Neuroprotective Effect of Pyritinol and Fluvastatin in Cerebral Ischemic Reperfusion Injury and Memory Dysfunction

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Abstract: Cerebral ischemia leads to neuronal damage in the hippocampus and cognitive decline. Reactive oxygen species play an important role in the neuronal loss after cerebral ischemia and reperfusion injury. In the present study, the effects of Pyritinol, Fluvastatin and co-administration of these drugs on cerebral ischemia-induced learning and memory impairment in rats were investigated. The animals were pre-treated with Pyritinol and Fluvastatin for a period of 10 days. Cerebral ischemia was induced by Bilateral Common Carotid Artery Occlusion (BCCAO) of rats for 30 minutes followed by reperfusion. The treatment was continued for another week after surgery. Rectangular maze test, Morris Water maze test, locomotor activity and pole climbing test were conducted to evaluate the learning and memory parameters. Various biochemical parameters such as acetyl cholinesterase (AchE), TBARS assay, Catalase activity and DPPH assay were also assessed. Donepezil was used as standard drug. The present study demonstrates that Pyritinol, Fluvastatin and co-administration of these test drugs had potential therapeutic effects on improving the memory in rats through inhibiting lipid peroxidation, augmenting endogenous antioxidant enzymes and decreasing acetylcholinesterase activity in brain. The memory enhancing capacity of the drugs was very significant when compared to disease control (p<0.001).

Key words: Cerebral Ischemia • Ache Estimation • Antioxidant Activity • Neuroprotective • Pyritinol • Fluvastatin

INTRODUCTION

Cerebral ischemia is caused by a deficiency in blood supply to a part of the brain, which in turn triggers various pathophysiological changes [1]. The brain requires continuous supply of oxygen and glucose to maintain normal function and viability. Interruption in this physiological process leads to cerebral ischemia, which initiates several cellular events such as depletion of ATP, release of inflammatory mediators, failure of the Na⁺/K⁺ ATPase [2]. Frequent incidence of cerebral ischemia has been seen in age-related disorders, hypoxic-ischemic brain injury, carotid artery pathologies, asphyxiation and shock [3]. Cerebral ischemia is a destructive event that is associated with high morbidity and mortality rates [4]. Stroke can cause deficits in a number of neurological domains leading to broad spectrum of neuropsychiatric complications, including emotional, behavioural and cognitive disorders [5].

Bilateral common carotid artery occlusion (BCCAO) causes moderate reduction of cortical and cerebral blood flow in diverse areas of brain [6]. Indeed, bilateral common carotid artery occlusion has been reported to produce memory impairment and cognitive deficit in rats [7]. Oxidative stress is one of the primary factors that exacerbate damage by cerebral ischemia. Several components of reactive oxygen species (ROS), (superoxide, hydroxyl radical, hydrogen peroxide and peroxynitrite radical) that are generated after ischemia-reperfusion injury play an important role in neuronal loss after cerebral ischemia [8]. Superoxide and hydroxyl radical are potent in producing destruction of the cell membrane by inducing lipid peroxidation [9]. Oxidative stress, a condition of cellular pro-oxidant-antioxidant disturbance in favour of the pro-oxidant state, also induces the production of reactive oxygen species ROS, leading to serious functional impairments [10].
The brain is particularly vulnerable to oxidative stress injury because of its high rate of oxidative metabolic activity, intense production of reactive oxygen species metabolites and high content of polyunsaturated fatty acids, reactivity of low antioxidant capacity, low repair mechanism activity and non-replicating nature of its neuronal cells [11]. Statins are HMG-CoA reductase inhibitors, which may have potential therapeutic benefit in Alzheimer’s disease (AD). Experimental studies revealed that lowering cholesterol levels with statins decrease the production of β amyloid plaque, which is the hallmark of AD [12].

Pyritinol, a vitamin B₆ analogue, has been taken as reference drug by basing on the possession of significant antioxidant activity. Pyritinol increased neuronal glucose uptake, its antioxidant abilities help to neutralize the reactive oxygen and nitrogen species, preventing them from oxidizing and damaging cell membranes. Pyritinol is used to treat stroke, dementia, traumatic brain injury, encephalitis, and even childhood learning disorders [13].

