

Original Article



Protective Effects of Combining Atorvastatin and Crocin on Hippocampal Neurodegeneration in Streptozotocin-Induced Alzheimer's Rats

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Summary

Introduction: The global prevalence of Alzheimer's disease (AD) continues to increase, posing a major public health challenge. Although currently available treatments provide limited symptomatic relief, no effective therapy has been shown to reverse disease progression. Consequently, exploring novel therapeutic approaches, particularly combination therapies with complementary neuroprotective mechanisms, has become increasingly important. Therefore, this study aimed to investigate the combined neuroprotective effects of Atorvastatin (Ator) and Crocin (Cro) on the hippocampus of rats with streptozotocin (STZ)-induced AD.

Methods: Thirty male Wistar rats were randomly assigned to five groups (n=6 per group): Sham, STZ, Cro (30 mg/kg), Ator (5 mg/kg), and Cro + Ator. AD was induced by intracerebroventricular injection of STZ (3 mg/kg). Following STZ administration, the treatment groups received their respective interventions orally by gavage once daily for 21 consecutive days. Therapeutic efficacy was subsequently evaluated by assessing neuronal density in both CA1 and CA2 regions of the hippocampus using Nissl body staining.

Results: STZ administration caused severe neuronal loss in the hippocampus (19.21% survival in CA1 and 44.39% in CA2). Monotherapy with Cro or Ator significantly improved both parameters, but the combined Cro+Ator treatment showed the most potent effect, restoring neuronal survival to 87.63%. The combination was significantly superior to either monotherapy.

Conclusion: The additive neuroprotective effects of the Cro-Ator combination are likely mediated by their complementary mechanisms of action, which preserve hippocampal integrity. By simultaneously targeting multiple pathological processes, this dual-agent therapy represents a promising therapeutic strategy for the management of AD.

Keywords: Alzheimer's disease, Crocin, Atorvastatin, Neuronal survival

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Introduction

Alzheimer's disease (AD) relentlessly erodes cognitive capacity, driving profound memory deficits alongside a spectrum of behavioral disturbances as the underlying neurodegeneration advances.^{1,2} The epidemiological burden of this condition is staggering; current global estimates of 55 million cases are projected to nearly triple to 152 million by mid-century.³ Beyond these overarching global metrics, regional profiles—such as the 2.3% prevalence documented in Iran—highlight specific clinical vulnerabilities, particularly linking clinical depression to an increased risk of disease onset.⁴ Anatomically, this pathology disproportionately ravages the hippocampus. Because this distinct brain region fundamentally dictates learning and memory consolidation, its early structural impairment remains a definitive clinical hallmark rather than a mere secondary symptom.⁵

At the molecular core of this regional collapse is the aggressive deposition of amyloid-beta (A β) plaques throughout both the cerebral cortex and hippocampal

networks. These neurotoxic aggregates emerge directly from the aberrant enzymatic cleavage of the amyloid precursor protein (APP).⁶ Once aggregated into insoluble fibrils, they initiate a cascade of synaptic dysfunction, severe oxidative stress, and inevitable neuronal apoptosis. This A β accumulation physically disrupts neuronal integrity while provoking localized inflammatory cascades. In tandem, it accelerates the intracellular formation of neurofibrillary tangles via tau hyperphosphorylation, locking the brain into a self-perpetuating cycle of structural decline.⁷ Disrupting this destructive loop requires interventions that move beyond symptom management to actively preserve hippocampal architecture and clear the neurotoxic burden.

Rather than relying on single-mechanism drugs, current pharmacological research increasingly focuses on repurposing agents with pleiotropic benefits. Atorvastatin (Ator), traditionally prescribed for lipid management, exerts substantial neuroprotection entirely independent of its systemic cholesterol-lowering mechanisms. By suppressing central neuroinflammation, Ator actively supports neuronal



viability and limits cognitive deterioration in experimental AD platforms.⁸⁻¹⁰ Concurrently, crocin (Cro)—the primary bioactive carotenoid extracted from *Crocus sativus* L.—provides robust, complementary antioxidant regulation capable of blunting hippocampal damage and mitigating localized AD pathology.^{11,12}

While both compounds demonstrate individual capability in combating A β -induced toxicity, evaluating their co-administration demands a reliable translational platform. The streptozotocin (STZ)-induced rat model fulfills this requirement; bilateral intracerebroventricular injection of STZ drives central insulin resistance, profound oxidative stress, and A β -linked cell death, accurately mirroring the metabolic and structural deficits of human AD.^{13,14} Despite the established standalone benefits of Ator and Cro, the structural neuroprotective potential of their concurrent administration remains largely unexplored. Because isolated interventions often fail to halt the multifaceted AD cascade, we hypothesized that this dual-agent strategy might offer superior histological preservation. Accordingly, using precise histopathological assessment via Nissl body staining, this study evaluates the combined (additive) effects of Cro and Ator on neuronal viability in the hippocampal CA1 and CA2 subfields of STZ-induced AD rats.

