Physiological indicators of Adenosine and Salidroside regulating the motor behavior in a *Caenorhabditis elegans* model via Ador-1 adenosine Receptor

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ABSTRACT

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| **Aims:** Adenosine and salidroside exert both preventive and therapeutic effects on neurodegenerative diseases, including Alzheimer's and Parkinson's diseases. However, the mechanism is unclear.  **Methodology:** N2 wild-type and EG6890 (Ador-1 gene knockout) *Caenorhabditis elegans* were used as models, and 0.03, 0.3, and 3 mM adenosine and salidroside were applied to systematically evaluate their effects on the nematodes' physiological indicators (including pharyngeal pumping, bending, head swinging, and swimming). The interaction between salidroside and Ador-1 adenosine receptors was further investigated using molecular docking.  **Results:** The results revealed that N2 wild-type treatment with different concentrations of salidroside and adenosine significantly downregulated pharyngeal pumping, bending, head swinging, and swimming. Comparison of the relative motor ability indices of the N2 and EG6890 strains indicated that adenosine modulated the nematodes' motor abilities via adenosine receptors. In addition to regulating motor behavior through adenosine receptors, salidroside may involve other receptors or demonstrate concentration-dependent effects. Molecular docking results revealed that salidroside exhibits a stronger affinity for the Ador-1 receptor compared to adenosine, suggesting that both compounds regulate motor function through this receptor pathway.  **Conclusion:** This study provides a novel experimental foundation for elucidating the neuroprotective mechanisms of salidroside and adenosine as adenosine receptor agonists, as well as for related drug screening. |

*Keywords: salidroside, adenosine, adenosine receptor, Caenorhabditis elegans*

1. INTRODUCTION

*Caenorhabditis elegans* is a multicellular organism with the entire genome sequenced [1]. Given the conservation in genes and signaling pathways between nematodes and humans, and as an experimental subject, non-ethical ease or regulatory concerns, makes it a commonly used biological model in biomedical research [2,3]. As a model system, *C. elegans* is widely used to study antioxidants, aging processes, age-related diseases, longevity mechanisms, and to identify drug compounds that may extend lifespan [4].

*Rhodiola rosea*, also known as Snow Rose, is a traditional Tibetan dietary supplement in China used to improve physical endurance and alleviate altitude sickness [5]. Salidroside, extracted from *R. rosea*, a perennial herb of the Crassulaceae family, is often referred to as "highland ginseng" or "snow mountain fairy grass" [6,7]. The medicinal effects of *R. rosea* include invigorating qi (in Chinese, 气), activating blood circulation, dredging meridians, and relieving asthma. Recent studies have shown that salidroside can protect dopaminergic neurons by regulating mitochondrial pathways associated with oxidative stress, and protect PC12 cells from Aβ1-40-induced cytotoxicity [8,9]. Parkinson’s disease, a common chronic degenerative disease of the central nervous system(CNS) affecting mid-aged and elderly individuals, is believed to result from the combined effects of genetic and environmental factors [10]. Salidroside holds significant potential for the treatment of Parkinson's disease. Numerous studies have demonstrated that salidroside has strong anti-inflammatory and antioxidant effects, effectively scavenges free radicals, and offers protective benefits against myocardial damage [6].

Studies have shown that adenosine has several roles in nearly all tissues and organs, exerting effects such as lowering blood pressure [11,12], inducing sedation [8], and regulating immune processes [13]. It also influences various physiological functions, including anxiety, schizophrenia, epilepsy, and drug addiction [14]. Adenosine acts as a neuromodulator, widely distributed across multiple organs, particularly in the central nervous system [15]. Its physiological effects are primarily mediated through binding to adenosine receptors (ARs) [11]. Adenosine exerts its effects through four distinct subtypes of adenosine receptors: A1R, A2AR, A2BR, and A3R, which four distinct AdoR isoforms have been identified in mammals [16]. AdoR-1, the single adenosine receptor homolog in *C. elegans*, which belongs to the superfamily of G-protein coupled receptors (GPCRs), mediates most of the physiological effects of extracellular adenosine [14].

By observing changes in physiological indicators of *C. elegans* treated with salidroside and adenosine, and comparing the results between wild-type (N2) and transgenic (EG6890, Ador-1 gene knockout) nematodes, we aim to provide a foundation for further research into the effects of drugs on adenosine receptors.

2. material and methods

**2.1 Nematode Strains**

Wild-type *C. elegans* strain N2 (var. Bristol) was provided by the Caenorhabditis Genetics Center (University of Minnesota, USA). EG6890 strain, ador-1(ox489), was kindly supplied from Dr. Erik Jorgensen laboratory (University of Utah, USA). This strain has a deletion from 1kb upstream and the first three exons of the ador-1 gene, and was outcrossed six times. All strains were maintained at 20°C and preserved in our laboratory.

