Human adipose-derived stem cells reduce receptor-interacting protein 1, receptor-interacting protein 3, and mixed lineage kinase domain-like pseudokinase as necroptotic markers in rat model of Alzheimer’s disease

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Abstract:
OBJECTIVES: Alzheimer’s disease (AD) is a constant, developing brain impairment that is described as the aggregation of misfolded amyloid-beta-peptide (Aβ) and abnormal tau protein in the brain. Stem cell therapy became a favorable candidate for the regeneration of neurodegenerative disorders like AD, but there is still shortage of knowledge about the underlying mechanisms. The goal of this survey was the determination of the necroptotic pathway as the possible mechanism for the effect of human adipose-derived stem cells (hADSCs) in the rat model of AD.

MATERIALS AND METHODS: Twelve rats were consumed, dividing into four groups: Control, sham, AD model and AD + stem cell groups. We utilized Nissl and Thioflavin S staining for determining histological changes and immunofluorescent techniques for evaluating necroptotic markers in different regions of the hippocampus.

RESULTS: The confirmation of AD model was approved with histological examination. The findings indicated more distinct Thio-S stain and an increased number of dead cells in AD rats comparing to other groups. Alternatively, the dead cells number in the CA3 area significantly lessened in AD + stem cell group comparing to AD group. Data showed that hADSCs significantly decreased the expression of necroptotic markers (receptor-interacting protein 1, receptor-interacting protein 3 and mixed lineage kinase domain-like pseudokinase (MLKL)) in AD + stem cell group compared to AD group in different regions of the hippocampus.

CONCLUSION: Our findings revealed that the intravenous injection of hADSCs reduced necroptosis and consequently declined the death of neuronal cells in the hippocampus of AD rats.

Keywords: Alzheimer disease, receptor-interacting protein 1 protein, receptor-interacting protein 3, mesenchymal stem cell transplantation, mixed lineage kinase domain-like pseudo kinase protein

Introduction
Neurodegenerative disorders are a set of long-lasting dysfunctions identified by neuronal impairment. Alzheimer’s disease (AD) is a constant, developing brain impairment which is described as the aggregation of misfolded amyloid

How to cite this article: Eftekharzadeh M, Simorgh S, Doshmanziari M, Hassanzadeh L, Shariatpanahi M. Human adipose-derived stem cells reduce receptor-interacting protein 1, receptor-interacting protein 3, and mixed lineage kinase domain-like pseudokinase as necroptotic markers in a rat model of Alzheimer’s disease. Indian J Pharmacol 2020;52:392-401.
beta-peptide (Aβ) and neurofibrillary tangles consist of hyperphosphorylated tau protein in the brain.\textsuperscript{[3]} Aβ is recognized as the common noxious element in the brain tissue of patients with AD. It was established that the accumulation of Aβ was capable of AD development and contributed to extensive neuronal impairment and cell necrosis.\textsuperscript{[2]} Furthermore, it has recently been reported that Aβ could promote the necrosome creation,\textsuperscript{[3]} which was composed of procaspase-8, FAS-associated death domain protein, RIP1, RIP3, and MLKL.\textsuperscript{[4]} Following the creation of the necrosome, it would proceed to necroptosis. Necroptosis is known as a form of cell death which has necrosis related conformation properties. Activation of necroptosis depends on the receptor-interacting protein (RIP) homotypic interaction motif (RHIM).\textsuperscript{[5]} The stimulation of RIP1 motivates the employment of RIP3 as a vital subsequent moderator of necroptosis. RIP1 associates with RIP3 in necrotic cells via the RHIM to create a network of amyloid-related signaling.\textsuperscript{[6]} RIP3 facilitates the phosphorylation of MLKL that promotes the oligomerization of MLKL at the kinase-like region of MLKL.\textsuperscript{[7]} The basic action of MLKL implicating in necroptosis has not been obvious yet. Previous research showed that MLKL might link to the transient receptor potential melastatin-related 7 ion channel in the plasma layer that could initiate the entry of calcium ions and eventually instigating cell extinction.\textsuperscript{[8]}

