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Thymosin β 4 for the Treatment of Acute Stroke: Neurorestorative or Neuroprotective?

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Abstract

Introduction: Thymosin β 4 (T β 4) is a 5K peptide which influences cellular migration by inhibiting organization of the actin-cytoskeleton. Treatment of acute stroke presently involves use of rt-PA and/or endovascular treatment with thrombectomy, both of which have time limitations. Therefore, development of a treatment beyond these times is necessary as most stroke patients present beyond these time limits. A drug which could be administered within 24 hours from symptom onset would provide substantial benefit.

Areas covered: This review summarizes the data and results of two in-vivo studies testing T β 4 in an embolic stroke model of young and aged rats. In addition, we describe in-vitro investigations of the neurorestorative and neuroprotective properties of T β 4 in a variety of neuroprogenitor and oligoprogenitor cell models.

Expert opinion: T β 4 acts as a neurorestorative agent when employed in a young male rat model of embolic stroke while in an aged model it acts a neuroprotectant. However evaluation of T β 4 as a treatment of stroke requires further preclinical evaluation in females and in males and females with comorbidities such as, hypertension and diabetes in models of embolic stroke to further define the mechanism of action and potential as a treatment of stroke in humans.

Keywords

Thymosin beta4; stroke; oligodendrocyte; aged

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Declaration of Interest

A US Patent has been granted for the use of TB4 in neurological injury: PCT No. 9,149,509 Method for Improving Neurological Outcome after Neural Injury and Neurodegenerative Disease, Oct 6, 2015. Henry Ford Health System has a Material Transfer Agreement with RegeneRX Biopharmaceuticals Inc, Rockville, MD. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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1. Introduction

Stroke is the third leading cause of death in the United States. Each year, approximately 795,000 people suffer a stroke and more than 140,000 people die each year from stroke in the United States. Stroke is also the leading cause of serious, long-term disability in the United States [1]. Clinically, stroke is treated with re-cannulation of the occluded artery with either rt-PA or endovascular treatment with thrombectomy [2, 3, 4, 5]. Both of these treatments are time-limited with administration of rt-PA given as quickly as possible from time of stroke symptom onset out to 4.5 hours and endovascular treatment with thrombectomy performed out to 6 hours of symptom onset for large artery occlusions. Recently, the DAWN trial demonstrated that time of thrombectomy could be extended out to 24 hours provided a mismatch of the severity of the clinical deficit and the infarct volume existed based on MRI or perfusion CT [6]. However these findings did not support extending the time window for all strokes unless a significant mismatch existed [7]. Moreover, thrombectomy can only be performed on large artery strokes and not on small artery strokes in which only rt-PA can be administered. Therefore, development of an agent that is independent of time is necessary as most acute stroke patients present beyond these time limits or have other exclusions. Two other strategies exist for treatment of stroke, neurorestoration and neuroprotection. Cell-based or pharmacological neurorestorative treatments stimulate coupled interactive events such as, neurovascular remodeling, including angiogenesis, dendritic/axonal plasticity to enhance remodeling processes in the injured brain and spinal cord and thereby promote neurological recovery [8]. Experimental data suggest that neurorestorative agents stimulate intact parenchymal cells of the central nervous system, specifically neuroprogenitor and oligodendrocyte progenitor cells, astroglial cells, and endothelial cells that work in concert to promote neurovascular remodeling in the injured brain. Preclinically, these treatments have shown improvement of neurological functional outcome in various animal models [9, 10, 11]. Neurorestorative therapies can be effective when given within 24 hours to 1 month from symptom onset. Conversely, Neuroprotective agents serve to reduce the ischemic damage within the lesion and penumbra in an attempt to salvage neurons and limit extension of the infarction [12]. Neuroprotective agents protect the affected cerebral tissue from a wide range of insults, e.g., oxygen free radicals, excitatory amino acids, mediators of inflammation and calcium overload. Attempts to translate neuroprotective strategies to the clinic have been met with disappointing failure because neuroprotective agents usually only work if administered within one hour of stroke onset and the affected ischemic area has some degree of reperfusion [13, 14].

