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Research Article

CRESYL VIOLET STAINING TO ASSESS NEUROPROTECTIVE AND NEUROREGENERATIVE EFFECTS OF HARUAN TRADITIONAL EXTRACT AGAINST NEURODEGENERATIVE DAMAGE OF KETAMINE

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ABSTRACT

Ketamine abuse is on the increase in Malaysia. Neurodegenerative change following ketamine use results in debilitating behavioural and cognitive dysfunctions. These changes are thought to be the consequences of network and cellular disorganization and degeneration in the brain involving areas such as hippocampus. Ketamine works by blocking NMDA receptor and hence ketamine research has value in study on NMDA-related neurological disorders such as schizophrenia. This work study the histological change in rats brain which was exposed to ketamine and the effect of treating ketamine-exposed rats with haruan traditional extract (HTE) a substance which has been shown to have positive neuroregenerative function in cell culture study. The changes were studied using cresyl violet and parameters such as pathological score, dead cell count and degenerative change were evaluated. It was demonstrated that ketamine influences these parameters in exposure length-dependent manners and degenerative change could be ameliorated by supplementation with haruan therapeutic extract (HTE) pre-damage induction but not post-damage induction. The result adds to the growing number of evidence on neuroplasticity, and the possible role of non-endogenous substances in protecting as well as inducing genesis of neurons for diseases that share similar pattern of neurological change as ketamine-abused subjects.

Keywords: Brain, Ketamine, Haruan, Cresyl violet, Neurodegeneration, Neuroprotection, Neuroregeneration

INTRODUCTION

Ketamine has been shown to influence glutamate activity of neurons in hippocampus[1] and induces hippocampal neurodegeneration after a prolonged exposure in developing rat brains[2]. Ketamine effects on hippocampus influences cognition and behaviour as the hippocampus region in the brain functions not only as a memory centre, but also regulates behaviour and is one of the known neurogenic zones in the brain[3]. Two large groups of associated behaviours are Type 1 which includes rearing behaviour, and Type II which includes grooming and active exploration[4]. Abnormality in behaviour likewise reflects the underlying neurodegenerative processes.

The neurotoxic property of ketamine is also probably due to the production of oxidative stress[5]. Drugs such as ketamine could also contribute to the neurodegenerative change by affecting brain inherent neurogenesis function adversely[6,7] however, the actual mechanism has not been completely elucidated, and these mechanisms appear to be dissimilar in different drugs of abuse.

The regions of hippocampus are each involved in behavioural control. The CA3 region in particular regulates activities related to novelty detection, working memory and recall for spatial information, reflecting operations of episodic memory which requires interactions between CA3 and dentate gyrus[8]. The CA3 is also important in the recovery of short term memory as well detecting changes in the geometry of the environment[9].

The neurodegeneration taking place in hippocampus could be assessed by staining hippocampal CA3 region with cresyl violet which provides estimation of neuronal cell death and degree of pathological change as well as effect of treatment of HTE, which has shown promising neuroregenerative function in in vitro study[10].

MATERIALS AND METHODS

Selection and preparation of rats

40 six-week old, defect-free male Sprague Dawley rats (150-200 grams) were kept individually in cages with stainless steel cover in the Animal House, Kulliyyah of Science, International Islamic University Malaysia (Kuantan Campus) for one week for acclimatization and reduction of post-travel stress. Temperature, humidity and lighting of the area were controlled at 23±2 °C, 50±10% humidity and 12 hours of light and dark cycle. Sawdust was used as bedding material. Rats were then classified to two large test groups: the short- and long-exposure to ketamine groups.

Neuroprotective and neuroregenerative assays

In the neuroprotective tests, 5 rats per group (n=10) were given HTE for 6 weeks orally, once per day at a constant dose of 100 µl per 100 mg body weight. In the 7th week, ketamine (Bioketan, Poland) (20 mg/kg body weight) was given intraperitoneally, 4 times per day at 2 hour-interval for a pre-determined period of time (1 day for short exposure or 5 days for long exposure).

In the neuroregenerative tests, 5 rats per group (n=10) were given ketamine intraperitoneally, 4 times per day at 2 hour-interval) for a pre-determined period of time (1 day for short exposure or 5 days for long exposure) before being given HTE (Mat Jais, UPM) for 6 weeks orally, once per day. The dose of HTE was constant at100 µl per 100 mg body weight.

For both groups, saline-treated rats (n=5) were used as negative control.

