patients by controlling blood sugar level, along with the administration of antibiotic and Debridement. Meropenem (Meronem) was found to be more effective than any other antibiotics used in our study in treating diabetic foot syndrome.

Keyword- *Immunocytochemical techniques; Neurotrauma; Axonal injured.*

INTRODUCTION

Neurotrauma is a major public health problem of modern life, with a high incidence of mortality and morbidity.[1]

Moreover, evidence is beginning to accumulate that the types of injury currently referred to as mild traumatic brain injuries (mTBI), where a patient may not even lose consciousness at the time of injury, result in long term deterioration.[2]

Traumatic brain injury is a result of both direct and immediate mechanical disruption of brain tissue, known as primary injury, and indirect delayed or secondary injury mechanisms.[3-6]

The most common mechanical input causing TBI, dynamic loading, is associated with a rapid acceleration/deceleration of the brain and the duration of this loading has been proven to be a significant factor in determining the severity of TBI.[7]

Diffuse axonal injury (DAI) is believed to occur from tissue distortion, or shear, caused by inertial forces present at the moment of injury.[5]

The importance of this widespread axonal damage has been confirmed by multiple approaches including routine postmortem neuropathology as well as advanced imaging.[8]

In the optic nerve stretch-injury model, transient (19-21 msec duration) mechanical loading is applied to the mobilized optic nerve by placing a sling around the globe and applying a load of 200-250g to the nerve along the longitudinal axis of the optic canal.[9]

Due to initial technical difficulties, this model was developed using guinea pigs but more recently has been refined to allow its use in mice.[10]

A range of ultrastructural techniques applied to the stretch-injury model has provided quantitative evidence in injured nerve fibers for structural and functional alterations in the axolemma at the nodes of Ranvier and internodes.[11,12]Abnormal accumulation of free calcium in the axoplasm and swelling of mitochondria with loss of cristae[13], loss of axonal microtubules[14], foci of increased packing density of neurofilaments[12], and disorganization of the myelin sheath with foci of dissolution of the axolemma and compacted neurofilaments at the site at which an injured axon has disconnected; that is has undergone secondary axotomy.[14]

However, perhaps most importantly, together with the fluid percussion model[6], the stretch-injury model has provided incontrovertible evidence for a time course extending to a minimum of several hours in experimental animal models and 12 hours in humans[15], before axonal disconnection occurs.

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MATERIALS AND METHODS

Traumatic axonal injury was induced by applying a controlled, transient mechanical loading (19-21 msec) to 9 right optic nerves of albino Duncan guinea pigs with a mean weight of 525 ± 46 g (Harlan UK).

Twelve animals were used for immune-cytochemical labeling.

The animals were subdivided into control/sham injured (n=3), stretch-injured and 1 week survival (n=3), injured 2 weeks survival (n=3) and injured 3 weeks survival (n=3).

In sham animals, the animals were anaesthetized and a peripheral canthotomy undertaken.

The animal was placed on the stretch-injury apparatus, Gennarelli *et al*.,[9] and injury to the right optic nerve was induced.

All animals were allowed to recover from anaesthesia in a warmed incubator set at 37°C.

Three animals were randomly selected from the whole group at 1 week, 2 weeks and 3 weeks, after injury. The animals were then euthanized by left ventricular transcardiac perfusion with 3% paraformaldehyde in 0.1M phosphate buffer.

Each animal was perfused with a fixative for about 30 minutes and then decapitated, and the optic nerve was removed and kept in the fixative for another 48 hours at 4°C.

Blocks were cut at a microtome setting of 7-8µm section,and collected on and mounted on 3-aminopropyltriethoxysilane coated, positively

charged slides (Superfrost Plus, VWR International, Poole, and U.K).

Prior to labeling, sections were heated in a Pro Line ST 45 (950W) microwave oven for 8 min at a power setting of 10. Sections were de-waxed, rehydrated

and incubated following one of three schedules of immunocy to chemical labeling. Either in primary antibody (β-APP) diluted 1:1,000,000 in 20% foetal calf serum (Boehringer-Mannheim, GE) in phosphate buffered saline overnight, RM014 (Cambridge Bioscience) 1:7500 in 20% foetal calf serum in phosphate buffered saline overnight or RM014 overnight followed with a second overnight incubation with β-APP.

