Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2026**; 31(6): 51780
https://doi.org/10.31083/FBL51780

Original Research

Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) Is Effective in Reducing Symptoms of Huntington's Disease in Females of the R6/1 Mouse Model

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Academic Editor: Antoni Camins
Submitted: 12 March 2026 Revised: 1 May 2026 Accepted: 13 May 2026 Published: 26 June 2026

Abstract

Background: Huntington's disease (HD) is an inherited (autosomal dominant) disorder caused by the occurrence of a pathogenic variant of the *HTT* gene. The genetic defect consists of an expansion of CAG repeats in exon 1, resulting in the production of a toxic misfolded huntingtin protein that forms aggregates. Currently, there is no registered therapy for this severe, neurodegenerative disease. Recent studies have demonstrated that treatment with genistein (4′,5,7-trihydroxyisoflavone or 5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), which acts by stimulating autophagy through a FOXO3-dependent process, can ameliorate behavioral abnormalities and improve biochemical parameters in the R6/1 mouse model of HD. However, although HD occurs in humans with similar frequency in men and women, the effects of high dose (150 mg/kg/day) of genistein on HD mice were tested previously only in males, because of concerns regarding potential ambiguity in interpretation of the results caused by structural similarities between genistein and estrogens. Nevertheless, as potential anti-HD therapies should be evaluated in both sexes, in this work the effects of genistein administration on behavior, levels of selected hormones, and biochemical parameters were evaluated in R6/1 female mice. **Methods**: Several behavioral tests, including the Rota-rod test, elevated-plus maze, open field test, and Morris water maze, were performed using R6/1 (HD) and control female mice treated with or without genistein at 150 mg/kg/day. We determined the levels of selected hormones and biochemical parameters in these animal groups. Genistein treatment was initiated at 16 weeks of age, shortly after the onset of symptoms at 14 weeks of age, and tests were conducted at 16, 22, and 30 weeks of age. **Results**: Genistein was effective in improving behavioral outcomes ($p < 0.05$ for all tests vs untreated animals) and improving hormonal and biochemical parameters ($p < 0.05$ for all tests) in female R6/1 (HD), similarly to results reported previously for males of the same line. **Conclusion**: Genistein effectively reduced symptoms of HD in both male and female R6/1 mice.

Keywords: Huntington's disease; genistein; laboratory mice; behavioral tests; hormones; reactive oxygen species

1. Introduction

Huntington's disease (HD) is caused by the presence of a variant in the *HTT* gene (encoding the huntingtin protein), revealing an expansion of CAG trinucleotide repeats. This leads to the production of a misfolded gene product prone to aggregation [1]. Clinically, HD manifests as progressive psychomotor abnormalities, including behavioral disturbances, cognitive decline, and choreiform movements. The average survival time following diagnosis is approximately 15–20 years [2]. Another hallmark of HD is the delayed onset of symptoms, which typically emerge during the third or fourth decade of life [1,2]. Recent findings suggest that delayed symptom onset may be linked to somatic instability of CAG repeats, leading to further expansion beyond the initial inherited length and potentially exceeding 150 repeats [3]. Indeed, expanded repeat numbers detected in peripheral blood cells have been correlated with the progression of neurodegenerative disease [4]. Although these observations provide new insights into disease pathogenesis, aggregation of mutant huntingtin protein remains a central driver of neuronal toxicity and degeneration.

HD remains an incurable neurodegenerative disorder, despite the fact that its primary genetic cause has been clearly identified and extensively characterized, and although the disease follows a monogenic inheritance pattern that might suggest a relatively straightforward pathogenic mechanism [1]. Numerous therapeutic strategies for HD have been investigated; however, no disease-modifying treatment has yet been successfully established. Many experimental approaches have shown limited effectiveness, partly because of the dominant inheritance pattern of HD, which complicates gene-replacement strategies aimed at restoring normal protein function. Additional challenges include the pronounced neurotoxicity associated with huntingtin aggregates, difficulties in delivering therapeutic agents efficiently to the central nervous system,

and the possibility that alternative pathogenic mechanisms, such as toxic RNA species generated from mutant *HTT* transcripts, contribute to disease progression [2,5].