Necroptosis deceptively performs an important function in the pathogenesis of different neurodegenerative disorders like AD.\textsuperscript{[9]} Besides, by reducing necroptosis stimulation, the cell impairment lessened in AD mouse model.\textsuperscript{[10]} In new researches, stem cell therapy became an encouraging candidate for the regeneration of neurodegenerative disorders like AD.\textsuperscript{[11]} Stem cells release various essential components such as growth elements and extracellular vesicles. Mesenchymal stem cells (MSCs) have angiogenic, anti-apoptotic and supportive (induction of mitosis or proliferation) results that are related to the secretion of trophic paracrine factors.\textsuperscript{[12]} Among the different adult MSCs, adipose-derived stem cells (ADSCs) are a feasible and suitable basis in clinical studies. These stem cells are an appropriate alternative for the remedy of neurodegenerative disorders since they are capable of crossing the blood-brain barrier (BBB) and transferring to the injured parts of the brain. They are also lacking any ethical issues, tumor genesis and immune rejection complications.\textsuperscript{[13]} Based on the earlier study, transplantation of ADSCs intensified neurogenic effects in the subventricular zone, reduced oxidative stress and relieved cognitive impairment.\textsuperscript{[14]} Furthermore, exosomes isolated from ADSCs (ADSC-Exo) were considered a beneficial basis for improving Aβ induced neuronal impairment in an in vitro model of AD.\textsuperscript{[15]} The goal of this survey was assessing the necroptotic pathway (by evaluating the expression of RIP1, RIP3, and MLKL in different regions of the hippocampus) as the possible mechanism for the effect of intravenous injection of human adipose-derived stem cells (hADSCs) in AD rats.

Materials and Methods

Chemical agents
Cresyl violet, DAPI, formaldehyde, formalin and phosphate buffer saline (PBS) were acquired from Sigma (St. Louis, MO, USA). Anti-RIP1 mouse monoclonal antibody, anti- RIP3 rabbit polyclonal antibody, Goat anti-mouse and Goat anti-rabbit secondary antibodies were provided from Abcam (Cambridge, MA, USA), anti- MLKL mouse monoclonal antibody was prepared from Santacruz (Dallas, Texas, USA).

Experimental design
Twelve male Wistar rats (250–320 g) were randomly separated into four groups (three rats in each group): Control group; rats with no surgery. Sham group; the Syringe of Hamilton entered hippocampus (CA1 region) through stereotaxic surgery. However, no injection was done. Lesion group; AD rat model (Aβ injected into hippocampal region [CA1]). AD + Sc; rats got intravenous infusion of hADSCs three weeks after injection of Aβ. Keeping the animals was according to standard circumstances (12-h light and dark period at 25°C). Water and nutriment were available for the rats during the experiments. This research was certified by the ethical council of Iran University of Medical Sciences (Ethical code: IR.IUMS.REC 1396.32058) for animal procedures and accomplished based on the university instructions.

Extraction, culture, characterization, and labeling of human adipose-derived stem cells
In this research, hADSCs were the cells which their procedures of extraction, cultivation and characterization were done in our former study. hADSCs Flow Cytometry results have been reported too. The stem cell labeling process was accomplished according to the prior procedure.\textsuperscript{[16]}

Histological analysis
Three months after intravenous infusion of hADSCs, anesthesia of rats was done by consuming ketamine and xylazine. The brain perfusion was done with paraformaldehyde (4%) in 0.1 mol/L solution of phosphate buffer (pH = 7.4). Subsequently, brain tissues were taken and fixed by means of 10% formalin solution for 24 h. After the regular process of parafinization, 5 µm coronal slices were made with microtome.
**Nissl staining**

Cresyl violet (1%) was utilized for Nissl staining of the sections. We consumed light microscope (Olympus optical co, Ltd, Japan) to calculate cells of three periodic Nissl-stained coronal brain slices. Data were evaluated by Image J software. Three fields in the hippocampus region of each section were calculated.

