Investigating the Effects of *Rosa canina* L. Fruit Extract in Animal Model of Alzheimer’s Disease

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**ABSTRACT**

This study was designed to evaluating the protective effects of RH extract (RHE) in a rat model of Alzheimer. Male rats were divided into three groups (n=8) including: Control (β-amyloid; 3 mµ ICV injection), RHE Vehicle (Normal saline) and RHE pre-treated groups (1350 mg/kg). Behavioural analysis Morris Water Maze (MWM) and histological study were done to reveal the protective potential of RHE in rats twelve days after Alzheimer induction. Enzyme-linked immunosorbent assay was used in the measurement of the Interleukine-6 (IL-6), Tumor necrosis factor (TNF-α), Malondialdehyde (MDA) and antioxidant enzymes. RHE markedly restored memory and learning impairments. RHE significantly decreased MDA, TNF-α and IL-6 levels in pre-treated group. In addition, RHE decreased the number of degenerated neurons in the hippocampal neurons of the RHE pre-treated rats. The results show that RHE has beneficial effects in a rat model of Alzheimer due to its anti-inflammatory and antioxidant properties.

Key words: Alzheimer’s disease, β-Amyloid, *Rosa hip*, Rat, Neuroprotection

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**INTRODUCTION**

*Rosa canina* (Rosaceae), a widespread plant in Asia and Europe, has fruits (rose hips) that are often used in nutrition because of their antioxidant content. Rose hip is traditionally used for prevention and treatment of inflammatory diseases such as common cold and osteoarthritis [1]. It has a rich composition because of its vitamin and mineral contents. Rose hips have a higher amount of vitamin C than any other commonly existing fruit or vegetable. Moreover, wild fruits have a high phenolic content [2]. Alzheimer's disease (AD) was first defined by Alois Alzheimer, in 1901 [3] and is now considered the most predominant progressive neurodegenerative disorder, responsible for 75% of all dementia cases [4]. AD is estimated that affect 36 million people worldwide. It will increase with population aging and will possibly affect nearly 107 million people by 2050 [5]. It causes cognitive, mental and physical deficits in elderly people [6]. Recent efforts have offered new insights into the underlying mechanism of AD and its treatment, providing new methods to prevent or cure it. But, in spite of these efforts, at this time, there is no prevention or cure for AD and new routes in AD are urgently needed. In view of the potent antioxidant and anti-inflammatory properties of Rosa hip, the current study was conducted to investigating the pre-treatment effects of RHE in the rat model of Alzheimer.
METHODS

Chemicals
Amyloid β (Aβ) Protein Fragment 25-35 (A 4559 Sigma) was bought from Sigma (St. Louis, MO). Folin-Ciocaltel reagent, Na₂CO₃, NaNO₂, AlCl₃·6H₂O, NaOH, sulphuric acid, sodium phosphate ammonium molybdate and cresyl violet were bought from Merck, Germany. TNF-α and IL-1β enzyme-linked immunosorbent assay (ELISA) kits were purchased from Bioassay Technology Laboratory (China).

Animals
Adult male Wistar rats (220 g-270 g) were purchased from Neuroscience Research Animal Center, Kerman, Iran. They were kept in a controlled environment at room temperature of 20°C ± 2.0°C and automatic day–night timetable (12 h cycle). The rats were allotted into three equal groups (n=8): Control group, animals which received β-Amyloid 25-35 [3 mµ intracerebroventricular (ICV) injection]; RHE vehicle group that undertook the same surgical methods as control, treated with vehicle (distilled water) for 7 consecutive days; RHE group that pre-treated by RHE for 7 consecutive days before β-Amyloid 25-35 administration (1350 mg/kg, gavage). The RHE dose used in this work was determined from a pilot study. All experiments were conducted after approval from the Kerman University Ethics Committee (EC/KNRC/94/15). Active form of amyloid β-peptide was added and homogenized by vortex. The absorbance of samples was compared with a standard curve made from quercetin (0 to 100 mg/l) at 506 nm. The data were presented as mg of quercetin equivalents per g of dry matter.