Methods

Chemicals and Reagents

Atorvastatin (PZ0001, Sigma-Aldrich, St. Louis, MO, USA) and Crocin (Sigma-Aldrich, St. Louis, MO, USA) were used for treatments. AD was induced using STZ (S0130, Sigma-Aldrich, St. Louis, MO, USA). For histopathological assessments, Cresyl Violet acetate (C5042, Sigma-Aldrich, St. Louis, MO, USA) was used for Nissl staining. Brain tissues were fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich, St. Louis, MO, USA). All other chemicals were of analytical grade.

Animals, Ethical Approval, and Treatment Protocol

Thirty adult male Wistar rats (weighing 250–300 g at baseline) were procured from the Research and Breeding Center of Laboratory Animals at Urmia University of Medical Sciences. The Institutional Animal Ethics Committee of Urmia University of Medical Sciences reviewed and approved all experimental procedures (Ethical approval code: IR.UMSU.AEC.1404.004). The study followed international standards, including the NIH Guide for the Care and Use of Laboratory Animals. The rats were housed in groups under standard laboratory conditions, maintained on a 12-hour light/dark cycle at 22 $\pm$ 2 $^{\circ}$ C, with unrestricted access to standard chow and water.

Rats were randomly divided into five experimental groups (n = 6 each). Drug treatments commenced one hour post-surgery. For the pharmacological interventions, Ator was suspended in a 0.5% carboxymethyl cellulose (CMC) saline solution and administered at a dose of 5 mg/kg/day [16]. Concurrently, Cro ($\geq$ 98% purity) was dissolved in

normal saline and administered at a dose of 30 mg/kg/day [17]. All oral treatments, including the vehicle for the control groups, were given via gavage once daily for 21 consecutive days in a standard volume of 5 mL/kg. The specific surgical and treatment regimens for each group were assigned as follows:

- Sham: Underwent the surgical procedure to receive vehicle (normal saline) via ICV injection (3 μ L per ventricle).
- STZ: Received a single bilateral ICV injection of STZ (3 mg/kg, 3 μ L per ventricle) to induce Alzheimer-like pathology.
- Cro: Received ICV STZ, followed by daily oral gavage of Crocin (30 mg/kg/day).
- Ator: Received ICV STZ, followed by daily oral gavage of Atorvastatin (5 mg/kg/day).
- Cro+Ator: Received ICV STZ, followed by concurrent daily oral gavage of both Cro (30 mg/kg/day) and Ator (5 mg/kg/day).

Induction of the AD Model

The Alzheimer-like phenotype was induced exclusively via bilateral intracerebroventricular (ICV) stereotaxic injections of STZ. Briefly, rats were deeply anesthetized using an intraperitoneal injection of ketamine hydrochloride 10% (80 mg/kg) and xylazine 2% (20 mg/kg). Each animal's head was secured in a stereotaxic apparatus. Following a midline scalp incision, the bregma landmark was identified, and burr holes were carefully drilled at the following coordinates relative to bregma: anteroposterior –0.8 mm, mediolateral $\pm$ 1.5 mm, and dorsoventral –3.6 mm. Freshly prepared STZ (dissolved in 0.1 M cold citrate buffer, pH 4.5) was then infused into each lateral ventricle (3 mg/kg in a volume of 3 μ L per ventricle) over 3 minutes using a microsyringe.¹⁵ To prevent backflow, the needle remained in place for 2 additional minutes before slow withdrawal. Sham-operated controls underwent the same surgical procedure but received only equivalent volumes of normal saline.

Tissue Processing and Sample Preparation

The rats were deeply anesthetized and euthanized (ketamine (80 mg/kg) and xylazine (10 mg/kg)), after which the brains were rapidly extracted. The hippocampus was fixed in 4% paraformaldehyde (PFA) for 48 hours, dehydrated through a graded ethanol series, cleared in xylene, and subsequently embedded in paraffin for histopathological analysis.