Transcardial Perfusion

Transcardial perfusion technique (Figure 1) was used to perfuse the brain according to established protocol[11]. Rats were made unconscious by exposure to chloroform (Sigma) in a large beaker and quickly put in a supine position on a dissection table. A Y-shaped incision was performed on the rat exposing the thoracic cavity. Saline was first introduced into the left ventricle using a cannula. Another small incision was performed on the right atrium as drainage outlet. 10% formalin (Sigma) was then introduced into the left ventricle via the same cannula, replacing the saline. Formalin was allowed to run until rigor was observed to set.

Brain Dissection

Successfully perfused brain was dissected out according to established protocol[12]. Briefly, the formalin-perfused rat was left in pronated position. An incision was made at the posterior of the neck. The skull was removed carefully to expose the brain. Left and right hemispheres of the brain were dissected out and kept in 10% formalin at room temperature and processed in a semi-enclosed bench top tissue processor (Leica TP1020) before being prepared as wax paraffin blocks.

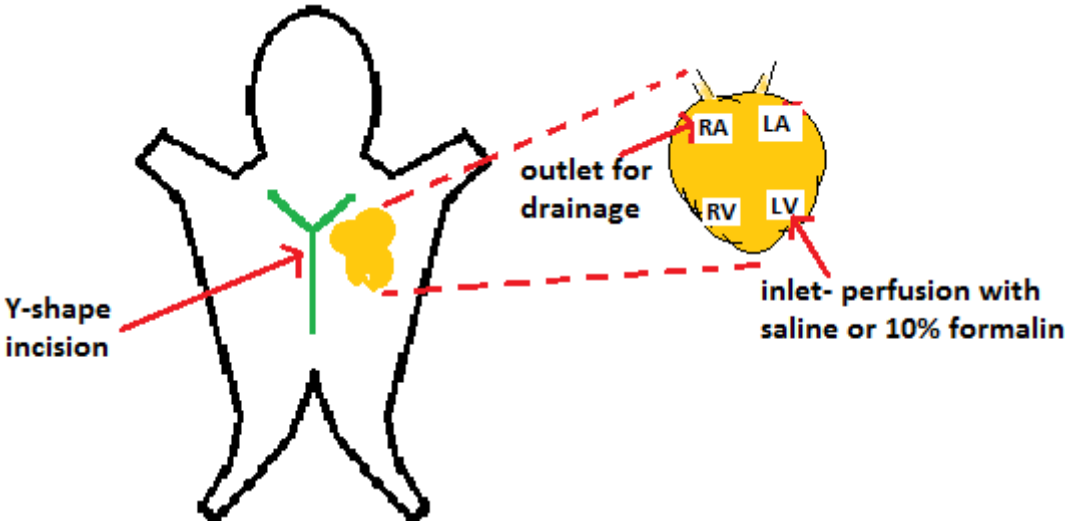

Fig. 1: Transcardial perfusion performed by first making a Y-shape incision to reveal the heart and then creating an inlet at the left ventricle and an outlet at the right atrium to perfuse and preserve the animal with formalin internally. (RA: right atrium; LA: left atrium; RV: right ventricle; LV: left ventricle)

Preparation of Hippocampal Brain Sections

Paraffin-embedded blocks of brain sections (bregma -1.8 mm to -3.8 mm) were cut at 6 µm thickness on rotary microtome (Leica RM2235). Ribbons of sections were floated in a water bath at 36 °C of temperature for 5-15 seconds and fished out and mounted on poly-L-lysine (Sigma) coated frosted microscope slides. The slide was left to dry first by vertical placement on a rack for at least 30 minutes and then deparaffinized on a hot plate at 56 °C for at least an hour. Slide was then kept in an incubator at 80 °C for at least overnight before being used in the staining procedure.

Preparation of Cresyl Violet Staining Solution

0.5 g cresyl violet acetate crystal powder (Sigma) is dissolved in 500 mL of distilled water together with 1.25 mL glacial acetic acid (Sigma) by magnetic stirrer at 60 °C until majority of crystals are dissolved. The solution is then filtered with Whitman paper and stored in an amber-glass bottle.