Total phenolic content (TPC) estimation
The phenolic compounds were calculated using the Folin-Ciocaltel method. One ml of sample (1 mg/ml) was blended with 1 ml of Folin-Ciocaltel reagent followed by addition of 10 ml of a 7% Na₂CO₃ and 13 ml deionized DW and mixed completely. The samples were homogenized and heated for 90 min at 23°C. The absorbance was measured at 765 nm after cooling at room temperature. The data were calculated by using a gallic acid standard curve. The data were presented as mg of gallic acid equivalents per g of dry matter.

Estimation of total antioxidant activity
The total antioxidant capacity of the contents was calculated by phosphomolybdenum method using ascorbic acid as a standard. A 0.1 ml aliquot of 100 µg/ml concentration of RHE and standard was mixed with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated in a water bath at 95°C for 90 min. After cooling, the absorbance of the mixture was determined at 765 nm. Total antioxidant capacity of the used extract, presented as mg/g of ascorbic acid equivalents. Antioxidant effect of the extract (%) was also calculated using following formula:

Antioxidant effect=[(control absorbance-sample absorbance)/control absorbance] × 100

EC50 values estimated to determine the 50% inhibition of Mo reduction by extract.

Learning and memory evaluation using Morris water maze (MWM) test
Spatial learning and memory was assessed using a modified version of the Morris water maze, in which rats learned to get away from the water onto a hidden platform [8]. The experiments were done in the blurrily light room with spatial cues that were attached to the walls around the maze at different sites. A smart video tracing system (Noldus Ethovision system, version 7.1) recorded the performance of rats. The maze made up of an iron circular (60 cm high and 1.36 m in diameter) filled with cloudy water with 20°C ± 1°C temperature. The rats were challenged to find a hidden platform (25 cm high and 10 cm in diameter) placed 1.5 cm below water level (26.5 cm) and in the same location during the entire trial. The platform was constantly placed 30 cm beyond the rim of the pool in the centre of one quadrant with respect to the distal visual cues. The test was done over 4 consecutive days to test their learning, when rats were placed into the water facing the rim from 4 different starting points in the pool, with the platform reserved in the same place and with an interval between each trial. The rats were trained in the maze during (16 trials), 4 sessions on successive 4 days except for the last day. The
animals had to swim to find the hidden platform in 60 s (maximum time), where they were let to stay for 30 s. If they failed to find the platform, rats were placed on it for 10 s to associate spatial cues of the room (light source, pictures, and cabinets). Swimming patterns were assessed and the following factors for spatial learning were calculated: Distance travelled to find the hidden platform (cm), time spent to find the hidden platform (Escape latency (s), and swimming velocity (cm/s). One day after the last learning trial, the rats underwent a probe trial to check their long-term spatial memory. For this aim, percent of distance, time spent and frequency in the target quadrant was recorded and analysed [7].

**Nissl staining**

The brain tissues were processed according to standard histological methods. Paraffinized samples were cut into 7 μm sections on a semi-automatic microtome and the sections were stained with Fast cresyl violet. Neuronal damage was then assessed for each animal as the rate of the number of degenerated neurons to that of both surviving and degenerated in three distinct regions of the CA1 sector (medial, middle and lateral) of hippocampus in coronal sections [8].

**Protein determination**

The total extracted protein was measured using Bradford assay.

**Lipid peroxidation assay**

Lipid peroxide levels were estimated by measuring malondialdehyde (MDA) in the hippocampus homogenate. The lipid peroxide level was ascertained spectrophotometrically at 532 nm by means of the thiobarbituric acid test and expressed as nmol/mg protein.

**Determination of inflammatory Cytokines and antioxidant enzymes**

Rat Tumor necrosis factor (TNF-α), Interleukine-6 (IL-6), Glutathione peroxidase (GSH-PX) and Superoxide dismutase (SOD) levels in brain tissue were measured by using enzyme-linked immunosorbent assay (ELISA) kits (TNF-α, eBioscience, Catalog #BMS622; IL-6, eBioscience, Catalog # BMS625; GSH-PX, Crystal Day Biotech, Catalog #E0814Ra; SOD, Crystal Day Biotech, Catalog #E0168Ra). Specific antibody against each antigen were coated in wells and our antigen was sandwiched between primary and secondary horse radish peroxidase (HRP)-coated antibody, then colour progress within the 10 min was assessed at 450 nm by ELISA reader instrument (ELM-2000).