Fluvastatin, an HMG-CoA reductase inhibitor, anti-hyperlipidemic drug, is known to have neuroprotective properties that may involve its regulatory effects on antioxidant enzymes. In addition Fluvastatin may acts as antioxidant in neurons and protect against glutamate receptor mediated excitotoxicity. Studies of Fluvastatin on aged male laboratory animals have showed delayed cognitive impairment and behavioural deterioration when compared with disease control animals [14].

The main purpose of the present study was to investigate the synergistic effect of Pyritinol and Fluvastatin in cerebral ischemia induced cognitive impairment and oxidative stress model.

**MATERIALS AND METHODS**

**Experimental Animals:** Male Wister albino rats weighing 200-250 g were used in the present study. They had free access to food and water and were maintained under standard laboratory conditions with alternating light and dark cycles of 12 h each. They were acclimatized to laboratory conditions for 2 days before behavioural studies. All the readings were taken during the same time of the day, that is, between 10 am and 2 pm [15]. The experiments were planned after the approval of Institution Animal Ethical Committee (IAEC), Vaagdevi College of Pharmacy, Warangal (A.P) and India (1047/ac/07/ CPCSEA, dated: 27/02/2007).

**Chemicals and Drugs:** Pyritinol (Merck Ltd), Fluvastatin (Novartis health care private Ltd) and Donepezil (Alkem Laboratories Ltd), Thiobarbituric acid (Himedia Private Limited), Hydrogen peroxide (Finar Chemicals), Acetyltiocholine iodide (Sigma Aldrich), Perchloric acid, Formalin 10% (Finar Chemicals), DTNB (5,5-dithiobis(2-nitrobenzoic acid)) reagent, DPPH (1,1-diphenyl-2-pircrylhydrazyl) radical reagent (Sigma Aldrich).

**Experimental Design:** Animals (36) were weighed and kept in cages accordingly and randomly divided into 6 groups (n=6). Drugs were prepared freshly and given daily for 10 days. On day 11, 60 min after last dose, all rats except normal control groups were subjected to 30 min bilateral common carotid artery occlusion with aneurysm clips and all rats were sacrificed after 7 days of induction, their brains were removed and subjected to biochemical analysis and histopathological evaluation. On day 1, the training sessions for all the animals were given. Drugs were administered, after 1 hr the retention time (RT) was calculated. This is followed on consecutive 5,7,9 of before induction and 3, 5, 7 days of after induction of cerebral ischemia. The doses of drugs were taken according to the body weights of the animals given in Table 1. The doses of Pyritinol and Fluvastatin were selected based on reported studies [16].

Donepezil, Fluvastatin, Pyritinol were dissolved in 0.1% CMC solution. All drugs were prepared freshly daily. Doses were given according to the respective rat weights [9, 17].

**Induction of Cerebral Ischemia:** Rats were anaesthetized with Thiopentone sodium at a dose of 50mg/kg, i.p [18]. A midline incision was made in the region between neck and sternum and trachea was exposed. Both the left and right common carotid arteries were located lateral to sternocleiamastoid, freed from the surrounding tissues and vagus nerve was separated. Cerebral ischemia was induced by clamping both the arteries with the help of aneurysm clips. After 30 min of cerebral ischemia, the clips were removed from both the arteries to allow the reflow of the blood through carotid arteries, the incision was sutured back in layers with the surgical suture. The sutured area was cleaned with spirit and was sprayed with antiseptic powder. After completion of surgical procedure, the body temperature was maintained at 37°C. All the surgical instruments used in the surgical procedure were sterilized prior to use [9].
Table 1: Experimental Design

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>Group-I</td>
<td>Normal Control Vehicle (0.1% CMC).</td>
</tr>
<tr>
<td>Group-II</td>
<td>Disease control Bilateral common carotid artery occlusion (BCCAO) for 30min</td>
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<tr>
<td>Group-III</td>
<td>Standard</td>
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<tr>
<td>Group-IV</td>
<td>Test-I Bilateral common carotid artery occlusion (BCCAO)+ Donepezil(5mg/kg)oral</td>
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<tr>
<td>Group-V</td>
<td>Test-II Bilateral common carotid artery occlusion (BCCAO)+ Fluvastatin(10mg/kg)oral</td>
</tr>
<tr>
<td>Group-VI</td>
<td>Test-III Bilateral common carotid artery occlusion (BCCAO)+ Pyritinol(100mg/kg)oral</td>
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</table>