Staining Procedure with Cresyl Violet

Slides were immersed in xylene (Sigma) twice, for 5 minutes each time, followed by two immersions in 100% ethanol (Sigma) for 5 minutes each. Then slides were immersed in 95% ethanol once and 70% ethanol once for 2 minutes each time before being placed in a container with distilled water for 2 minutes. The slides were then immersed in cresyl violet staining solution for 15-17 minutes and washed again in a container with distilled water for another 2 minutes. The previous steps were repeated in reverse, first by dipping in 70% ethanol for 2 minutes, then 90% ethanol for 2 minutes, followed by two immersions in 100% ethanol for 5 minutes each. The final two immersions were in xylene solution for 5 minutes each. Slides were then coverslipped with permanent mounting medium and left air dried overnight.

Cell counting and pathological score.

The slides were examined with microscope (Nikon T*i*) equipped with epifluorescence and image-capture software. Images of section were examined and cell counting was done using ImageJ software 1.45 with Java pre-installed. (National Institute of Health, available free on-line).

The following parameters were assessed in each section stained with cresyl violet:

(a). the pathological change in the overall area of CA3. The sections were scored using a semi quantitative grading system (Table 1).

(b). the number of viable cells within a 1mm^2 area. Well-rounded cells with no pycnosis were counted as live cells. All of the assessments were made in comparison to the cresyl violet-stained sections of the HTE-treated and saline-treated groups.

Table 1: Description of pathological score in cresyl violet-stained sections

Pathological score	Description
0	Normal appearance
1	Dispersed population of cells in 1-5% area of CA3
2	Dispersed population of cells in 5-15% area of CA3
3	Dispersed population of cells in more than 15% area of CA3

Statistical Analysis

Data obtained were analyzed using Sigma Plot 11.0 for Windows. Firstly, data was tabulated as a line plot graph with error bar. Then Analysis of Variance (ANOVA) tests were conducted at 95% confidence level and differences of $P<0.05$ were considered to be significant.

Ethical statements

Animal care, handling and experiments were approved by the International Islamic University's ethical committee.

RESULTS

The saline- (Image A, Figure 2) and HTE-treated groups (Image B, Figure 2) showed normal appearance of cell and normal structure of the CA3 region in corresponding to pathological score 0 for structure of CA3. Live cell count in both saline and HTE-treated were similar to each other implying HTE-exposure for 6-weeks did not produce degenerative change in terms of cell survival and live cell numbers in the CA3 region.

Neuroprotective assay

In the neuroprotective assay, pathological scores were 0 for SEK (Table 2) respectively which gave the impression that little structural change was affected and the gross structure of the hippocampus remained close to normal despite exposure to ketamine (Image C, Figure 2). The LEK produced a dispersed distribution of cells, in which about 1-5% change to the general appearance of the cell distribution could be observed (Image D, Figure 2) denoting a pathological change of 1 (Table 2).

Counting of the live cells by looking at the cresyl violet stained sections at CA3 revealed that the reduction of cell count in SEK and LEK (Figure 3), in comparison to HTE and saline-treated groups, was not significant (ANOVA, $p>0.05$). These observations imply that providing HTE prior to the exposure to ketamine could protect against structural and cellular damage in the CA3 region but is still influenced by the length of exposure.

Neuroregenerative assay

Significant pathological change could be observed in the neuroregenerative assay as the SEK group scored a pathological score of 3 while LEK scored the pathological score of 2 (Table 2). In both SEK and LEK, dispersal of cells and structural disorganization could clearly be seen. (Image E and F, Figure 2).

Cell count in SEK and LEK in the neuroregenerative assay showed differences which imply that exposure to HTE for 6 weeks post-ketamine exposure could affect neurodegenerative changes. In the SEK group, live cell counts remained below the normal count as seen in the HTE and saline groups). Statistically, this difference is significant ($p<0.05$), suggesting that regeneration did not take place in the SEK group.

Despite the lengthened exposure, live cell count in the LEK showed a higher cell count than both the HTE and saline (Figure 3) but the difference is not significant difference ($p>0.05$). Given the results in SEK, this suggests that some level of neuroregeneration might have taken place in the LEK group when HTE-treatment was given.