After optimal staining for 3-6 mins, sections were washed for 5 mins with water and mounted in non-aqueous mounting medium (Vectamount).

The number of labeled/damaged axons within each optic nerve was counted using routine stereological techniques.

Unbiased random sampling was used to count numbers of labeled axons.

The appropriate section from each nerve was selected and the total numbers of labeled axons were counted at X 100 under oil immersion.

Digital photomicrographs were taken from ten non-overlapping fields where each field was 84 x 53µm on a Lieca microscope.

Within each field all labeled axonal profiles were counted.

The top left hand corner of the optic nerve was selected and a series of 10 photographs were taken across the entire diameter of the nerve. This provided a sample field of 44,520 µm.^{2}

Photographs were printed onto A4 sized photographic paper and all photographs were printed in a single session such that a single enlargement factor was used. On a piece of graph paper a grid with lines spaced at the equivalent spacing of 10µm was drawn and a square cut out of the paper to provide a counting frame. This sized counting frame provided a sample of labeled axons up to five in any one field. The top left-hand corner of each photograph was found, the counting frame placed over that area and the number of labeled axons counted. The counting grid was then moved a distance equivalent to two frames to the right and the number of labeled axons were counted again. Thus the number of labeled axons in 0.33 of the total field was counted. The mean and SEM for the number of labeled axons was calculated and this value was multiplied by the total number of counting frame areas across each photograph. The areas of all photographs for each nerve were summated and the cross-sectional area of each nerve was divided by that value. This provided an estimate of the total number of labeled axons in each experimental animal.

TS sections were labeled for β-APP as an indicator of foci in which fast axonal transport had been lost; sections labeled for NRO14-9 indicated foci at which post-traumatic compaction of neurofilaments had occurred, and sections labeled both for β-APP and MR014.

This labeling strategy was used to test the hypothesis that labeling for β-APP and MR014 occurs in the same axon. The hypothesis may be expressed through the formulae given below:

Number of labeled axons for RM014= (A).

Number of β-APP labeled axons = (B)

Number of double labeled axons= (C)

Thus, if B= C = A this means that the same axons are labeled for β-APP and RM014.

And If B + C = 2A then a completely different population of axons are labeled.

Analysis of Variance (ANOVA) was used to determine whether the differences in the number of labeled axons were statistically significant across all experimental groups. ANOVA demonstrated a difference in the number of RM014 labeled axons across the whole group. But no difference for the number of axons labeled for β-APP.

Comparison of differences in total number of RM014 labeled axons was carried out by comparison of pairs of sets of data.

However, since there is only one set of control animals, the use of the *Student's t* test would have been inappropriate. Rather the Dunnett comparison's test was used.

RESULTS

***Results of Immuno-histochemical labeling*:**

Transverse sections (n = 10 for each animal) of the middle segment from the control (n=3) and injured (n=9) right optic nerves were labeled with antibodies for β-APP, RM014 and both antibodies in combination. From a randomly selected

Table 1: Estimates for numbers of axons labeled either for β-APP, RM014 or with a combination of both antibodies.

Label	Control	1 Wk	2 Wks	3 Wks
β-APP	0	0	0	0
RM014	1365 ± 56	29568.54 ± 1563.7	39441.8 ± 1270.6	52861.6 ± 1563.8
RM014 and β-APP	1436.2 ± 64.7	32165.6 ± 1636.9	39102 .4 ± 1987.2	52672. 8 ± 1930.8

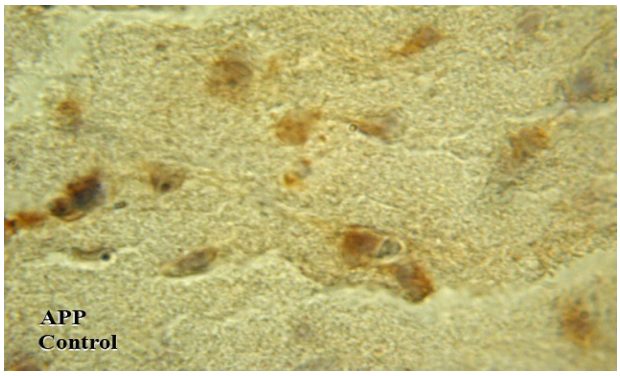


Figure 1: A field of a transverse section of optic nerve from a control animal labeled with β-APP.