Thymosin beta4 (T β 4) is a pleiotropic 5K peptide isolated from the thymus. T β 4 sequesters G-actin monomers which inhibit organization of the actin-cytoskeleton [15, 16]. Administration of T β 4 improves functional neurological outcome in a mouse model of multiple sclerosis (EAE, experimental autoimmune encephalomyelitis) and a rat model of traumatic brain injury [17, 18]. A common observation in these neurological disease models is that T β 4 promotes axonal repair and myelination by promoting differentiation of oligodendrocyte progenitor cells (OPCs) in the subventricular zone (SVZ) and in the intact white matter, providing evidence to support the hypothesis that T β 4 is a neurorestorative agent [19, 20]. In this brief review describing our procedures and results testing T β 4 in a

young and an aged rat model of embolic stroke, we found different treatment effects with regard to infarct volumes and functional outcome between the young and aged populations suggesting the T β 4 may act differently when taking age into consideration. We also describe in-vitro data describing upregulation of specific microRNAs (miRs) and other factors that promote cell restoration and survival.

2. Embolic stroke rat model and treatment strategy

We used a clinically relevant embolic stroke rat model that produces a reproducible infarct volume and reproducible neurological deficits that are measureable using standardized testing. The middle cerebral artery (MCA) of male Wistar rats was occluded by placement of an embolus at the origin of the MCA [21]. Young rats (3–4 months old) were randomized and subjected to MCAo and divided into one of 4 groups of 10/group: control, 2, 12 and 18 mg/kg. T β 4 (RegeneRx Biopharmaceuticals Inc) was administered intraperitoneally 24 hrs after MCAo and then every 3 days for 4 additional doses [22]. In a separate study performed later, randomized aged rats (18–21 months old) were treated in a similar fashion using the 12 mg/kg dose [23]. This dose was chosen because it showed the greatest efficacy in the younger rats. For labeling proliferating cells, 5-bromo-2'-deoxyuridine (BrdU, 100 mg/kg; Sigma, St. Louis, MO) was injected IP into rats 24 hours after MCAo and then daily for 7 days.

3. Neurological functional tests

In this embolic stroke rat model, a battery of behavioral tests were performed at 1, 7, 14, 21, 28, 35, 42, 49 and 56 days after MCAo by an investigator who is blinded to the experimental groups. The battery of tests consisted of the adhesive-removal test (ART), the modified Neurological Severity Score (mNSS) and foot fault test (FFT) [24, 25, 26]. Briefly, the ART involves placing two small pieces of adhesive-backed paper dots on the wrist of each forelimb (of equal size, 113.1 mm²) to act as bilateral tactile stimuli occupying the distal-radial region. The time required for the rat to remove both stimuli from each limb was recorded in 5 trials per day. Each animal received 5 trials on all testing days after MCA occlusion and the mean time required to remove both stimuli from limbs was recorded. In order to increase sensitivity of the test, the adhesive-backed paper dots were reduced in size by one-half at day 35. The mNSS test is a composite score in which motor, sensory, balance and reflex measures are used to calculate a value ranging from 1 to 18, with the higher score implying greater neurological injury. Points are awarded for the inability to perform the tasks or for the lack of a tested reflex. (normal score 1, maximal deficit score 18). The FFT measures the ability of the rat to walk along an elevated hexagonal wire grid and the number of times the paw slips (foot fault) between the wires. The total number of foot faults is divided by the total number of steps the rat uses to cross the grid. The average number of foot faults of a normal rat is 2–3%. Fifty-six days after MCAO, rats were anesthetized with ketamine (80 mg/kg) and xylazine (13 mg/kg) and transcardially perfused with saline followed by 4% paraformaldehyde. The rat was then decapitated and brain removed and fixed in 4% paraformaldehyde and the brains embedded in paraffin and cut into seven equally spaced (2 mm) coronal blocks. A series of adjacent 6 μ m-thick sections was cut from each block in the coronal plane and was stained with hematoxylin and eosin. Seven

brain sections were traced using a microcomputer imaging device (MCID) image analysis system (Imaging Research, St. Catharines, Canada) by laboratory associates who were blinded to the dose and functional testing results. The indirect lesion area, in which the intact area of the ipsilateral hemisphere was subtracted from the area of the contralateral hemisphere, was calculated. Lesion volume is represented as a volume percentage of the lesion compared with the contralateral hemisphere.