**Statistical analysis**

Data were offered as mean ± SEM. The means were evaluated using one-way analysis of variance (ANOVA) followed by the Tukey’s post hoc test and p<0.05 was determined statistically significant.

**RESULTS**

**Total flavonoids contents and total phenolics estimation**

RHE flavonoids content was 40 mg as quercetin equivalents/g fraction. Total phenolic compounds content as presented in Table 1 in RHE was 43 mg/g gallic acid (GAE) equivalent per one gram of dried extract.

**Assessment of antioxidant activity of RHE**

Antioxidant activity of RHE is shown in Table 1. RHE showed free radical scavenging activity against DPPH radicals, with an EC50 value of 82.50 μg/ml.

**Effects of Aβ injection on learning parameters in rats**

There was a statistically significant difference in distance travelled to find the hidden platform (p<0.05) and escape latency (p<0.01) between control and RHE vehicle groups. Pre-treatment with RHE (1350 mg/kg) significantly improved the aforementioned parameters (p<0.01) (Figure 1A and 1B). There was no statistically significant difference in swimming speed among all groups (p>0.05) (Figure 1C).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total phenolics content mg (GAE)/ gram extract</th>
<th>Total Flavonoids content mg (quercetin)/ gram extract</th>
<th>EC50 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHE</td>
<td>43</td>
<td>40</td>
<td>82.5</td>
</tr>
</tbody>
</table>

RHE: Rose hip extract

<table>
<thead>
<tr>
<th>Sample</th>
<th>MDA (nmol/mg protein)</th>
<th>GSH-PX (u/mg protein)</th>
<th>SOD (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>39.72</td>
<td>89.29</td>
<td>3.47</td>
</tr>
<tr>
<td>Control</td>
<td>51.45*</td>
<td>33.41*</td>
<td>1.38*</td>
</tr>
<tr>
<td>RHE</td>
<td>45.30*</td>
<td>50.57</td>
<td>1.7</td>
</tr>
</tbody>
</table>

R*: statistically significant difference (p<0.05)
Effects of Aβ injection on memory parameters in rats

There was a statistically significant difference in percent of distance (p<0.001), time (p<0.01), and frequency spent in target quadrant (p<0.01) between control and RHE vehicle groups. Administration of RHE (dose of 1350 mg/kg) improved the toxic effect of Aβ on the memory parameters (p<0.05, p<0.05 and p<0.01 respectively) (Figures 2A-2C).

Neuronal degeneration

In the histological study, the morphology of neurons in the vehicle group was intact including round nuclei with prominent nucleolus and a clear cytoplasm. In contrast, most pyramidal neurons in the control group displayed severe degenerative alterations; dark piknotic nuclei and an eosinophilic cytoplasm. The degenerative alterations observed in the cytoplasm and nucleus of CA1 neurons was less in the RHE-treated group comparing to control. Neuronal counting (the percentage of degenerated neurons) in CA1 area of hippocampus illustrated significant differences between the sham (8.6%) and β-amyloid group (68.24%) (p<0.001). Administration of RHE significantly reduced the percentage of degenerated neurons in CA1 area of pre-treated animals (36.48%) (p<0.001) (Figure 3).

Table 3: The effects of RHE on hippocampal inflammatory cytokines in male rats exposed to β-amyloid

Effects of Aβ injection on inflammatory cytokines of hippocampus in male rats

There was a significant elevation in TNF-α, and IL-6 (0.5 ng/mg and 0.4 ng/mg protein, respectively) levels in control group compared with vehicle (0.33 ng/mg and 0.21 ng/mg protein, respectively) (p<0.001) and these elevations were markedly decreased by RHE pre-treatment (0.42 and 0.29 ng/mg protein, respectively) (p<0.05) (Table 3).
DISCUSSION

In our study, using the dosage of 1350 mg/kg RHE significantly improved spatial memory in MWM test in comparison to control group. Spatial learning and memory was disturbed in rats by Aβ 25-35 however, pre-treatment using RHE reserved learning and memory. In addition, RHE pre-treatment markedly reduced neurodegeneration and inflammatory factors and which all these symptoms have been reported in hippocampus of clinical AD patients.