Nissl Body Staining (Cresyl Violet)

To assess neuronal survival and structural integrity, paraffin-embedded sections (5–7 μ m thick) were stained with 0.1% Cresyl Violet. This method enables visualization of Nissl bodies within the cytoplasm of viable neurons. In each section, the hippocampal subfields (CA1 and CA2) were identified. Morphologically normal neurons (characterized by relatively large, light-colored nuclei and prominent Nissl bodies) and damaged neurons (exhibiting nuclear pyknosis, shrinkage, or fragmented

cytoplasm) were examined. The results were expressed as the percentage of Nissl-positive neurons relative to the total cell count in the specified areas.¹⁶ All histological sections were examined using a light microscope (Olympus, BH2, Japan).

Statistical Analysis

All data are expressed as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed using the Python programming language. We first verified the normal distribution of the variables using the Shapiro-Wilk test. To evaluate differences among the five experimental groups, we conducted a one-way analysis of variance (ANOVA), followed by Tukey's HSD post-hoc test for pairwise comparisons. A p-value of less than 0.05 was considered to indicate statistical significance.

Results

Histopathological Assessment and Neuronal Morphology Using Cresyl Violet (Nissl) staining

Histopathological examination of the hippocampus

using Cresyl Violet (Nissl) staining revealed distinct morphological differences across the experimental groups. In the Sham group, pyramidal neurons in both CA1 and CA2 regions exhibited a normal histological profile, characterized by a regular, densely packed arrangement, prominent nuclei, and abundant Nissl substance in the cytoplasm (Figure 1, arrowheads). In contrast, the STZ group showed severe structural disintegration and significant neuronal loss. A substantial number of neurons appeared shrunken with pyknotic, hyperchromatic nuclei and fragmented cytoplasm (Figure 1, arrows), indicating advanced neurodegeneration.

Quantitative analysis confirmed this observation. In the CA1 subfield, the percentage of viable neurons dropped significantly to $19.21 \pm 8.72\%$ compared to the Control group ($P < 0.001$). Oral administration of Cro or Ator as monotherapies significantly increased neuronal survival compared to the STZ group ($P < 0.01$ for both), although the hippocampal architecture remained partially disrupted. Notably, the Cro+Ator group demonstrated the most significant neuroprotective effect. In the CA1 region, the

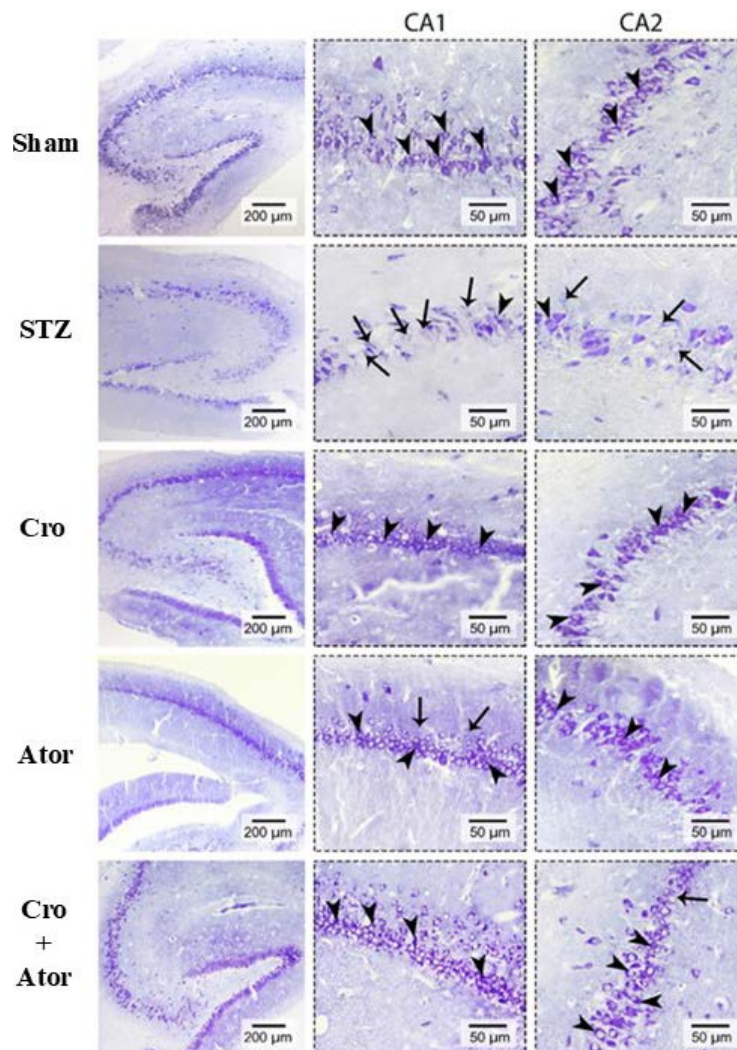


Figure 1. Representative photomicrographs of Cresyl Violet (Nissl) staining in the hippocampal CA1 and CA2 subfields