4. Infarct volumes and outcomes

Figure 1 shows the distribution of infarct volumes in both young and aged rats. No significant differences in infarct volumes were observed in the young rats treated with T β 4 but a large difference was observed in the aged T β 4 treated rats. However, functional neurological outcomes measurements showed different results. Young rats treated with T β 4 showed a significant improvement in overall functional outcome (Figure 2), while the T β 4 treated aged rats showed no improvement (data not shown). The 2 mg/kg dose demonstrated a 24.2, 32.1, and 29.6% relative global improvement in the ART, mNSS and FFT, respectively, while the 12 mg/kg showed a similar improvement of 33.0, 26.0, and 35.5% when compared to controls ($p < 0.05$).

5. Histological and immunohistochemical assessment

Standard paraffin blocks were obtained from the center of the lesion, corresponding to coronal coordinates for bregma -1 – 1 mm. A series of 6 μ m thick sections at various levels (100 μ m interval) were cut. Immunostaining was performed on these sections. Antibodies used for identification of OPCs and OLs were NG-2 (chondroitin sulfate proteoglycan) (1:800, polyclonal rabbit, incubated overnight at 4°C, Chemicon, CA) and APC (CC1) (adenomatous polyposis coli) (1:20, monoclonal mouse, incubated 60 minutes at room temperature, Genway, CA), respectively. To identify proliferating OPCs and OLs, double immunostaining of NG2 / BrdU and APC / BrdU was performed, respectively. Antibodies used for identification of Aquaporin4 (AQ4) and endothelial cells were AQ4 (1:1500, polyclonal rabbit, incubated overnight at 4°C, Millipore, Germany) and Endothelial Barrier antigen (EBA) (1:1000, monoclonal mouse, incubated overnight at 4°C, Covance, NJ), respectively. Double immunolabeling was visualized by secondary antibodies conjugated to FITC and Cy3 (Jackson Immuno).

Myelin basic protein (MBP) antibody (1:1000, monoclonal mouse, incubated 60 minutes at room temperature, Covance, CA) was used to identify myelinated axons. Following sequential incubation with biotin-conjugated anti mouse or rabbit IgG (dilution 1:200, Vector laboratories, INC), the sections were treated with an ABC kit (Vector laboratories, INC). Diaminobenzidine was then used as a sensitive chromogen for light microscopy. Isotype matched non-immune IgG controls were performed for each antibody.

6. Image acquisition and quantification

Coronal sections were digitized using a 40x objective using a three-CCD color video camera (Sony DXC-970MD) interfaced with a MCID system. Laboratory associates performing the

quantitative analysis were blinded to the dose and functional testing results. For quantitative analysis of proliferating OPCs, the numbers of double labeled NG2 and BrdU immunoreactive cells were counted throughout the ipsilateral SVZ (ranging from 13–17 fields of view), ischemic boundary zone (IBZ) (8 fields of view) and the corpus callosum (CC) (4 fields of view). The number of positive cells within three coronal sections per rat was averaged to obtain a mean number of cells. The total measured area of the field of views were calculated. A similar analysis was performed using double labeling of APC and BrdU cells. AQ4 and EBA were analyzed using a computer program to count and auto-threshold double labeled immunoreactivity throughout the IBZ of the cortical area. Data are presented as the density of immunoreactive cells (cells/mm²) for NG2 and APC. For AQ4/EBA, the average percentage of increase or decrease is shown.