**2.2 Nematode Treatment**

Nematodes were synchronized using the method described by Xie et al. [17] and cultured at 20oC until reaching the L4 stage. Both strains were treated in parallel with salidroside (HPLC ≥ 98%, Aladdin, Shanghai, China) and adenosine (Sigma-Aldrich, St. Louis, MO, USA). The L4 nematodes were exposed to low, medium, and high concentrations of salidroside and adenosine (0.03, 0.3, and 3 mM, respectively) and M9 buffer (control) for 48 hours.

**2.3 Physiological indicators observation**

**Pharyngeal Pump Frequency**

At room temperature, nematodes of similar age and health status from each group were selected and observed under a MZ7.5 stereomicroscope (Leica Microsystems). The pharyngeal pumping frequency of each nematode was recorded three times at 20-second intervals. Each group consisted of 15 nematodes, and the experiment was repeated across three independent trials.

**Bending Frequency**

Nematodes of similar age and health status were selected from each group and placed on NGM medium without OP50. After allowing them to move for 1 minute, the number of body bends within that time frame was recorded. A single body bend was defined as one complete sinusoidal movement of the nematode. Each group tested 15 nematodes, and the experiment was repeated across three independent trials.

**Head Swing Frequency**

Nematodes from each group, selected for similar age and health status, were placed on NGM medium without OP50 and allowed to move for 1 minute. The number of head swings within 20 seconds was recorded. A head swing was defined as the movement of the nematode's head from one direction to the opposite and back. Each group tested 15 nematodes, and the experiment was repeated across three independent trials.

**Swimming Ability**

Nematodes from each group were selected based on similar age and health status, and transferred to a 96-well plate containing 200 μL of M9 buffer. After allowing the nematodes to swim freely for 10 seconds, their body swings were recorded over a 30-second period. Each group tested 15 nematodes, and the experiment was repeated across three independent trials.

**2.4 Molecular Docking of Salidroside and Adenosine Receptor**

Chemical structures of the components to be docking were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). The 3D structures of the compounds were then imported into Open Babel 3.1.1 software and transformed into PDB format. In AutoDock Tools 1.5.6, the obtained 3D structure was modified by adding hydrogen, performing protonation, charging calculation, charging assignment, and setting of rotatable bonds. Subsequently, the modified structure was saved as a PDBQT ligand file. The PDBQT structures of the receptor and ligand were imported into AutoDock Tools 1.5.6 to create a docking binding site. During the construction of the binding site, the spacing (e) was set to 1. The DeepSite protein prediction website (https://playmolecule.com/deepsite/) was utilized to predict the centroid of each protein's binding site. The Ador-1 adenosine receptor was downloaded from the Protein Data Bank (PDB) (https://www.rcsb.org/) using its PDB ID. Using PyMOL2.3.0, the protein's crystal water and original ligands were removed. The protein structure was then imported into AutoDockTools for hydrogenation, charge calculation, charge assignment, and atomic type assignment.

Protein binding sites were predicted using POCASA 1.1, and AutoDock Vina 1.1.2 was used for molecular docking. The docking calculations were performed using AutoDock Vina, with all other parameters set to their default values. Upon completion of the Vina operation, the binding affinity score between the target protein receptor and the small molecule ligand was determined based on the calculated docking binding free energy.

**2.5 Statistical Analysis**

Statistical analysis and data visualization were performed using GraphPad Prism 8.0.2 software. Data were analyzed using a t-test, and results are expressed as the mean ± SEM. A p-value < 0.05 was considered statistically significant and is denoted by an asterisk (\*), while a p-value < 0.01 indicates a highly significant difference and is denoted by two asterisks (\*\*).

3. results

**3.1 Observation and Comparison of the physiological indicators of N2 and EG 6890 Strains Treated with Adenosine**

The pharyngeal pumping frequency of N2 strain nematodes decreased with increasing adenosine concentration (Figure 1-A). No significant difference was observed between the 0.03 mM treatment group and the control group (P > 0.05), while the 0.3 mM and 3 mM treatment groups showed significant decreases (P < 0.01). In contrast, the pharyngeal pumping frequency of EG 6890 strain nematodes decreased at all three concentrations, with significant differences observed only between the high concentration (3 mM) and the control group (P < 0.01). The bending ability of N2 strain nematodes decreased with increasing adenosine concentration, with the most notable decline at 3 mM (Figure 1-B). The bending ability of EG 6890 strain nematodes only decreased significantly at the high concentration (3 mM). The head-swinging ability of N2 strain nematodes declined with increasing adenosine concentration, with a significant reduction compared to the control (Figure 1-C). Similarly, EG 6890 strain nematodes exhibited a significant decrease in head swinging at the 3 mM concentration (P < 0.01). The swimming ability of N2 strain nematodes was significantly reduced at 0.03 mM compared to the control, and further decreased with higher adenosine concentrations (Figure 1-D). EG 6890 strain nematodes also showed a significant reduction in swimming ability at the high concentration (3 mM).