In current study, Aβ 25-35 impaired learning and memory in rats. Rats which received Aβ displayed more prolonged escape latency compared to the rats in the sham group. Also RHE treatment significantly diminished escape latency in treated group, which suggests that long-term memory was impaired by Aβ. In addition, during the probe trial session, the Aβ induced declined swimming times within the target quadrant which was markedly improved by RHE, indicating a positive effect on spatial memory. This positive effect is due to its anti-inflammation and anti-oxidative stress actions. Research has shown that phenolic compounds can block early assembly processes of β-amyloid by binding to different sites. Hence, these compounds are introduced as therapeutic candidates for AD [9].

AD is known as the deposition of fibrillary forms of Aβ in the brain and the compaction of the Aβ fibrils into senile plaques [10]. The deposits of Aβ cause a significant inflammatory response due to microglial activation [11]. Activation of microglia is accompanied by the elaboration of an extensive range of pro-inflammatory molecules that mediate the fulminating auto-activation of these cells and a concomitant astrocyte activity. The inflammatory response is simultaneous with neuronal apoptosis next to the plaques [12]. TNF is known as one of the main pro-inflammatory cytokines that plays a key role in initiating and regulating the cytokine cascade during an inflammatory response [13]. It participates in local and systemic events involving inflammation. TNF, being a potential paracrine stimulates other inflammatory cytokines, including interleukin-1 (IL-1), IL-6, and granulocyte-monocyte colony-stimulating factor (GMCSF). In an inflammatory condition, TNF along with a variety of pro-inflammatory mediators and neurotoxic factors are produced by activated microglia [14]. TNF increases the production of Aβ and inhibits the secretion of neuroprotective, soluble amyloid precursor proteins (sAPPs) [15]. TNF has also been shown to upregulate cyclooxygenase-2 (COX2) expression [16], which would increase the level of free radicals [17]. In Alzheimer patients, COX2 expression increases [18], especially within neurofibrillary tangles [10]. Recent data have also demonstrated rises in interleukin-6 (IL-6) and the IL-6-inducible acute phase protein in Alzheimer’s disease (AD) brains [19]. Moreover, TNF-α disrupts cognitive functions and regulates neuronal death [20]. On the other hand, it has been documented that non-steroidal anti-inflammatory drugs (NSAIDs) treatment decreases AD risk, slows disease progression, as well as reducing microglial activation [21]. In agreement with this data, administration of Aβ resulted in significant elevation in TNF-α, and IL-6 levels in rats and these elevations were significantly decreased by RHE pre-treatment.

Rose hip fruits (RHFS) are known to be rich source of antioxidants including flavonoids, carotenoids and anthocyanins [22]. Our data also showed that RHE has potent antioxidant activities. Another healthy function of RHF is their essential fatty acids such as linolenic, linoleic and oleic acids. Some studies demonstrated that these fatty acids have potent anti-inflammatory properties [23,24]. Our used extract also demonstrated the anti-inflammatory activities through TNF-α and IL-6 suppression. In addition, RHE pre-treatment decreased MDA level, as a lipid oxidation factor, in treated group compared to control group this is probably due to anti-oxidative stress properties of the used extract. In the current work, injection of Aβ resulted in attenuation of antioxidant enzymes (GSH-PX and SOD) in control group and RHE pre-treatment increased antioxidant enzymes levels, but failed to be significant possibly due to low sample size.

Neural degeneration is one of the main characters of AD. According to our obtained data, Aβ can cause severe neuronal damage in hippocampus of rats and RHE pre-treatment decreased neuronal death. Our results suggest that the beneficial effects of RHE can be related to potent anti-inflammatory and antioxidant activities of used extract.

CONCLUSION

We found that RHE pre-treatment in Alzheimer’s-induced rats provided protection against deterioration in CA1 area of the hippocampus. It may be concluded that anti-inflammatory property is one of the key profiles that are involved in neuronal protection. Thus our findings recommend that RHE may be considered as a potential approach for Alzheimer’s disease induced by Aβ in male rats.

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CONFLICT OF INTEREST
The authors declared no conflicts of interests.

REFERENCES