**Thioflavin S staining**

The brain slices were placed in formalin (10%) for 10 min and then rinsed by PBS. Incubation was done in potassium permanganate (0.25%) for ten minutes. After washing in PBS, the slices were maintained in potassium metabisulfite (2%) and oxalic acid (1%) till they looked like white. Afterward, the slices were rinsed with water and staining was accomplished by Thioflavin-S solution (0.015%) (Thermofisher) in ethanol and water (50%) for ten minutes. The segments were wiped and covered by Histo-Clear. Fluorescence microscope (Labomed microscope provided with an Invenio6EIII camera) was used for examination of sections.

**Immunofluorescent staining**

For immunostaining, brain sections were processed based on standard protocols. For analyzing of RIP1, RIP3, MLKL protein expression, three coronal sections from each rat were immunostained. Concisely, brain slices were placed in formaldehyde (4%) for twenty minutes and then immunostained by employing of 1:500 dilutions of primary anti-RIP1, RIP3, MLKL antibodies followed by goat anti-mouse/rabbit FITC-conjugated secondary antibody (1:200 dilutions). Counterstaining of the nuclei was done with DAPI. Finally, the sections were placed under fluorescence microscope for evaluating the expression of necroptotic markers in three regions of hippocampus (CA1, CA3, and DG) in all studied groups (Labomed microscope with an Invenio 6EIII camera). Image J software was utilized for the evaluation of the data.

**Statistical analysis**

One-way analysis of variance (ANOVA) and Tukey’s post hoc test were utilized for examining histological parameters. Furthermore, we used two-way ANOVA and Bonferroni post hoc test for analyzing the molecular results. The results were evaluated by means of Graph Pad Prism program (Graph Pad software, Inc., USA). Statistics were specified as mean ± standard error of the mean. Statistically, $P < 0.05$ were considered as significant.

**Results**

**Nissl staining**

Neurons (nonbasophilic) which had light nuclei were noted as healthy neurons, whereas those with huge and shrunken (hyper basophilic) characteristics were calculated as dead neurons (Dark neurons). Hippocampus (CA3) Nissl staining was displayed in all the groups with ×4, ×10 and ×40 [Figure 1A]. The analysis [Figure 1B] showed that there was not a significant difference in dead cells number between sham and control groups. Dead cells in AD group significantly raised compared to the control group ($***P < 0.001$); conversely application of hADSCs significantly reduced the dead cells number in AD group ($**P < 0.001$). It is noted that CA1 Nissl analysis was reported in our previous study.\[16\]

**Thioflavin staining**

The results of Thioflavin S staining (green) confirmed the AD model in the hippocampus region (A displays ×40, B displays ×400) [Figure 2]. There was more distinct Thio-S stain in AD rats comparing to other groups. In addition, Thio-S stain was less distinct in hADSCs treatment group compared to AD group.

**Immunofluorescent staining**

Immunofluorescent staining was accomplished to study necroptotic markers in different regions of hippocampus (CA1, CA3, and DG) in all studied groups. Figures 3-5 (a-c) indicated immunofluorescent staining for primary anti-RIP 1, Anti-RIP3 and Anti-MLKL antibodies followed by Goat Anti-Mouse/Rabbit IgG secondary antibody (Green), Nuclei (DAPI – blue) and merge images in CA1, CA3 and DG regions of hippocampus in different groups.

Our data showed that there was more distinct RIP1, RIP3, and MLKL immunoreactivity in AD rats in different regions of hippocampus (CA1, CA3, and DG) comparing to other groups [Figures 3a-5c].

Figures 3-5d explained the statistical analysis of the previous results and compared the expression of each of the necroptotic markers in CA1, CA3, DG regions of hippocampus.

Figure 3d shows that there was a significant raise in RIP1 expression in AD group comparing to the control group in each of the three regions ($***P < 0.001$). Unlike, transplantation of hADSCs significantly reduced RIP1 expression in AD group in each region of hippocampus ($**P < 0.01$ in CA1, $**P < 0.001$ in CA3 and DG).