For quantification of myelinated axons, MBP immunoreactive area within the IBZ and CC and the contralateral homologous area were digitized. Three coronal sections (100µm interval) from the center of the ischemic core (bregma 0.2 mm) per animal in each hemisphere were analyzed. The fields of view of IBZ and CC of each section were analyzed. Comparing the measured area of the field of view within the ischemic hemisphere to its corresponding homologous area in the contralateral hemisphere, the average percentage of increase or decrease of MBP or AQ4 and EBA reactivity within both the IBZ and/or CC regions of each section is presented. In addition, reactive gliosis was quantified by measurement of GFAP immunoreactivity in eight fields of view within the IBZ. Data are presented as the percentage of increase in area at the IBZ compared with the contralateral homologous region on the same section.

7. Neurorestoration: Markers of oligodendrogenesis

Histological data indicated that Tβ4 promotes oligodendrogenesis in young rats in previous studies [27]. Analysis of brain sections were performed on the 12 mg/kg group. An increase of proliferating oligoprogenitor cells (OPCs) in the striatal ischemic boundary as well as the subventricular zone (SVZ) and corpus callosum was observed (Figure 3) resulting in increased myelination in the striatum (Figure 4). These observations suggest that Tβ4 stimulates generation of OPCs in the SVZ and in the intact white matter, and this increase in OPCs may contribute to the observed recovery. These results support the observed improvement in functional outcome of the Tβ4 treated young rats. In contrast, the Tβ4 treated aged rats showed no histological improvement regarding oligodendrogenesis (data not shown). Because Tβ4 treatment reduces infarct volume in aged rats, we measured blood brain barrier (BBB) integrity by quantifying by the area of AQ4 and EBA double staining in cortical IBZ. AQ4 is a water channel protein while EBA is a marker of endothelial cells [28, 29]. AQ4/EBA reactivity was marginally significantly increased in the treated group ($0.06 \pm 0.04\%$ vs $0.11 \pm 0.010\%$, $p=0.056$) (control vs treated) (mean $\pm$ std) (Figure 5). Collectively, these results suggest that Tβ4 may act as a neurorestorative agent in younger rats but as neuroprotectant in aged rats.

8. MicroRNA expression and cell survival

T β 4 upregulates microRNA expression in both in-vitro and in-vivo models of neural damage [30, 31]. Using both an immortalized OPC cell line (N20.1) and a primary OPC cell isolated from 17-day old rat embryos, T β 4 (25 and 50 ng/ml) increased expression of miR-146a and attenuated its targets, IRAK and TRAF6, components in the TLR inflammatory pathway, which was associated with an increase in myelin expression. Moreover, T β 4 increased expression of miR-200a in neuroprogenitor cells isolated from the SVZ of embolic stroke rats and primary OPC cells isolated from 17-day old rat embryos. A target of miR-200a, Mig-6, a potent EGFR kinase inhibitor, was found to be down-regulated which has implications for oligodrogenesis, as the EGFR signaling is required for MBP expression [32, 33]. T β 4 mediated upregulation of miR-200a also activated AKT, a cell survival, growth, differentiation, migration, and metabolism factor [34]. Activation of AKT not only regulates cell cycle and cell survival of OPCs, but also induces MBP synthesis and differentiation of OPCs [35]. Anti-miR-200a transfection reversed the T β 4 effect on AKT activation, indicating that T β 4 treatment induced AKT activation in a miR-200a dependent pathway, and suggested that miR-200a may target endogenous PI3K/AKT inhibitors. Two miR-200a targets, FOG2 and PTEN negatively regulate PI3K/AKT pathway, and were observed to be downregulated after T β 4 treatment. Data has also demonstrated that T β 4 reduced apoptosis in both the N20.1 and primary OPC cells line in both normal and oxygen and glucose deprivation conditions.