No axons are labeled. But astrocyte cell bodies (A) containing nuclei are labelled.

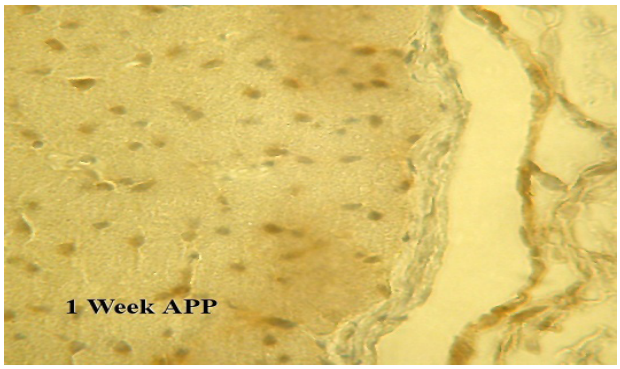


Figure 2: A representative field from a transverse section of a right optic nerve labeled for β-APP at 1 week after injury.

There are no labeled axons, but some astrocytes are lightly labeled (arrow).

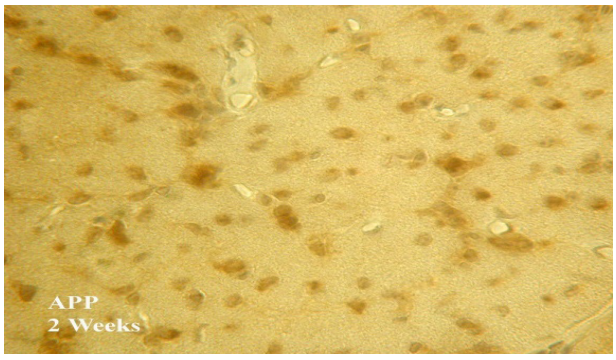


Figure 3: A field of part of a right optic nerve β-APP at 2 weeks after injury.

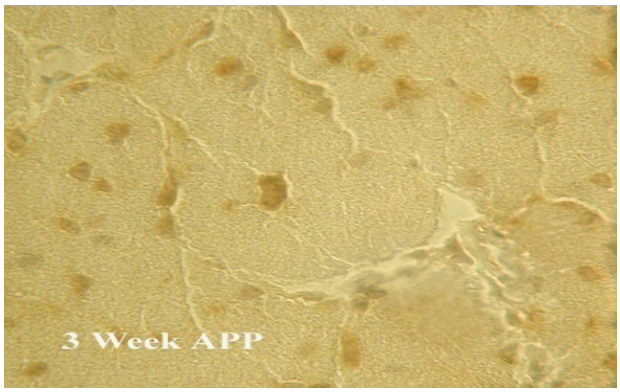


Figure 4: A field from a right optic nerve 3 weeks after injury labeled with β-APP, illustrate the lack of labeling of axons.

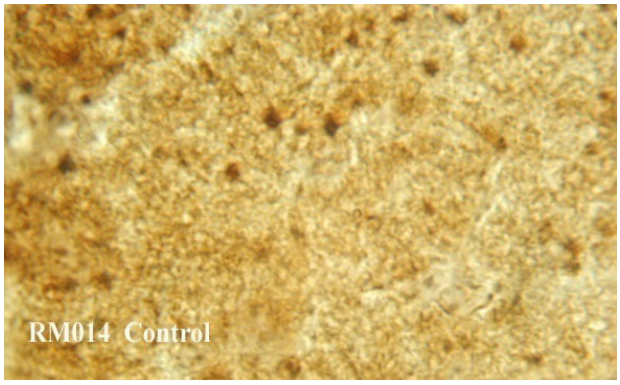


Figure 5: A field taken from a transverse section of a right optic nerve from a control animal.