**Behavioural Tests:** All the animals were trained for 7 days before drug administration.

**Rectangular Maze Test:** Assessment of learning and memory can be effectively done by this method. The maze consists of completely enclosed rectangular box with an entry and reward chamber appended at opposite ends. The box is partitioned with wooden slats into blind passages leaving just twisting corridor leading from the entry to the reward chamber. Animals were trained prior to the experiment by familiarizing with the rectangular maze for a period of 10 min for 2 hrs. Transfer latency (time taken to reach the reward chamber) was recorded. For each animal, four readings were taken and the average is taken as learning score (transfer latency) for that animal. Lower scores of assessment indicate efficient learning while higher scores indicate poor learning in animals. The time taken by the animals to reach the reward chamber from the entry chamber was noted on day 1, 3, 5, 7 and 9 [17, 19].

**Morris Water Maze Test:** Morris water maze was used to assess learning and memory in experimental rats. There are several advantages of learning and memory including absence of motivational stimuli such as food and water deprivation, electrical stimulations and buzzer sounds [20, 21]. Briefly, it consists of a circular water tank, filled with opaque water and one centimetre submerged platform. First animals were trained to locate the platform. During acquisition, trials escape latency time (ELT), time measure to locate the hidden platform, was noted as an index of acquisition. Each animal was subjected to the four acquisition trials per day for 4 consecutive days. During each trial, the escape latencies of rat were recorded. The point of entry of rat into the pool and the location of the platform [22] for escape between trials changed each day thereafter. The time spent by the animal, searching for the missing platform in target quadrant Q2 with respect to other quadrant (Q1, Q3 and Q4) on 5th day, was noted as an index of retrieval. For studying the effect of drug on acquisition, the drug solution was administered before acquisition trial [17].

**Locomotor Activity:** Locomotor activity is influenced by most of the CNS drugs in both man and animals. The locomotor activity of drug can be studied using actophotometer which operates on photoelectric cells which are connected in circuit with a counter when the beam of light falling on photocell is cut-off by the animal and then a count is recorded [23]. Animals are placed individually in the activity cage for 10 min and the activity was monitored. The test is done before the 30 mins and after the drug administration. The photocell count is noted and decrease or increase in locomotor activity is calculated [17, 20].

**Pole Climbing Test:** When an electrical stimulus is given to animal, it tries to escape from it and move to the near safe place. This equipment is designed in such a way to climb the pole when stimulus is generated. Prior to the experiment, animals were trained. Training and testing is conducted in a 25×25×40cm chamber that is enclosed in a dimily light, sound attenuated box. Scrambled shock is delivered to the grid floor of the chamber. A smooth wooden pole, 2.5 cm in diameter, is suspended by a counter balance weight through a hole in upper centre of the chamber. A micro switch is activated when the pole is pulled down by 3 mm with weight greater than 200 gm. A response is recorded when rat jumps on the pole and activate micro switch. The activation of light and speaker together is used as conditioned stimulus. Each animal was placed six times per day [24, 20].

**Histopathological Studies:** 7 days after induction of ischemia, the brains of different groups were perfusion fixed with 10% formaldehyde in 0.1M phosphate buffer. The brains were removed and postfixed in the same fixative overnight at 48°C. The brains were then routinely embedded in paraffin and stained with Heamtoxylin-Eosin. The hippocampus lesions were assessed microscopically at 40x magnification [24].

**Dissection and Homogenization:** After behavioural assessments, animals were sacrificed by cervical dislocation. The brains were removed. Each brain was
separately put on ice and rinsed with ice-cold isotonic saline. A (10% w/v) homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 3000 rpm for 15 minutes and aliquots of supernatant were separated and used for biochemical estimation [25].