9. Discussion

Treatment of acute stroke has significantly evolved over the last 25 years. Treatment modalities designed to re-cannulate occluded cerebral arteries include both rt-PA and endovascular treatment with thrombectomy [2, 3, 4, 5]. Although the treatment window of rt-PA is presently 4.5 hours from time of symptom onset for most patients with acute stroke, evidence supports that rt-PA should be administered as soon as possible upon arrival to the Emergency Department. Efficacy of rt-PA decreases significantly after 2 hours from symptom onset and guidelines for Emergency Physicians is to administer rt-PA 45 minutes from the time the patient arrives to triage, a significant feat for most busy Emergency Department as CT of the head, consultation with stroke services and consent must be obtained in this narrow time window [36]. If large artery deficits exists on head CT angiography, then endovascular treatment with thrombectomy may be initiated within 6 hours for those select patients and some patients can be treated out to 24 hours provided a significant mismatch between clinical deficit and infarct volume exists on MRI or perfusion CT [6]. However these findings do not support extending the time window for all strokes unless a significant mismatch exists. In addition, small artery strokes can only be treated with rt-PA. Although public awareness has increased the number of patients being treated over the last decade, the vast majority of patients arrive past these critical time treatment windows necessitating development of a drug that is independent of time or at least has a treatment window of 24 hours [37].

Two other strategies exist for the treatment of acute or sub-acute stroke, neuroprotection and neurorestoration [8, 12]. Neuroprotection attempts to protect the infarcted area and the

surrounding penumbra from excitatory amino acids, calcium and electrolyte overload, preserve cell membranes, reduce cellular apoptosis and activate cell survival pathways (ie, AKT). Neuroprotective agents must be administered very early from time of symptom onset (< 1 hour), and some degree of reperfusion must exist so the drug can be delivered to the affected area. Neurorestorative agents, on the other hand, target intact parenchymal cells, including neuroprogenitor cells, to promote an endogenous repair process of neurogenesis, angiogenesis, oligodendrogenesis, synaptogenesis and axonal remodeling. Progenitor cells typically reside in the SVZ and dentate gyrus so they can preserve blood flow and can migrate to the area of injury [38, 39]. More importantly, time is not a significant factor as administration has been delayed out to one week with observed improvement in experimental models [8].

Over the past 25 years, numerous neuroprotective agents have shown efficacy in pre-clinical models, yet none showed any efficacy in randomized controlled clinical trials. The causes of these failures primarily relate to not strictly adhering to the pre-clinical conditions demonstrated in the animal model. For instance, not adhering to specific time limitations from symptom onset, not establishing reperfusion and testing the drug in young animals which is not reflective of the actual clinical conditions. Because of these clinical failures, the Stroke Treatment Academic Industry Roundtable (STAIR) Consensus Conference was created to make recommendation and establish guidelines of pre-clinical studies destined for clinical trials [40, 41]. Among the recommendations include use of aged animals, females and animal models with co-morbid conditions such as diabetes and hypertension.

Our investigations to date on the treatment of acute and sub-acute stroke using T β 4 involve both young and aged rats. These two embolic stroke models demonstrated different results verifying the need to test candidate drugs in different models and conditions. The young rats showed functional improvement but no significant change in infarct volume suggesting that T β 4 is a neurorestorative agent, while the aged rats showed opposite results, no improvement in functional outcome, but a significant and vast reduction in infarct volume. Clinically, most strokes occur in the aged and most stroke victims have some type of co-morbid condition such as diabetes, hypertension, or obesity [42]. The aged rats showed no histological improvement when investigating oligodendrogenesis. However, we did observe a possible explanation for the reduced infarct volume, as it appears that T β 4 increased, or preserved expression of the water channel protein, aquaporin4 and endothelial barrier antigen, suggesting that T β 4 maintains a degree of stability of the blood brain barrier.

The aging BBB is susceptible to dysfunction and dysregulation and it has been identified as a potential cause of many age related diseases [43]. Because it is an important size selective regulatory barrier that serves to restrict exogenous toxins, compromise of the BBB may be responsible for the initiation of many age-related neurological diseases. Aging of the neurovascular unit that comprises the BBB shows decreased expression of AQP4, tight junction breakdown and increased expression of matrix metalloproteins [28]. This pathological alteration that occurs over time may indeed be responsible for many neurodegenerative diseases. T β 4 treatment upregulated AQP4 in this aging stroke model, so future studies investigating the effect of T β 4 on the BBB may be warranted.