The left column shows the general view of the hippocampus (Scale bar: 200 μ m). The magnified panels for CA1 and CA2 (Scale bar: 50 μ m) highlight the morphological details. Arrowheads indicate healthy, intact neurons with distinct Nissl bodies, while arrows point to damaged, shrunken, and pyknotic neurons. The STZ group (Alzheimer) shows extensive neuronal loss and nuclear condensation, which is remarkably attenuated in the combined treatment group (STZ: Alzheimer / STZ, Cro: Crocin, Ator: Atorvastatin)

Combined group showed a remarkable recovery, achieving $87.63 \pm 8.14\%$ survival, which was significantly higher than the STZ group ($P < 0.001$) and both the Cro and Ator monotherapy groups ($P < 0.05$ for each), (Figure 2). In the CA2 region, although the damage was relatively less severe, the STZ group still exhibited a significant reduction in neuronal density ($44.39 \pm 3.23\%$) compared to the Control group ($94.07 \pm 2.49\%$, $P < 0.001$). The combination therapy effectively maintained neuronal integrity at $84.82 \pm 3.79\%$, which was significantly improved versus the STZ group ($P < 0.01$) and versus each monotherapy ($P < 0.05$). These findings suggest that the combined application of Ator and Cro provides robust additive protection against STZ-induced neurotoxicity across multiple hippocampal subfields (Figure 3).

Discussion

The present study demonstrated that co-administering crocin (Cro) and atorvastatin (Ator) yields a robust, additive neuroprotective response in the STZ-induced rat model of AD. Rather than offering marginal improvements, this dual treatment fundamentally rescued hippocampal architecture, driving neuronal survival in the CA1 region from a mere 19.21% to 87.63%, alongside a parallel recovery in the CA2 subfield (from 44.39% to 84.82%). This pronounced structural preservation likely stems from disrupting the bidirectional positive feedback loop that tightly couples oxidative stress to A β pathology.¹⁷ Under typical pathological conditions, oxidative stress aggressively upregulates β -secretase (BACE1) expression via the JNK/c-jun pathway, triggering toxic A β overproduction. These accumulated plaques subsequently drive localized lipid peroxidation and reactive oxygen species generation, effectively dismantling cellular membranes.¹⁷ Because STZ administration heavily initiates this self-amplifying cascade,¹⁸ which is clearly reflected by the severe neuronal degradation in our untreated STZ group, breaking the cycle at multiple molecular checkpoints appears necessary for meaningful tissue preservation. The marked morphological recovery observed with the Cro+Ator combination suggests this dual approach successfully intercepts these intersecting neurotoxic pathways.

Situating these structural findings within the existing literature reveals consistent patterns of efficacy for both agents. Naghizadeh et al. primarily linked oral Cro administration (100 mg/kg) for 21 days to improved cognitive performance and lowered biochemical oxidative markers in ICV-STZ-lesioned rats. Our histological data confirms that these biochemical benefits translate directly into the physical preservation of hippocampal architecture.¹⁹ This aligns directly with subsequent work utilizing Cresyl Violet staining, which established crocin's capacity to shield Nissl granules from STZ-induced neuroinflammation.²⁰ Similarly, Piermartiri et al demonstrated that Ator prevents hippocampal cell death, neuroinflammation, and oxidative stress following A β_{1-40} administration in mice. Their histopathological assessments corroborate our observation that Ator monotherapy actively suppressed pyknotic cell formation and partially preserved hippocampal architecture.²¹ Recent evaluations further reinforce this trajectory; Ferak et al reported that Ator improves long-term memory by reducing A β formation and neuronal damage in STZ-induced diabetic rats, supporting its neuroprotective role in STZ-based AD models.²² Rather than viewing these single-target successes in isolation, our data integrates them, confirming that both Cro and Ator independently mitigate AD-related neurodegeneration while establishing a solid empirical baseline for their combined application.