Biochemical Tests

AchE Estimation: AchE levels in whole brain were estimated according to Ellman’s method [26]. To 0.4 ml tissue homogenate 2.6ml of phosphate buffer (0.1M, pH 8.0) was added. To this 100µl of DTNB (0.01M) was added and incubated for 5 minutes. To that 20µl of acetylthiocholine iodide (0.075M or 21.67 mg/dl) was added and change in absorbance per minute was calculated by observing for 5 minutes at 412nm [27]. By using the following formula the AchE levels in µmol/l/min/mg tissue were calculated [28].

\[
R = 5.74 \times 10^{-4} \times \frac{\Delta A}{C_0}
\]

where,

\[ R = \text{AchE level in } \mu \text{mol/l/min/mg tissue.} \]
\[ \Delta A = \text{Change in absorbance per min} \]
\[ C_0 = \text{Original concentration of tissue (mg/dl)} \]

Thiobarbituric Acid Reactive Substances (TBARS) Assay: This assay is used to determine the lipid peroxidation. Aliquots of 0.5 mL distilled water were added with 1 ml of 10% trichloroacetic acid and were added with 0.5 ml of brain tissue homogenate [29]. This is centrifuged at 3000 rpm for 10 min. To the 0.2 mL supernatant, 0.1 mL Thiobarbituric acid (0.375%) was added. Total solution is placed in water bath at 80°C for 40 min and cooled at room temperature. Absorbance was read at 532 nm [30]. The levels of lipid peroxides were expressed as nano moles of TBARS [31].

Catalase Activity: Catalase activity was assessed by the method of Luck, where in the breakdown of hydrogen peroxide is measured. In this 3 mL of \( \text{H}_2\text{O}_2 \) phosphate buffer was added to 0.05 mL of the supernatant of the tissue homogenate. The absorbance was recorded at 240 nm using Perkin Elmer \( \lambda \)20 spectrophotometer. The percentage scavenging activity was determined [32]. The results were expressed as micromoles of \( \text{H}_2\text{O}_2 \) decomposed per minute per mg protein [33].

DPPH Radical Scavenging Assay: The free radical scavenging activity of the test drug was measured in vitro by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay [34]. In this, measurement is made from the bleaching of purple-coloured methanol solution of DPPH. To the 1000 µL of diverse concentration of the homogenate, 4mL of 0.004% methanolic solution of DPPH was added. After 30 min incubation in dark, absorbance was read at 517nm [35]. Inhibition of free radical by DPPH in percentage was calculated in the following way:

\[
\% = \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100
\]

\( A_{\text{blank}} \): absorbance of control reaction. \( A_{\text{sample}} \): absorbance of test sample. Values of inhibition were calculated [24, 30].

Statistical Analysis: The statistical analysis of data was done by the one way analysis of variance (ANOVA) followed by the Dunnett’s test. p< 0.05 was considered as significant. Results were expressed as Mean±SD.

RESULTS AND DISCUSSION

The present investigation showed the neuroprotective potential of Pyritinol and Fluvastatin against ischemia induced oxidative stress as well as histopathological alterations. The activities of these drugs to work by restored the altered catalase, as well as decreased the lipid peroxidation in various brain regions induced by BCCAO. There is considerable evidence which supports the role of reactive oxidative species (ROS) in pathogenesis of ischemia induced oxidative stress in brain [2].

Brain reperfusion after ischemia frequently results in neuronal death, which occurs preferentially in some regions. This neuronal degeneration has been associated with ROS, which reacts with cellular macromolecules such as lipids, proteins and nucleic acids leading to oxidative damage of the neurons. Thus the endogenous antioxidant enzyme activity of the brain impaired by ischemia is particularly important and measurement of those antioxidant enzymes after reperfusion can assess the vulnerability of the particular areas of the brain [1]. Many studies reported that memory impairment in the cerebral ischemia induced animal model is associated with increased oxidative stress within the brain. Oxidative stress is the cytotoxic consequence of oxyradical and oxidant formation and the reaction with cellular constituents [36]. ROS were generated continuously in nervous system during normal metabolism and neuronal activity [9]. The nervous system is particularly vulnerable to deleterious effects of ROS. Because the brain has a high consumption of oxygen, large amount of...
polyunsaturated fatty acids (PUFs), high contents of free ions and low levels of antioxidants defence were compared to other organs. Increased MDA levels as one of the ROS has been shown to be an important marker for \textit{in vivo} lipid peroxidation [17].