Age has a profound influence on disease recovery, as the ability of a mammalian tissue to replace mature cells declines with age [44]. A central hypothesis of aging is that homeostatic maintenance and regenerative potential of tissues decrease as an organism progresses through time [45]. Neurogenesis declines with increasing age in rats. Stroke induces neurogenesis, and in the aged rat the proliferative potential of the SVZ is conserved; however, the ability of the cells to migrate to the area of injury in the rat is diminished [46]. The majority of the decline (80%) occurs in the subgranular zone over 3 to 12 months of age [47, 48]. Many factors have been implicated in this decline. Most notably, is the reduced responsiveness of neural stem cells to environmental cues and the accumulation of inhibitory factors [49, 50, 51]. Certain diseases that commonly affect the elderly, such as osteoporosis or sarcopenia, are theorized to occur because cell loss exceeds renewal. Adult progenitor cells have become central to aging because of their ability to self-renew or engage in repair or replacement of diseased tissue. The aging process is associated with loss of self-renewal in adult stem cells and acquisition of defects in differentiation [44, 45]. These change over time of the plasticity and potentiality that are characteristic of adult progenitor cells are hypothesized to occur because of cumulative DNA mutations, oxidative damage, and accumulation and aggregation of abnormal proteins and lipids [44]. Therefore, the ability of a neurorestorative agent to stimulate recovery may be limited in the aged due to a lack of viable progenitor cells, the biological substrate of recovery, to induce a repair process.

Aged animals recover more slowly with reduced functionality when compared to younger animals [52]. The functional testing used in this study has only been validated in young rats and not aged rats. It is possible that the functional tests used in this study are not sufficiently sensitive to detect differences in neurological outcome. Motor function decreases significantly during aging and the movements required in these tests may require a more sensitive evaluation of motor function to detect differences between groups.

Remyelination generally occurs only from OPCs and not from surviving oligodendrocytes (OLs), or from mature surviving OLs adjacent to the injured axons [53, 54, 55, 56, 57]. Mature OLs are for the most part, unable to migrate or divide, and therefore, new OLs must originate from OPCs. OLs are highly vulnerable to focal cerebral ischemia and the lack of observed oligodendrogenesis in the aged rats is consistent with the theory of the decline of regenerative potential of aging tissues and the loss of self-renewal in adult stem cells and acquisition of defects in differentiation [58].

Our in-vitro data also demonstrated results consistent with both a neurorestorative and neuroprotective mechanism of action, even though our cell culture models, employed SVZ neuroprogenitor cells isolated from young animals and OPCs isolated from day 17 embryonic pups. For instance, T β 4 treatment induced cell survival by inhibiting apoptosis in N20.1 and SVZ cells similar to the reduction in apoptosis seen in cardiac and cornea injury models [59, 60, 61]. In a similar fashion, T β 4 treatment down-regulated p53, an inducer of pro-apoptotic genes resulting in a reduction of ischemic-induced cell death in both rat SVZ neurospheres and primary OPCs under oxygen glucose deprivation (OGD) conditions. T β 4 treatment also activated AKT, a serine/threonine kinase which phosphorylates more than 30 downstream targets/substrates and regulates cell survival, growth, differentiation, migration, and metabolism in a cell-specific manner [34]. Activation of AKT in both rat SVZ

neurospheres and primary OPCs not only regulates cell cycle and cell survival of OPCs, but also induces MBP synthesis and differentiation of OPCs into myelinating mature oligodendrocytes. Inhibition of apoptosis and activation of AKT suggests that T β 4 is a neuroprotectant by inhibiting cell death and promoting cell survival.