Critically, combining Cro and Ator produced a distinct additive advantage that consistently outperformed either monotherapy. Although individual treatments yielded partial neuroprotection, neuronal survival in the CA1 region under the co-administration protocol (87.63%) statistically eclipsed the single-agent outcomes ($P < 0.05$). We attribute this enhanced efficacy to the complementary, parallel mechanisms engaged by the two compounds. Cro operates fundamentally as a robust radical scavenger, neutralizing reactive oxygen species and reinforcing endogenous antioxidant networks.²² Concurrently, Ator drives pleiotropic responses independent of cholesterol modulation, explicitly limiting central inflammation and stabilizing mitochondrial function.²³ As highlighted by Doig et al, anti-inflammatories and antioxidants may

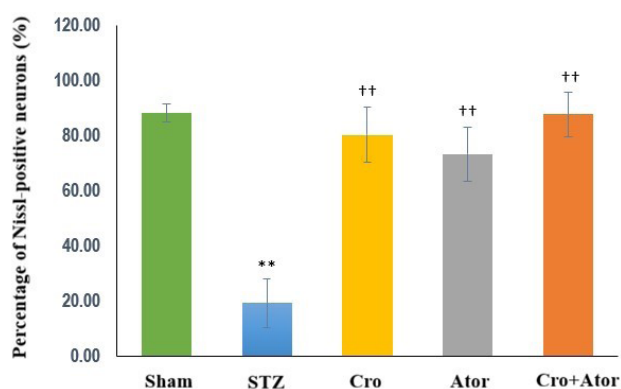


Figure 2. Representative photomicrographs and quantitative analysis of Nissl-stained neurons in the hippocampal CA1 subfield
Data are expressed as Mean $\pm$ SD. (** $P < 0.001$ vs. Control. †† $P < 0.001$ vs. Alzheimer) (STZ: Alzheimer / STZ, Cro: Crocin, Ator: Atorvastatin)

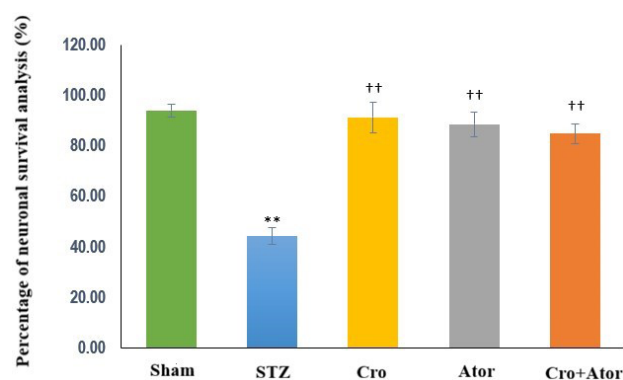


Figure 3. Histological changes and neuronal survival analysis in the hippocampal CA2 subfield using Nissl staining
Data are expressed as Mean $\pm$ SD. (** $P < 0.001$ vs. Control. †† $P < 0.001$ vs. Alzheimer) (STZ: Alzheimer / STZ, Cro: Crocin, Ator: Atorvastatin)

be especially valuable in AD because these processes are involved in multiple positive feedback loops and hence affect numerous pathological processes simultaneously.²⁴ By simultaneously buffering the cellular redox state and suppressing inflammatory signaling, this combination plausibly dampens BACE1 expression, thereby limiting the neurotoxic A β burden and enhancing clearance mechanisms. The near-normal Nissl staining patterns observed in the treatment group physically validate this microenvironmental shift, positioning this dual-agent strategy as a highly rational countermeasure against AD's complex pathophysiology. Expanding upon these histological foundations, the restoration of neuronal density observed in the Cro+Ator group warrants further experimental validation across diverse preclinical models to map the precise molecular pathways governing this structural recovery. Consistent reproducibility of these additive benefits could eventually provide a strong empirical basis for early-phase clinical evaluations in patients facing mild cognitive impairment.

Conclusion

Co-administering atorvastatin and crocin provides a highly effective, additive therapeutic strategy against STZ-induced Alzheimer's pathology. By shifting the hippocampal microenvironment from a state of active degeneration to one of structural preservation, this dual intervention fundamentally rescued CA1 pyramidal neurons and reversed severe cellular damage. The histopathological data strongly imply that simultaneously targeting inflammatory cascades and antioxidant pathways offers superior neuroprotection compared to isolated treatments, plausibly by restricting A β -mediated toxicity. To transition these preclinical observations toward clinical relevance, future investigations must map the precise molecular interplay affecting tau hyperphosphorylation and specific synaptic markers. Furthermore, validating this combinatorial approach across diverse in vivo models alongside developing targeted nano-delivery systems to optimize crocin's blood-brain barrier permeability will be crucial steps before initiating early-phase clinical trials for patients with mild cognitive impairment (MCI).

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Artificial Intelligence Use Disclosure

Artificial intelligence (AI) tools were used solely to improve the language, grammar, and readability of the manuscript. The authors take full responsibility for the content, interpretation of the results, and conclusions presented in this manuscript.

Authors' Contribution

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Competing Interests

There is no conflict of interest.

Ethical Approval

Ethical approval for the present study was granted by the Research Ethics Committee at Urmia University of Medical Sciences (Approval No. IR.UMSU.AEC.1404.004).

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