The major antioxidant and oxidative free radical scavenging enzymes like glutathione, SOD and catalase play an important role to reduce oxidative stress in brain. In this study, from the DPPH assay antioxidant levels are estimated. These antioxidant enzyme levels are decreased in the disease control group compared to the normal control group and the standard group. Individual test drug treated groups are showing less than combination group. It supports the antioxidant action of drugs.

In the present study rats of disease control group showed a significant increase in the brain levels of malondialdehyde, which is measure of lipid peroxidation and free radical generation. In the drug treated groups, there is significant decreased in the levels of malondialdehyde which is nearly equal to standard group. From the results it is clear that Statins of Fluvastatin decreased the disease progression. The antioxidant activity of Pyritinol is clear from biochemical tests, which includes the estimation of antioxidant enzymes.

From the behavioural tests, that is, rectangular maze test and Morris water maze test, it is clearly seen that there was a decrease in the transfer latency in all treated groups compared to the disease control group. The memory loss in disease control group is more prominent compared to the normal control group. In comparison with Donepezil, the test drugs treated groups had almost equal performance which indicates synergistic effect of Pyritinol and Fluvastatin against memory loss, meanwhile pole climbing test is done which also indicates the learning ability.

**Pyritinol and Fluvastatin Improved of Behavioral Alteration in Disease Rats**

**Rectangular Maze Test:** The activity of Pyritinol and Fluvastatin were evaluated by using Rectangular maze. The transfer latency measured for all the group of animals before and after induction of ischemia compared against disease control group of that day which was given in Fig. 1. Results showed that there is a significant (p<0.05) decrease in transfer latency time for standard and test compounds in comparison with the disease control using Dunnet’s test. The co-administration of Pyritinol and Fluvastatin showed synergistic effect which is comparable with the standard group than individual drug treated groups.

**Pole Climbing Test:** Pole climbing was used to know the positive avoidance in the animals and to assess the memory enhancement activity of test compounds. The escape latency time was measured for the animals of all the groups showed significant (p<0.05) decrease in standard and test compounds in comparison with the disease control group using Dunnet’s test which was given in Fig. 2. The co-administration of Pyritinol and Fluvastatin showed synergistic effect which is comparable with the standard group than individual drug treated groups.

**Locomotor Activity:** The activity of Pyritinol and Fluvastatin were evaluated by using actophotometer. The standard, Pyritinol and Fluvastatin showed significant (p<0.05) increase in locomotor activity in comparison with the disease control group using Dunnet’s test which was given in Fig. 3. The co-administration of Pyritinol and Fluvastatin showed synergistic effect which is comparable with the standard group than individual drug treated groups.

**Morris Water Maze Test:** The special learning ability of animals was assessed by Morris water maze test. The learning abilities of all the groups of animals compared before and after induction of cerebral ischemia against disease control group of that day. The animals of standard, Pyritinol and Fluvastatin showed significant (p<0.05) learning abilities compared to disease control and control groups using Dunnet’s test which was given in Fig. 4. Synergistic effect was observed which is comparable with the standard group than individual drug treated groups.

**Acetylcholinesterase Inhibitory Effects of Pyritinol and Fluvastatin:** Disease control group significantly increased the brain AchE level compared to control group which was given in Fig. 5. Standard drug (Donepezil) and test drugs (Pyritinol, Fluvastatin) treatment significantly inhibited the brain AchE level compared to their corresponding disease control groups.

**Antioxidant Effect of Pyritinol and Fluvastatin in Disease Rats**

**Catalase Activity:** Catalase levels were decreased in disease control group compared to normal control group which was given in Fig. 6. Significant (p<0.05) difference has been found in drug- treated groups. Synergistic effect was observed with drug treated groups which is comparable with the standard group.
Rectangular Maze Test:

Fig. 1: Effect of Pyritinol and Fluvastatin on latency time compared to the disease control group (Mean ± SD, n = 6). Values are expressed as Mean±SD of latency time in seconds. *p<0.05, *p<0.01, *p<0.001 as compared with corresponding values of disease control group.