A common mechanism observed for both inhibition of apoptosis and activation of AKT is the T β 4 mediated upregulation of miR-200a [30]. A drug which upregulates microRNA suggests that the drug is acting as a neurorestorative agent. In this regard, T β 4 would be considered a neurorestorative agent. Transfection of anti-miR-200a in both the rat SVZ neurospheres and primary OPCs reversed the anti-apoptotic effect of T β 4, reversed the activation of AKT and reversed the MBP expression, suggesting that T β 4 mediated upregulation of miR-200a definitely activates pathways which promote neurorestoration as well as neuroprotection. The distinct power of miRs is the ability to regulate gene expression by targeting mRNA destined to be translated into receptor inhibitor and/or transcription factors. T β 4 mediated upregulation of miR-200a demonstrates this neurorestorative effect by inhibiting the inhibitor of EDGF-receptor mediated oligodendrogenesis, Mig-6, thus allowing expression of MBP.

10. Conclusion

Stroke is a devastating neurological disease and treatment currently relies on symptom time of onset restricted rt-PA and/or recannulation using IA therapy. However, since most patients present beyond the time window of these therapies, development of a recovery agent is necessary. T β 4 was tested in two different embolic stroke models as required by STAIR recommendations, a young and an aged model. T β 4 was administered 24 hours after MCA occlusion and differing results were observed for each model. In the young rat embolic stroke model, T β 4 did not significantly reduce infarct volume but improve functional outcome at 2 and 12 mg/kg suggesting the T β 4 was acting as a neurorestorative agent. On the other hand, T β 4 reduced infarct volume in the aged embolic stroke model but did not improve functional outcome suggesting that T β 4 was acting as neuroprotectant. In-vitro data also demonstrated a bilateral mechanism of action. T β 4 promoted expression of miRs, specifically miRs 146a and 200a, resulting in myelination and reduction of inflammation suggesting neurorestoration. Conversely, in-vitro data also suggested that T β 4 acted as a neuroprotectant by reducing apoptosis and activating AKT, a cell survival protein. Collectively, this review suggests the T β 4 should be continued to be tested in female, hypertensive and diabetic models of embolic stroke to further define the mechanism of action and its potential as a treatment of stroke in humans.

11. Expert Opinion

Thymosin β 4 (T β 4) is a 5K peptide which influences cellular migration by inhibiting organization of the actin-cytoskeleton [62]. Treatment of acute stroke presently involves use of rt-PA and/or endovascular treatment with thrombectomy, both of which have time limitations of 4.5 hours and 6 hours from onset of symptoms, respectively [3, 5]. Some patients with large artery strokes who show a mismatch between clinical deficit and infarct volume can be treated with thrombectomy out to 24 hours of symptom onset [6]. However

these findings do not support extending the time window for all strokes unless a significant mismatch exists. In addition, small artery strokes can only be treated with rt-PA. Therefore, development of a treatment beyond these times is necessary as most stroke patients present beyond these time limits. A drug which could be administered within 24 hours from symptom onset would provide important benefit. Preliminary studies suggested that T β 4 is a candidate for the treatment of stroke and therefore, we tested the peptide in two different rat models of embolic stroke, a young and an aged model as recommended by the STAIR committee for the translation of pre-clinical models to the human model. We chose to test T β 4 using a 24 hour treatment window to develop a drug that could treat the majority of stroke patients rather than time-limit treatment that can only help a small minority of patients. Our results suggest that T β 4 acts differently when using age as a variable. In the young rat model of embolic stroke, T β 4 did not significantly reduce infarct volume but did significantly improve outcome on neurological functional tests. Moreover, histological analysis did suggest that T β 4 promotes oligodendrogenesis which is an important finding as myelin is sensitive to ischemia. In the aged rat model of embolic stroke, T β 4 significantly reduced infarct volume by 50% but did not improve outcome on neurological functional tests. In addition, oligodendrogenesis was not observed on histological sections. Why the difference in results using two models using age as a variable? These results provide an example of the difficulties in treating neurological disorders in the aged is that the regenerative potential of tissues decrease as an organism progresses through time; neurogenesis declines with increasing age in rats [51]. Neurorestorative agents work by stimulating progenitor cells, in this case neuroprogenitor cells in the SVZ or dentate gyrus, to promote neurogenesis, oligodendrogenesis, synaptogenesis and angiogenesis [8]. In young models, these cells are abundant and drugs such as T β 4 are able to stimulate this process. In the aged however, neurorestorative effects may be limited as the organism approaches its natural lifespan [63]. This is not to say that T β 4 cannot be used in the aged, however, further testing is needed such as, possibly administering the drug earlier using a 12 hour treatment window or increasing the dose. In addition, as we mentioned earlier, different types of neurological testing, such as cognitive testing, may be necessary in aged rat rather than the motor/sensory neurological testing used in younger rats. We also suggest that T β 4 be tested in the aged females and rat models with co-morbid conditions such as diabetes and hypertension to further mimic the human condition. T β 4 also has potential for treatment of neurological diseases that affect myelin, specially, multiple sclerosis. Using a mouse EAE model of multiple sclerosis, T β 4 was shown to improve outcome and remyelination [18]. Therefore, development of T β 4 for treatment of any of the demyelinating diseases in humans should be considered.