Some RM014 labeled axons are indicated by arrows.

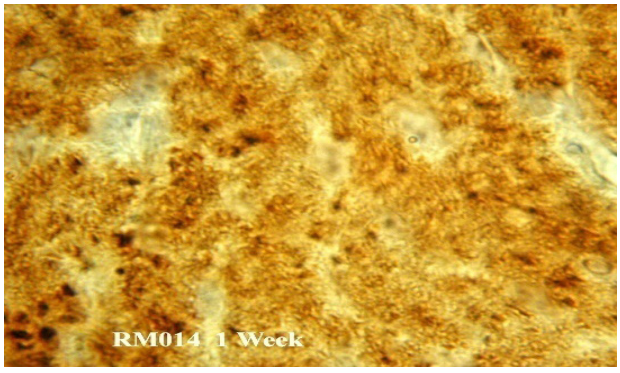


Figure 6: A field taken from a right optic nerve of an injured animal 1 week after injury. Some RM014 labelled axons are indicated by arrows.

transverse section in each animal, the number of labeled axons was estimated using stereological techniques.

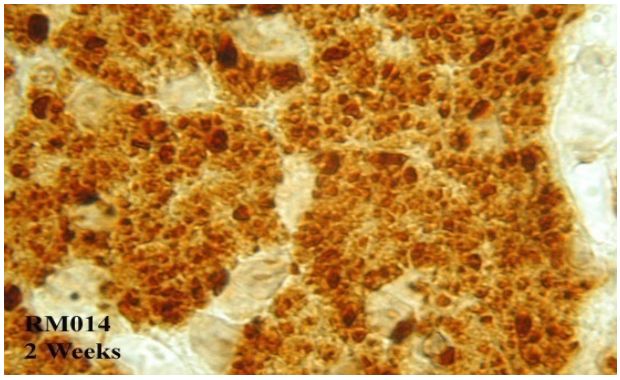


Figure 7: A section labeled for RM014 taken from an injured, right optic nerve 2 weeks after injury.
Numerous axons are labeled – brown coloration.

A second set of transverse sections of right optic nerve from control and 1 week, and 2 weeks after TAI were labelled with antibody RM014. This antibody labels axons within which pathology of the NF cytoskeleton has developed following the injury.

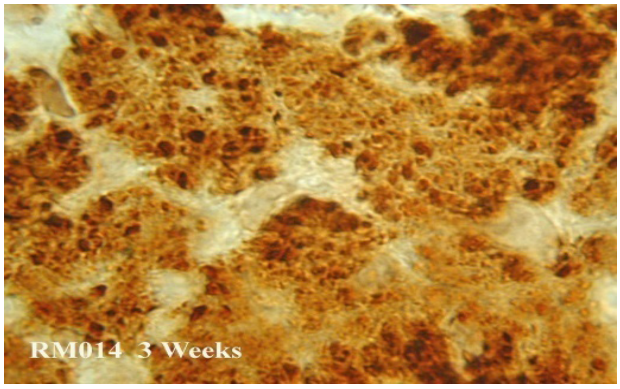


Figure 8:TS section labeled for RM014, taken from an injured right optic nerve 3 weeks after injury, with an increase in the number of labeled axons -brown coloration.

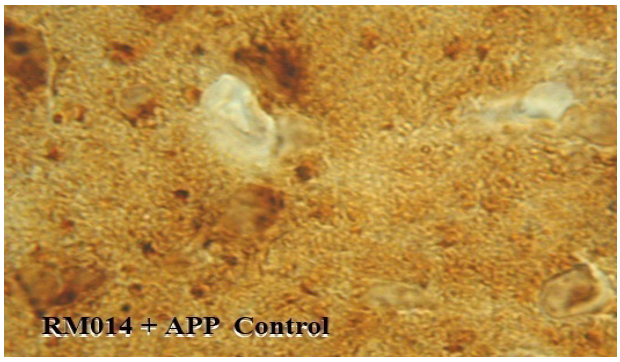


Figure 9: A field of a section from a control optic nerve labeled with RM014 and APP.
A small number of axons are labeled (arrows).