The results presented the significant effect of groups (F (3, 24) = 169.9, $P < 0.0001$) and regions of hippocampus (F (2, 24) = 42.05, $P < 0.0001$) on RIP1 expression. Nevertheless, the interaction of these two parameters was insignificant (F (6, 24) = 0.5166, $P = 0.7899$).
Figure 4d revealed that there was a significant raise in RIP3 expression in the AD group compared to the control group in each of the three regions ($***P < 0.001$). Transplantation of hADSCs significantly reduced RIP3 expression in AD group in each region of hippocampus ($***P < 0.001$).

Statistics revealed the significant effect of groups ($F (3, 24) = 368.2, P < 0.0001$) and regions of hippocampus ($F (2, 24) = 4.011, P = 0.0314$) on RIP3 expression. The interaction of these two parameters was significant ($F (6, 24) = 2.944, P = 0.0268$).

Figure 5d indicated that there was a significant raise in MLKL expression in AD group comparing to the control group in each of the three regions ($***P < 0.001$). Transplantation of hADSCs significantly decreased MLKL expression in AD group in each region of hippocampus ($^{*}P < 0.05$ in CA3, $^{***}P < 0.001$ in CA1, DG).
Data displayed the significant effect of groups (F (3, 24) = 155.2, P < 0.0001) and regions of hippocampus (F (2, 24) = 9.407, P = 0.0010) on MLKL expression. But, the interaction of these two parameters was not significant (F (6, 24) = 1.463, P = 0.02329).

Besides, we compared RIP1, RIP3 and MLKL expressions of AD and AD + Sc groups between CA1, CA3 and DG regions of hippocampus which were shown in Figure 6a-c. There was a significant difference in RIP1 expression of AD + Sc groups between (CA1 and DG) and (CA3 and DG) (P < 0.05) (A).

Analysis indicated significant impact of groups (F (1, 12) = 44.70, P < 0.0001) and regions of hippocampus (F (2, 12) = 15.37, P = 0.0005) on RIP1 expression. The interaction of these two parameters was not significant (F (2, 12) = 0.2506, P = 0.7823).

Figure 6b shows a significant difference in RIP3 expression of AD + Sc groups between CA3 and DG regions (P < 0.01).

Data analysis indicated significant impact of groups (F (1, 12) = 172.5, P < 0.0001) and regions of hippocampus (F (2, 12) = 6.759, P = 0.0108) on RIP3 expression. Nevertheless, the interaction of these two parameters was insignificant (F (2, 12) = 3.603, P = 0.0595).

Besides, there was a significant difference in MLKL expression of AD + Sc groups between (CA1 and CA3) and (CA3 and DG) (P < 0.05) regions (C).

There was significant impact of groups (F (1, 12) = 71.26, P < 0.0001) and regions of hippocampus (F (2, 12) = 7.696, P = 0.0071) on MLKL expression. The interaction of these two parameters was insignificant (F (2, 12) = 2.286, P = 0.1442).

**Discussion**

AD is a progressive degeneration of nerve cells and is considered as the current manifestation of dementia in old people. It is defined using numerous pathological signs in the brain such as neuronal loss and creation of neurofibrillary tangles. Several evidence support that Aβ peptide has prominent role in the diagnosis of memory impairment.\(^{[19]}\) Whereas Aβ is formed from its progenitor peptide through the lifetime, this particle is notably identified as the major fragment of amyloid plaques. Decreasing the amount of Aβ in the brain has been the principal aim of current investigational treatments against AD.\(^{[20]}\) In this study, there was more distinct Thio-S stain in AD rats comparing to sham and control groups. Thio-S stain was less distinct in hADSCs treatment group comparing to AD group.