Pole climbing Method:

Fig. 2: Effect of Pyritinol and Fluvastatin on latency time compared to the disease control group (Mean ± SD, n = 6). Values are expressed as Mean±SD of latency time in seconds. *p<0.05, *p<0.01, *p<0.001 as compared with corresponding values of disease control group.

Locomotor Activity:

Fig. 3: Effect of Pyritinol and Fluvastatin on mobility of animals compared to the disease control group (Mean ± SD, n = 6). Values are expressed as Mean±SD of latency time in seconds. *p<0.05, *p<0.01, *p<0.001 as compared with corresponding values of disease control group.
Morris Water Maze Test:

Fig. 4: Effect of Pyritinol and Fluvastatin on latency time compared to the disease control group (Mean ± SD, n = 6). Values are expressed as Mean±SD of latency time in seconds. *p<0.05, **p<0.01, ***p<0.001 as compared with corresponding values of disease control group

AchE Assay:

Fig. 5: Effect of Pyritinol and Fluvastatin on AchE Estimation compared to the disease control group (Mean ± SD, n = 6). Values are expressed as Mean±SD of AchE levels. **p<0.01 compared with corresponding values of disease control

Catalase assay:

Fig. 6: Effect of Pyritinol and Fluvastatin on % H$_2$O$_2$ scavenging activity compared to the disease control group (Mean ± SD, n = 6). Values are expressed as Mean±SD of % H$_2$O$_2$ scavenging activity. *p<0.01 compared with corresponding values of disease control.
DPPH Assay:

![DPPH assay chart]

Fig. 7: Effect of Pyritinol and Fluvastatin on DPPH assay compared to the disease control group (Mean ± SD, n = 6). Values are expressed as Mean±SD of % Inhibition of DPPH. *p<0.01 compared with corresponding values of disease control.

TBARS assay:

![TBARS assay chart]

Fig. 8: Effect of Pyritinol and Fluvastatin on malondialdehyde levels compared to the disease control group. (Mean ± SD, n = 6). Values are expressed as Mean±SD of malondialdehyde levels. *p<0.01 compared with corresponding values of disease control.

DPPH Method: Antioxidant levels were decreased in disease control group compared to the control group which was given in Fig. 7. Drug treated groups showed significant (p <0.05) difference compared to the disease control group.

Thiobarbituric Assay: Disease control group significantly increased the brain MDA level compared to control group which was given in Fig. 8. Standard drug (Donepezil) and test drugs (Pyritinol, Fluvastatin) treatment significantly (p < 0.05) decreased brain MDA level compared to their corresponding disease control groups.

Histopathological Studies: From Fig. 9, it is clearly visible that in control group brain showed optimum sized, normal neuronal cells. In disease control group showed neuronal swelling, shrinkage and more degenerated cells. The test drugs treated groups were in between the normal control and disease control groups. The standard drug treated group and combinations group is mostly near to the control group compared to the individual drug treated groups.

These results indicated that the memory and cognition-enhancing effects of Pyritinol and Fluvastatin might be related to factors such as antioxidant and anti acetylcholinesterase activities.

In conclusion, the results of this study suggested that Pyritinol and Fluvastatin showed the neuroprotective effect on cerebral ischemia induced oxidative stress and cognitive impairment. Co-administration of these Pyritinol and Fluvastatin had potential therapeutic effect on improving the anti oxidant and anti amnesic activity in rats.
Histopathological Studies:

Fig. 9: Histopathological studies. The figures (a), (b), (c), (d), (e) and (f) are normal control, disease control, Pyritinol, Fluvastatin and Pyritinol+Fluvastatin, respectively, responding the histological sections of the brain tissue showing neurological lesions.

which was assessed by various behavioural assessment tests, reducing lipid peroxidation, increasing endogenous antioxidant enzymes and decreasing acetyl cholinesterase activity in brain. Pyritinol and Fluvastatin thus prevent special memory deficits and neuronal loss.

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REFERENCES