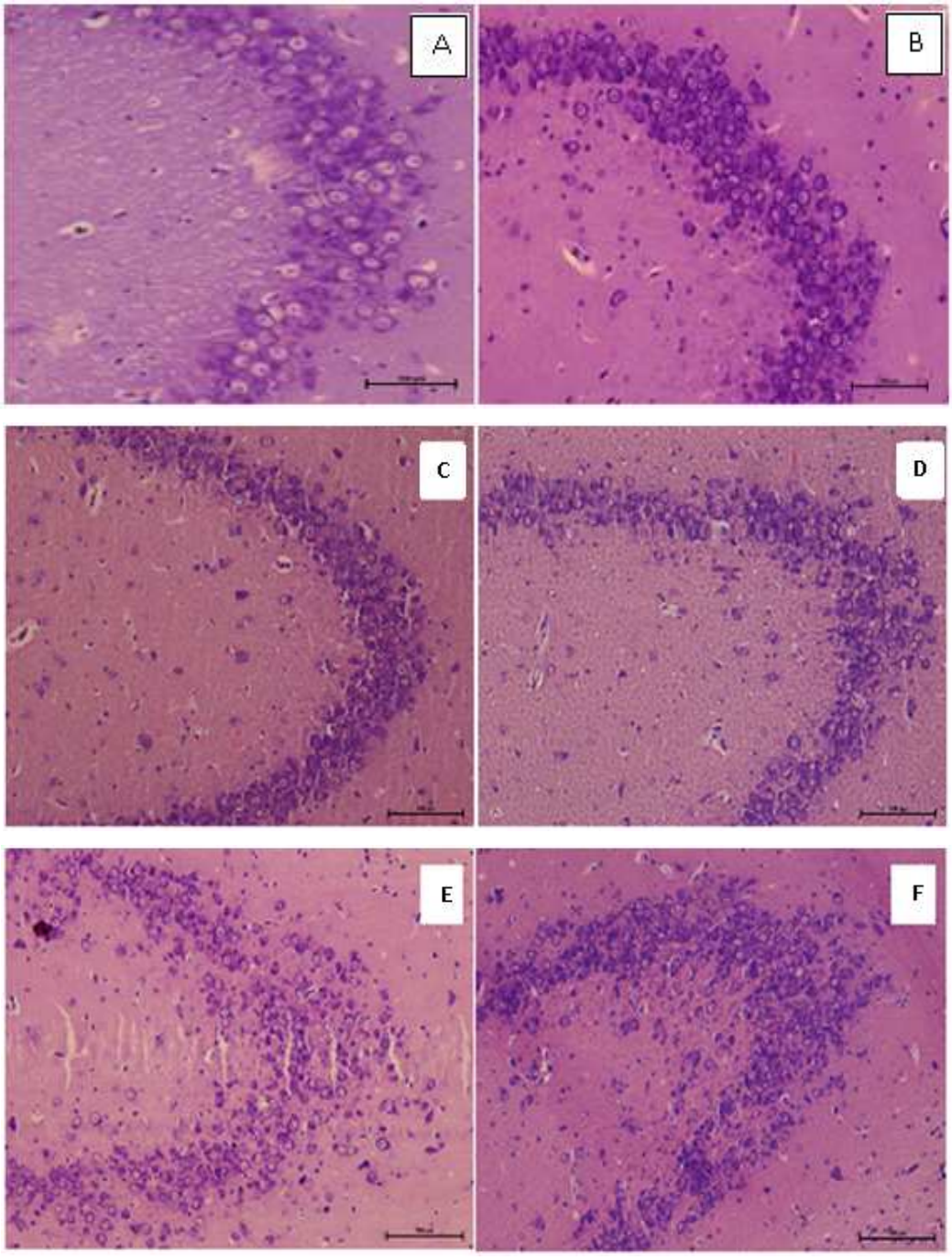

Fig. 2: Cresyl violet stained sections of CA3 of the hippocampus. (A: Saline only; B: HTE only, C: SEK-NP, D: LEK-NP, E: SEK-NR, F: LEK-NR). Bar represents 100µm.

Table 2: Pathological score in cresyl violet-stained hippocampal sections in the neuroprotective (NP) and neuroregenerative (NR) assay.

Neuroprotective assay	Pathological score
Saline	0 (n=5, mean=0, SD=0)
THE	0 (n=5, mean=0, SD=0)
SEK-NP	0 (n=5, mean=0, SD=0)
LEK-NP	1 (n=5, mean=1.2, SD=0.447)
SEK-NR	3 (n=5, mean=2.8, SD=0.447)
LEK NR	2 (n=5, mean=1.8, SD=0.447)