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**Fig. 1. physiological indicators of N2 and EG 6890 nematodes treated with varying concentrations of adenosine. (A) pharyngeal pumping, (B) bending, (C) head swinging, and (D) swimming (\*P < 0.05, \*\*P < 0.01).**

The relative index of EG 6890 strain nematodes, compared to the N2 strain, treated with different adenosine concentrations is shown in Figure 2. This comparison highlights the regulatory role of adenosine receptors. As shown in Figure 2-A, only the high concentration (3 mM) of adenosine significantly reduced pharyngeal pumping frequency through adenosine receptors (P < 0.01), while no significant differences were observed at the lower concentrations. Additionally, all three concentrations significantly reduced bending and head swinging frequencies (Figures 2-B, C), but no effect on swimming ability was observed through adenosine receptor regulation (Figure 2-D).



**Fig. 2. Comparative analysis of adenosine's regulation of nematode physiological indicators through adenosine receptors. (A) pharyngeal pumping, (B) bending, (C) head swinging, and (D) swimming.**

**3.2 Observation and Comparison of the physiological indicators of N2 and EG 6890 Strains Treated with Salidroside**

The pharyngeal pumping frequency of the N2 strain nematodes decreased in response to salidroside, with a mild reduction observed at concentrations of 0.3 mM and 3 mM (Figure 3 -A). In contrast, the pharyngeal pumping frequency of the EG 6890 strain showed no significant difference between the 0.03 mM treatment and the control, but exhibited a dose-dependent decrease at the 0.3 mM and 3 mM concentrations. As shown in Figure 3 -B, the bending ability of the N2 strain nematodes decreased at all three concentrations of salidroside, with a pronounced reduction at 3 mM. For the EG 6890 strain, the bending ability initially increased significantly at 0.03 mM, followed by a decline at 0.3 mM. The head-swaying ability of the N2 strain nematodes decreased with increasing salidroside concentration, and the overall decline was significant when compared to the control (Figure 3 -C) (P < 0.01). The head-swaying ability was notably variable for EG 6890 strain, which increased at 0.03 mM, weakened at 0.3 mM, and further declined at 3 mM. The swimming ability of nematodes in both the N2 and EG 6890 strains decreased with increasing salidroside concentration, and this overall decline was significant when compared to the control (Figure 3 -D) (P < 0.01).

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**Fig. 3. physiological indicators of N2 and EG 6890 nematodes treated with different concentrations of salidroside (\*P < 0.05, \*\*P < 0.01). (A) Pharyngeal pumping, (B) Bending, (C) Head** **swinging, and (D) Swimming.**

Using the physiological index of the N2 strain as a baseline, the relative indices of the EG 6890 strain nematodes treated with varying concentrations of salidroside were shown in Figure 4. Comparison of the physiological indicators between the two strains at different concentrations highlights the regulatory role of adenosine receptors. As shown in Figure 4-A, 0.03 mM salidroside significantly reduced the frequency of pharyngeal pumping via adenosine receptors, while the control group exhibited minimal changes. Additionally, all three concentrations significantly reduced the bending frequency of nematodes through the adenosine receptors, with 0.03 mM having a comparatively weaker effect (Figure 4-B). Among the three concentrations, both the low and medium concentrations significantly decreased the head swinging frequency, while the high concentration showed no significant difference from the control (Figure 4-C). Interestingly, salidroside's effect on swimming ability through adenosine receptors was not observed (Figure 4-D), with only a slight decrease at the highest concentration (3 mM). These findings suggest that salidroside does not regulate the swimming ability of nematodes exclusively through adenosine receptors, or that it only exerts an effect at high concentrations.



**Fig. 4. Comparative verification of salidroside regulating nematode physiological indicators through adenosine receptors. (A) Pharyngeal pumping, (B) Bending, (C) Head swinging, and (D) Swimming.**

**3.3 Molecular docking**

The binding affinity between salidroside and the adenosine receptor was predicted through protein sequence analysis and simulated using molecular docking. As shown in Figure 5, salidroside forms hydrogen bonds with amino acids GLU169 and ASN253 respectively of the adenosine receptor. It also exhibits a π-Sigma interaction with PHE168 and a π-alkyl interaction with amino acids ALA63, VAL84, ILE274, and ILE66. The binding energy between salidroside and the adenosine receptor is -7.4 kcal/mol, while the binding energy between adenosine and the receptor is -6.5 kcal/mol, suggesting that salidroside has a stronger binding affinity.