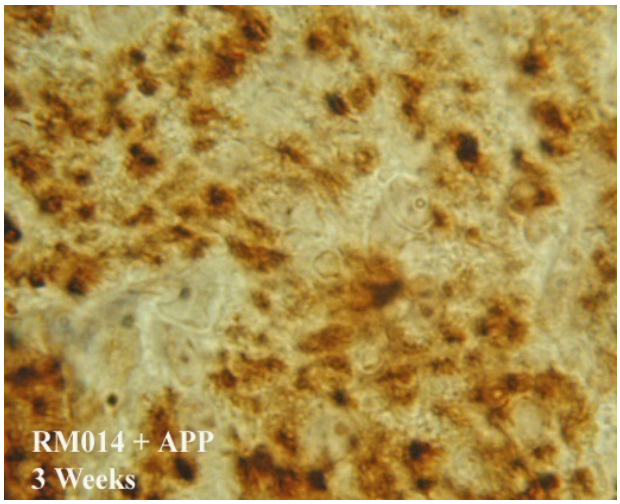
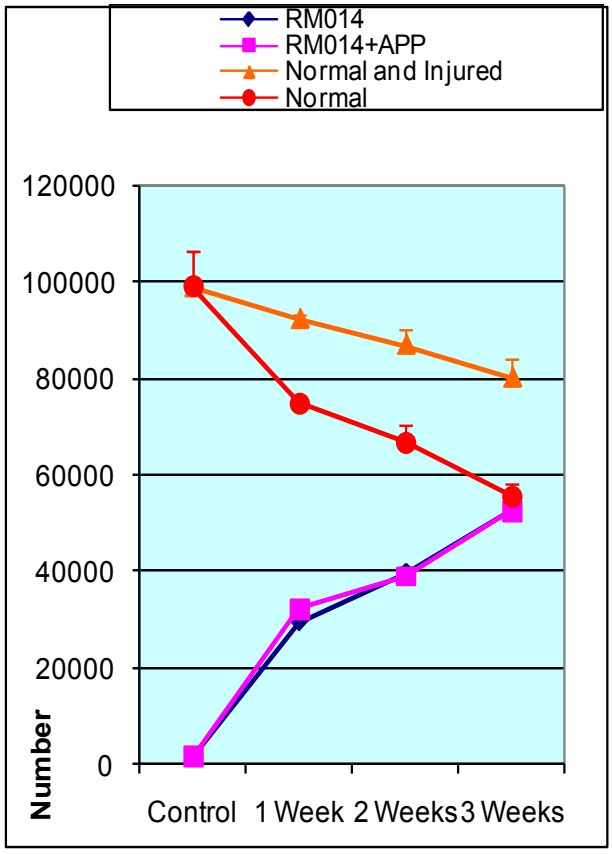


Figure 10: A field of a transverse section of a right optic nerve 3 weeks after injury labeled for RM014 and APP.
Many axons are labeled.



RM014+ βAPP

Figure 11: A graphical illustration of the difference in number of normal/intact axons, normal and intact axons, RMO14 labeled and RM014+APP labeled axons in control, 1 week, 2 weeks and 3 weeks survival animals after injury to the right optic nerve.