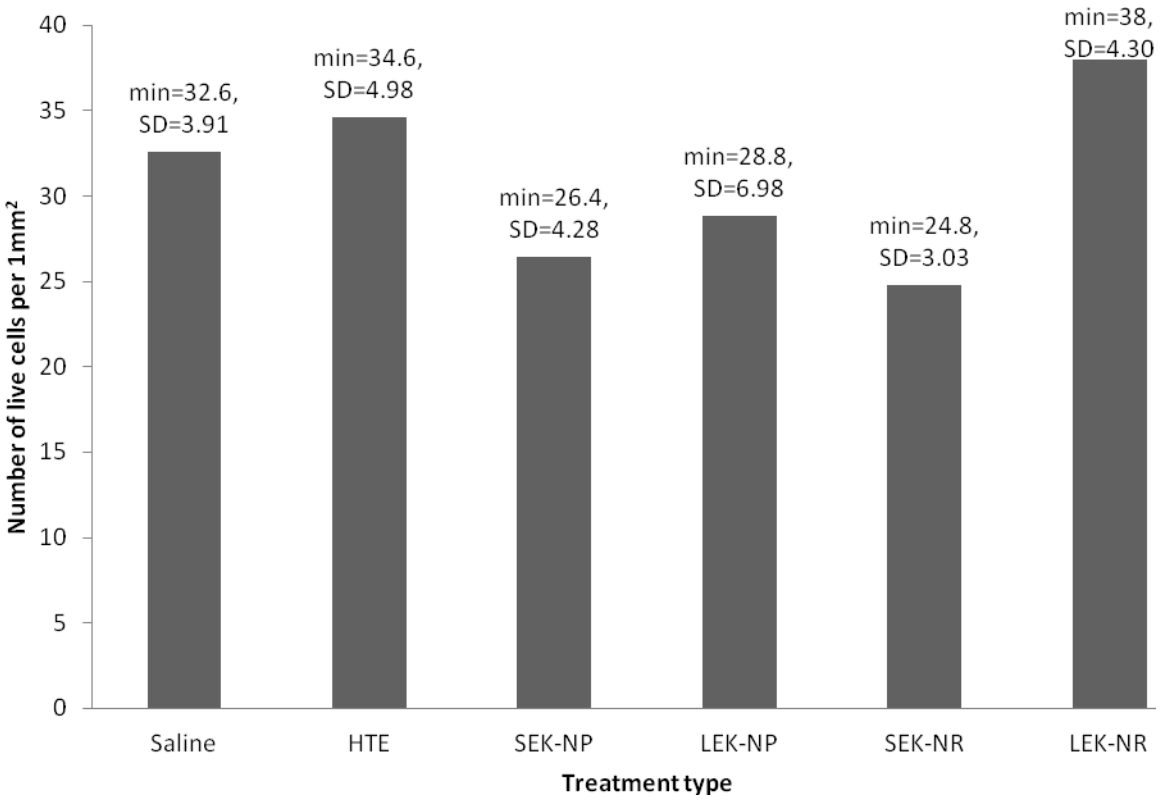

Fig. 3: The number of live cells in 1mm² area of CA3 in the neuroprotective assay. (SEK-NP: Short exposure ketamine in neuroprotective assay; LEK-NP: Long exposure ketamine in neuroprotective assay; SEK-NR: Short exposure methamphetamine in neuroregenerative assay; LEK-NR: Long exposure methamphetamine in neuroregenerative assay; HTE: Haruan traditional extract)

DISCUSSION

Haruan traditional extract (HTE) has exhibited the ability to provide neurite protection and regeneration[10]. In this study, the effect of HTE on brain structure is studied further using in vivo model by calculating live neuron cell number, degenerative cells and the degree of pathological change by staining with cresyl violet. This would give some idea on neurogenesis and neuronal survival affected by the drugs as well as the HTE.

The results of the neuroprotective assay are showing that HTE is able to protect against both number and structural damage caused by ketamine. This is reflected by the preservation to or close to normal in terms of live cell count and pathological score in both SEK and LEK groups. The protection may be greated had the treatment with HTE is lengthened to more than six-weeks. This would translate in real life situation as a continuous, long duration use of haruan-based supplement or nutritious food prior to the initiation of a damaging event.

When the experiment was carried out in the neuroregenerative assay, it appears that the structural damage which took in these groups were not rescued or restored by 6-weeks treatment with oral HTE. Interestingly, live cell count in the SEK was statistically significantly low suggesting that recruitment and survival of neuron cells have not been affected positively by HTE. In the LEK, the count of live neuron cells was also surprisingly higher than the LEK. This however did not register as a significant change ($p>0.05$) when it is compared to the HTE- and saline-treated groups. This could mean that despite the longer exposure to LEK, HTE was able to induce sustained production and stimulate production of live cells. In other words, HTE may provide the exogenous, neurogenesis cue that drives the recruitment and maintenance of neuronal cells.