**Fig. 5. Binding interactions between salidroside and the Ador-1 adenosine receptor of *C. elegans* (A: structure of the adenosine receptor protein; B: hydrogen bond interactions of salidroside).**

4. discussion

This study used *C. elegans*, an indicator model organism, to investigate the effects of adenosine and salidroside on the motor movement parameters of two nematode strains: N2 and EG 6890. In *C. elegans*, adenosine receptor ortholog (ADOR-1) can be studied to evaluate the purinergic system for protection from oxidative stress [14,18,19]. The study also compared the responses of *C. elegans* to two drug environments: salidroside and adenosine. Salidroside affected both nematode strains, with results indicating that motor movement characteristics changed in response to drug concentration. Low concentrations of adenosine regulated pharyngeal pumping, bending, and head swing movements through adenosine receptors, while high concentrations of adenosine likely modulated these movements through alternative signaling pathways. These findings align with our previous research on adenosine [20]. The *C. elegans* neuromuscular junctions show a high degree of molecular and functional conservation with the cholinergic transmission of the autonomic, central and neuromuscular synapses in mammals [21]. Therefore, pharyngeal pumping, bending, and head swing movements provide a whole organism bio-assay to investigate the neuroprotective mechanisms. Although salidroside demonstrates scavenging activity against DPPH free radicals, hydroxyl radicals, and superoxide anions, its overall antioxidant activity is modest, and its effect on the lifespan of *C. elegans* is limited [22,23].

Adenosine receptor agonists reduce the response to external stimuli by binding to adenosine receptors [24], leading to a decrease in motor activity compared to control groups. In this study, salidroside exerted its motor inhibitory effects through the Ador-1 receptor. The movement of nematodes was progressively slowed proportional with the concentration. When compared to the effects of adenosine, these findings suggest that salidroside functions as an adenosine receptor agonist. Although there is no universally agreed-upon conclusion regarding the specific mode of action of salidroside on adenosine receptors, most studies suggest that it exerts an agonist-like or positive regulatory effect rather than acting as an antagonist. Rat models such as myocardial ischemia/reperfusion [25] and neuroprotection [26] has shown that salidroside can reduce cell damage through A₁ or A₂A receptor-mediated pathways, demonstrating effects similar to agonism or enhancement of adenosine signaling [27]. Additionally, limited data indicate that salidroside may function as an "agonist" or "enhancer" by modulating adenosine receptor expression or associated signaling proteins, without necessarily binding directly to the receptors [28]. Although both adenosine and salidroside downregulated the swimming ability index, comparative analysis between the N2 and EG 6890 strains revealed no significant differences. This suggests that the swimming index may serve as a composite measure, potentially influenced by receptors other than adenosine receptors.

Molecular docking technology is a commonly employed research method in drug discovery and screening, particularly in the investigation of active components of TCM [29]. It is widely accepted that a lower energy indicates a higher likelihood of stable binding between the ligand and receptor. A binding energy ≤ -5.0 kJ/mol suggests good binding activity, while a binding energy ≤ -7.0 kJ/mol indicates strong binding activity [30] The binding energy between salidroside and the adenosine receptor is -7.4 kcal/mol, predicted a strong binding interaction between salidroside and adenosine receptors, with a binding mode distinct from that of the natural ligand, adenosine. This study contributes to understanding the medicinal properties of salidroside in *C. elegans* by exploring its interaction with adenosine receptors. Salidroside exhibited a significant impact on the movement ability of *C. elegans*, particularly through its binding to adenosine receptors, consistent with the neuroprotective effect of salidroside in *C. elegans* models involves its antioxidant capabilities [31]. Therefore, the overall trend suggests that salidroside primarily acts as a "positive regulator" in the adenosine pathway. To definitively determine whether it is a direct agonist, partial agonist, or indirect enhancer, further specialized pharmacological and molecular-level experiments are required.

5. Conclusion

Our study demonstrates that both adenosine and salidroside interact with the adenosine receptors of *C. elegans*. Molecular simulations confirmed that salidroside exhibits a strong affinity for the nematode adenosine receptor (Ador-1). Additionally, by comparing the homology between the adenosine receptors of *C. elegans* and human adenosine receptor subtypes, this research highlights the significance of the *C. elegans* N2 wild-type and EG6890 (Ador-1 gene knockout strains) in the development of adenosine receptor drug screening models.

**Disclaimer (Artificial intelligence)**

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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