T β 4 is a pleiotropic peptide that is presently being developed for treatment of dry eye and corneal healing [15, 61, 62]. T β 4 has also been shown to have some benefit in wound healing [64]. The 5th International Symposium on Thymosin in Health and Disease in Washington DC on November 15–17th demonstrates the wide potential of T β 4 as a healing and anti-inflammatory agent. Our results presented at this conference, summarized in this review, suggest that T β 4 has potential for treatment of stroke and other neurological diseases, such as multiple sclerosis, and further development should be pursued.

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Highlights

- T β 4 was tested in young and aged rat models of embolic stroke
- In young rats, T β 4 acted as a neurorestorative agent promoting oligodendrogenesis and improving neurological outcome
- In aged rats, T β 4 acted as a neuroprotectant reducing infarct volume but did not improve neurological outcome
- Cell culture models of neuroprogenitor cells and oligoprogenitor cells showed that T β 4 upregulated miRs-146a and miRs-200a and reduced apoptosis and activation of AKT survival pathway
- T β 4 should be tested in other rat models of embolic stroke, such as aged female and rats with co-morbid conditions of diabetes and hypertension to further define its potential as a treatment for stroke

Figure 1.

Infarct volumes of embolic stroke young rats treated with control 2, 12 and 18 mg/kg of T β 4. There were no significant differences in volumes among the control, 2, 12 and 18 mg/kg groups. On the right, infarct volume of embolic stroke aged control rats and T β 4 treated rats (12 mg/kg). A significant difference in lesion volumes was observed, 26.04 $\pm$ 4.29% and 12.77 $\pm$ 3.61%, (mean $\pm$ SE), (p <0.05). Reprinted with permission from [22, 23]

Figure 2.

Tβ4 (2 mg/kg and 12 mg/kg) treated young embolic stroke rats showed significant improvement on the adhesive-removal test, the modified Neurological Severity Score and foot fault test when compared to control, ($p < 0.05$). Reprinted with permission from [22]

NG2+BrDu Labeling

Figure 3.

NG-2+BrdU double-labeled cells is increased in the ipsilateral SVZ, striatum and corpus callosum of Tβ4 (12mg/kg) treated rats when compared to saline control. Quantitative data show significantly increased double-labeled cells in these areas in the Tβ4 treated rats compared to the saline control ($p < 0.05$). Reprinted with permission from [22]

MBP Staining

Figure 4.

MBP staining shows the myelin in the white matter bundles of the ipsilateral striatum of control and Tβ4 (12mg/kg) treated rats. There is an increased density in the Tβ4 treated rats compared to the demyelination of the saline control. Quantitative data show significantly increased staining in the striatum of Tβ4 treated rats compared to the saline control ($p<0.05$). Reprinted with permission from [22]

Figure 5.

Photograph shows AQ4+EBA double staining in the IBZ of control and T β 4 (12mg/kg) treated rats. T β 4 treatment marginally increased staining when compared to control ($p=0.056$). Arrows point to double-labeled area (AQ4+EBA). White bar is 25 μ m in length. Reprinted with permission [23]