Time factor may have played an important role in the difference seen in the SEK and LEK of the neuroregenerative assay in terms of their cell count and pathological score.

The difference between all these groups was in the length of time the rats were allowed to survive following first exposure to ketamine. It has already been noted that neurogenesis, one of the key factors which may be involved, could only happens a few days after the event of injury[13]. Time as a factor is a definite interest in neurodegenerative studies particularly as longevity has been touted as a contributor to the susceptibility of human to some neurodegenerative disease as the brain is rendered more vulnerable to neurotoxicity[14].

In the neuroregenerative assay, HTE treatment took place for another 6 weeks following the last injection of ketamine allowing

ample time for both endogenous mechanism as well as HTE to influence repair to damage. In the neuroprotective, similar explanation could be applied in the case of comparable performance of the LEK group to saline- and HTE-treated groups where rats continued to receive treatment for the next five days. Treatment with HTE which preceded exposure to ketamine may have preconditioned neuronal cells and neurogenic zones to optimum level and initiate modulatory and repair mechanism upon initiation of insult, both of which may have latent time to produce full effect. In the SEK of neuroprotective assay however, the short time span between initiation of insult and sacrifice may not provide ample window of time for the neurorepair and neuromodulatory mechanisms to exert their effects.

A postulation is that the overall result is in effect is a result arising from reactionary process to the presence of ketamine, or the effect of haruan, or both. Firstly the presence of ketamine might actually be a key to initiate internal process that optimises neuron cells and conditions them to prepare for damaging event. In the SEK, the abrupt withdrawal of ketamine may disrupt the optimization process and whatever recuperative mechanism already initiated may not be enough to overcome the overall sum of degenerative effect even when treatment with HTE commenced. In the LEK on the other hand, prolonged exposure to ketamine allows optimization of natural internal process of recuperation and repair. The treatment of HTE may have sustain and even helpful in boosting the number of live cells hence explaining the higher number of live cells seen. HTE treatment was not able to restore structural integrity in the LEK too. Nevertheless the lessening pathological lesion seen in the LEK may indicate that structural repair may also been underway.

The results of live cell count, pathological score and gross structural change would be better understood by works that assess level of degeneration and functional change in terms of memory, learning and behaviour. It has been appreciated that indication of change in neurogenesis, represented by cell number, alone cannot be reliably used to represent regenerative event as it could also happen as direct consequence of pathological phenomenon of neurodegeneration[15]. Data shows that reduced live cell, reduced neurogenesis and increased cell death can all cause or be the results of neurodegenerative changes[16]. On the other hand there are empirical observations in which behavioural dysfunctions and symptoms of neurodegenerative diseases were exhibited in the light of what appear to be increase in live cell number and neurogenesis and reduction in cell death[17,18,19,20,21,22]. To add to the confusion, however, there are also evidences in which physical, structural and disease symptoms appear without changes in neuronal number. One example is the transgenic mice model of AD which show that the neurofibrillary tangles and abnormal plaques of AD could occur in the absence of any neuron loss[23,24].

What is not known is whether, in an adult neuron, the cellular event is part of the pathology or rather a desperate attempt of a neuron under stress to protect itself[28]. What could be suggested about neurogenesis is a rise in the rate of new neuronal cell recruitment but in itself, it could not guarantee that each cell would be functionally normal. Erratic rate of cell proliferation has been a hallmark of some other diseases for example anaemia, in which the insufficient numbers of haematocytes in the bloodstream drives haematogenesis forward producing immature and dysfunctional haematocytes. In the same vein, higher level of neurodegeneration could be the initiating factor which drives neurogenesis. The presence of neurogenic agent, such as the proposed HTE, could ameliorate the process by providing a proper positive stimulation while dampening the effect from the neurodegenerative change and prevent an erratic, uncoordinated stimulation. It could also make things worse by increasing the erratic stimulation further.

CONCLUSION

The results showed how varied are the ability of brain to adapt to structural change and damaging insults to preserve function and memory. It appears that the variation is dependent on the type of insult and length of exposure as well as the manner in which the therapeutic agent is given. The results also underscore the need for a more complete study involving degenerative cells assessment, cognitive and behavioural assessment as well as genomics and proteomics study to help provide a better conclusion on the protective and regenerative capacity of HTE.

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