Our previous research revealed the same findings.\(^{[16]}\) Investigations suggest that there are many reasons for AD; therefore, aiming the neurodegeneration process is important, as it may have useful effects in developing new strategies for the treatment of AD. Neuronal loss is a crucial characteristic of AD and is constantly found in various regions of the brain. In recent studies, the identification of mechanisms that have caused the loss of neurons was considered very important in AD.\(^{[21]}\)

Apoptosis has a major role in neurodegenerative diseases, while DNA fragmentation has been discovered in brains of human beings with AD.\(^{[22]}\) However, DNA fragmentation is notable in most of the neurons in AD, apoptotic hallmarks like apoptotic bodies and/or chromatin condensation have not clearly been described in this disease.\(^{[23]}\)

Assessments exhibited that apoptosis has taken up to 24 h for happening in a neuron. If apoptosis was the lonely critical issue needed for neuronal damage in AD, this dysfunction would result in an acute disorder, instead of the chronic, gradually developing one.\(^{[24]}\) The fragmentation of DNA in the cells without the formation of apoptotic bodies and chromatin condensation would alternatively be indication of necroptosis initiation.
Certainly, new documentation emphasized on general relation between apoptosis and necroptosis.\[25\]

Data demonstrated that necroptosis was stimulated in the brains of human with AD and also AD mouse model and could promote the neuronal damage in the brain.\[10\] Investigations demonstrated that necroptosis had starring role in neuronal death in neurodegenerative diseases as AD. Studies reported that necroptosis had an effective contribution in neuroblastoma cell death caused by Al, and inhibition of necroptosis was more effective than inhibition of apoptosis and autophagy in improving the viability of Al-treated cells in AD model. Inhibition of the necroptosis pathway could delay the development of cognitive problems related to AD.

Necroptosis is encoded necrosis that may be influenced using various extracellular and intracellular features. RIP1-RIP3-MLKL necrosome has imperative function in necroptosis launch. In addition, bimolecular attachment between RIP kinase and Aβ has confirmed the cell death pathology in AD. There are also some investigations which show that RIP1, RIP3 and MLKL are not only implied in necroptosis but also are indicators of inflammation.\[3\]

Excitotoxicity has an important role in chronic neurodegenerations like AD. Necroptosis is involved in neuronal excitotoxicity. Nec-1 as a RIP1 kinase inhibitor could keep hippocampal HT-22 cells from oxytosis, which was induced by glutamate. Furthermore, necroptosis is implicated in excitotoxicity induced by NMDA in neocortex of rat.\[5\]

An investigation indicated that the operation of RIP1 kinase (RIP1K) could be controlled through various signaling ways, such as chronic tumor necrosis factor α as a pathogenic cause of AD.\[26\] Furthermore, RIP3 kinase (RIPK3) had a high correlation with RIPK1 and MLKL in brains of patients with AD. In fact, RIPK1 attached to RIPK3, which sequentially made linkage with MLKL to create the necrosome. After the necrosome
creation, RIPK3 phosphorylated MLKL. Phosphorylated MLKL was accumulated to produce homodimers attaching to phosphatidylinositol phosphate that could permeabilize membranes and provoke cell death.\(^{[10]}\)

Our study verified for the first time that the markers of necroptosis such as RIP, RIP3, and MLKL raised in AD rat group in three regions of hippocampus (CA1, CA3 and DG) compared with other groups. It seemed that the formation of A\(_\beta\) plaques in AD group contributed to necroptosis and increased the neuronal death in the hippocampus.

More investigations have supplied substantial evidence for MSCs administration as an alternative approach for tissue revival in the central nervous system disorders like AD.\(^{[27]}\) There are different scenarios for curative results of stem cells in neurodegenerative disorders. Stem cells can treat the lesions by reducing the extent of cell death. \textit{In vitro} studies indicated the presence of anti-apoptotic markers in MSC-conditioned medium. An investigation revealed that in the first stages of tissue injury and hypoxia, adipose tissue-derived MSCs might express anti-apoptotic markers like VEGF, transforming growth factor-beta (TGF-\(\beta\)), HGF, granulocyte-macrophage colony-stimulating factor (GM-CSF) and basic fibroblast growth factor (bFGF, aka FGF2) that could decrease the cell death in damaged portions of the tissues. Apoptosis has pivotal role in physiological cell death, unlike; necrosis is more frequent in pathological conditions.\(^{[12]}\) hADSCs as a source of MSCs could reduce stress oxidative, which might occur due to neurogenesis in a cell, after transplantation in AD rat model.\(^{[14]}\) hADSCs with paracrine effects could modify the cell signaling of adjacent cells\(^{[29]}\) and prevent their apoptosis. As we displayed in this study, these stem cells led to reduction of necroptosis markers such as RIP1, RIP3, and MLKL in three regions of hippocampus (CA1, CA3 and DG) after intravenous injection to AD rat model that might be through secretion of anti-apoptotic or