Table 2 : Comparison of the numbers of β-APP and RM014 labeled axons

Comparison	Value for q	Value for p
Against RM014 Control		
RM014 control *vs* RM014 1 week	13.73	<0.01
RM014 control *vs* RM014 2 weeks	18.54	<0.01
RM014 control *vs* RM014 3 weeks	25.07	<0.01
RM014 control *vs* RM014+APP control	0.03	>0.05 (ns)
RM014 control *vs* RM014+APP 1 week	14.99	<0.01
RM014 Control *vs* RM014+APP 2 Weeks	18.37	<0.01
RM014 control *vs* RM014+APP 3 weeks	24.98	<0.01
Against RM014+APP control		
RM014+APP control *vs* RM014 control	0.03	>0.05 (ns)
RM014+APP control *vs* RM014 1 week	13.69	<0.01
RM014+APP control *vs* RM014 2 weeks	18.50	<0.01
RM014+APP control *vs* RM014 3 weeks	25.04	<0.01
RM014+APP control *vs* RM014+APP 1 week	14.96	<0.01
RM014+APP control *vs* RM014+APP 2 weeks	18.34	<0.01
RM014+APP control *vs* RM014+APP 3 weeks	24.95	<0.01

DISCUSSION

This study provides novel evidence that with post-traumatic survival up to 3 weeks, the number of β-APP labeled axons is reduced between 8 hours and 7 days after injury and remains very low thereafter (Figure 1).

On the contrary, the number of RM014 labeled axons increases between 8 hours and 7 days and increases further until the end of the present experimental time frame of 21 days

(Figure 8; Table 1).

ANOVA demonstrated that the differences between controls, 1, 2 and 3 weeks survival groups are extremely significant and much greater than expected by chance (P<0.0001).

Furthermore, there are significant increases in the number of labeled axons between controls and 1 week, controls and 2 weeks, and finally control and 3 weeks survivals (Figure 11).

As demonstrated using the Dunnett Multiple Comparisons Test, the data obtained using the latter test,as provided in Table 2, shows that the value for q is greater at 2 weeks than at 1 week and greater at 3 weeks than at 2 weeks.

The hypothesis that there is an increase in the number of labeled axons between 1 week, 2 weeks, and 3weeks was tested using the Tukey-Kramer Multiple Comparisons Test.

There were increased numbers of labeled axons at 1 week compared with controls (P<0.001, q = 22.11), between 1 week and 2 weeks (P<0.001, q = 7.74) and between 2 weeks and 3 weeks (P<0.001, q=10.52).

Thus, in the guinea pig optic nerve stretch injury model of TAI, as the survival time increases larger numbers of axons undergo compaction of neurofilaments and this reflects a continuing and ongoing pathology.

The conclusion can thus be drawn that the extent of or number of axons demonstrating NF compaction in the first several hours after injury,[17,18]increase in number over at least 3 weeks after injury (Figure 11).

Thus there is no support for either the hypothesis that compacted neurofilaments remain unchanged, or that axons recover.

The lack of labeling of axons for β-APP is both puzzling and disappointing. As reviewed in the introduction, the use of APP labeling has become accepted as an important aid to the clinical diagnosis of DAI.

APP labeling has been used to identify damaged axons in several clinical studies.[19,20]

Comparison of the numbers of β-APP and RM014 labeled axons (Table 2) shows that there is no difference in their numbers.

Thus, a definite statement that β-APP and RM014 label the same or different axons following TAI cannot be made at present.

Since the number of axons did not differ between the two experimental groups above, and since sections were not labeled for β-APP alone in the current series of experiments it cannot be definitively concluded that β-APP labeling occurred at all in the double labeling experiment.

However, results obtained at shorter survivals, 8 hours after injury,[21]do provide evidence that β-APP and RM014 do identify different axons in the guinea pig optic nerve. In most white matter tracts, axonal APP accumulation was observed at an earlier time point (6h) than the appearance of RM014 immunoreactivity.[22]

However, other data by Marmarou *et al*.[23], does suggest that the same axons are labeled although their results clearly show that the axons are in different CNS tracts, including the corticospinal tract and the medial lemniscus pathway label differently.

CONCLUSION

The present study provides a novel result in that use of the RM014 antibody demonstrates a steady increase in the number of labeled axons over the period of one to three weeks post-traumatic survival. This steady increase indicates that the pathology which started at the time of trauma somehow continues to recruit more axons. Therefore, treatment of head injured patient should not be stopped early, and we should think of novel treatment strategies.

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