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**Figure 4:** Immunofluorescent staining for primary Anti-RIP3 antibody followed by Goat Anti-Rabbit IgG secondary antibody (green). Nuclei (DAPI – blue) and merge image in CA1, CA3 and DG regions of hippocampus in different groups (a-c). RIP3 expression was significantly increased in AD group compared to the control group in all three regions. Intravenous transplantation of hADSCs significantly decreased RIP3 expression in AD group in all three regions of hippocampus. Each value explains mean ± standard error of mean. (**P < 0.001 different from control group, ###P < 0.001 different from AD group)** (d)
Figure 5: Immunofluorescent staining for primary anti-MLKL antibody followed by Goat anti-mouse IgG secondary antibody (green). Nuclei (DAPI – blue) and merge image in CA1, CA3 and DG regions of hippocampus in different groups (a-c). MLKL expression was significantly increased in AD group compared to the control group in all three regions. Intravenous transplantation of hADSCs significantly decreased MLKL expression in AD group in all three regions of hippocampus. Each value explains mean ± standard error of mean (**P < 0.01 different from control group, #P < 0.05, ###P < 0.001 different from AD group) (d).

Figure 6: Comparison of RIP1, RIP3 and MLKL expressions of AD and AD + Sc groups between CA1, CA3 and DG regions (a-c). There was a significant difference in RIP1 expression of AD + Sc groups between (CA1 and DG) and (CA3 and DG) regions (a). (b) showed a significant difference in RIP3 expression of AD + Sc groups between CA3 and DG regions. There was also a significant difference in MLKL expression of AD + Sc groups between (CA1 and CA3) and (CA3 and DG) regions (c) (*P < 0.05, **P < 0.01, ###P < 0.001 different from AD group, #P < 0.05, ##P < 0.01 difference between AD + Sc groups of various regions).
anti-inflammatory factors.\textsuperscript{29} Additionally, hADSCs could also improve the behavioral tests in Alzheimer’s rat model (Data not published yet).

As a research has proven, there were distinct features for various parts of the hippocampus comprising dentate gyrus (DG), CA3, and CA1. Each of the specified parts of the hippocampus had its particular cellular organization and typical operation. There were principal distinctions among CA1 and DG regions concerning the morphology of the cells, plasticity of synapses, diverse pathways of signaling, the capability of neurogenesis, susceptibility to different pathologies, and reaction to pharmacological mediators. One of the important differences between CA1 and DG regions was the capacity of the DG area for manifesting neurogenesis under physiological and pathological conditions while this feature was not seen in the CA1 area. Therefore, this specific area of the brain could supply the brain with new cells.\textsuperscript{30}

Based on this perception, immunohistochemistry analysis of this study presented that after hADSCs administration, there was a significant difference between the DG with CA1 and CA3 areas in the expression level of necroptotic markers. By administration of stem cells, we had the maximum effect of these cells on reducing the necroptotic markers in DG region which might be due to the higher ability of this area for neurogenesis comparing to other regions.

**Conclusions**

Consequently, we can conclude that inhibition of necroptosis is a possible underlying mechanism for the therapeutic effects of hADSCs in AD rats. From a therapeutic aspect, our data recommended that reducing necroptotic factors by the administration of hADSCs might be an interesting opportunity for developing new treatments in AD.

**Financial support and sponsorship**

Nil.

**Conflicts of interest**

There are no conflicts of interest.

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