Enhancement of autophagy has emerged as a promising therapeutic avenue, because activation of this pathway may facilitate degradation of mutant huntingtin aggregates and other pathogenic structures, including aberrant nucleoprotein complexes formed by mutant *HTT* transcripts [6,7,8,9]. Although several autophagy-inducing agents, including rapamycin and related compounds, have demonstrated efficacy in cellular models, significant limitations remain. These include the need to avoid excessive autophagy activation that could lead to cellular self-digestion, the requirement for efficient penetration of the blood–brain barrier, and long-term safety considerations, given that treatment for a genetic disease such as HD would likely need to be lifelong. Unfortunately, many known autophagy activators do not fully meet these criteria [10]. Recent studies have highlighted genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), a naturally occurring isoflavone (4′,5,7-trihydroxyisoflavone), as a potential therapeutic candidate based on autophagy stimulation. This compound has been shown to reduce mutant huntingtin aggregation in engineered cellular models [11] as well as in patient-derived fibroblasts *in vitro* [12]. Importantly, genistein can cross the blood–brain barrier [13] and has demonstrated a favorable safety profile during long-term clinical use [14]. Further mechanistic studies revealed that genistein activates autophagy through a FOXO3-dependent pathway, leading to decreased levels of mutant huntingtin in the brains of animals used as an HD mouse model; improvements in behavioral outcomes were also observed when treatment was initiated after symptom onset [15].

One limitation of prior investigations demonstrating genistein efficacy in HD animals was the exclusive use of male R6/1 mice [15]. This choice was partly justified by evidence indicating that female animals exhibit a more severe disease phenotype, characterized by accelerated progression and more pronounced symptoms [16,17]. Sex hormones are likely to play a significant role in these differences, and structural similarity between genistein and estrogens [13] could potentially influence experimental outcomes. Nevertheless, HD affects men and women with comparable frequency in the human population [1,2], underscoring the need for studies that include both sexes. Accordingly, the present study aimed to evaluate whether oral administration of genistein at a previously established [15] dose of 150 mg/kg/day, initiated shortly after the appearance of disease-specific symptoms, can also improve behavioral, hormonal, and biochemical parameters in female R6/1 HD mice.

2. Materials and Methods

2.1 Animals

The R6/1 transgenic mouse line (B6.Cg-Tg(HDexon1)61Gpb/J; Jackson Laboratories, USA) served as the experimental model of HD [16], while C57BL/6J mice were used as controls. Female animals from both strains were included in the study. Animals were tested by genotyping (to confirm or exclude the presence of the mutation) before including to the appropriate experimental group. Because all qualified animals revealed typical features (including weight, behavior, and ability to collect blood), no individuals or data points were excluded. Housing conditions were maintained in a ventilated animal facility with approximately 15 air exchanges per hour, under a controlled 12-hour light/12-hour dark cycle, at a temperature of 22 ± 2 °C and relative humidity of 50 ± 5%. Animals had unrestricted access to tap water and a rodent diet formulated with reduced isoflavone content. Standard laboratory cages (15 cm high, with a total floor area of 400 cm^2) were enriched with environmental accessories appropriate for rodents. All procedures complied with the Polish Act on the Protection of Animals Used for Scientific or Educational Purposes (Journal of Laws, February 26, 2015) and adhered to European Commission guidelines regarding animal welfare in scientific research. The study was carried out in accordance with the ARRIVE guidelines (see **Supplementary Material**). Experimental protocols were approved by the Local Ethics Committee for the Care and Use of Laboratory Animals in Bydgoszcz (approval no. 48/2021).

Animals were randomly assigned to experimental groups comprising six individuals each (the same animals were used in all tests). The group size was determined using power analysis (considering the following parameters: significance level (α) = 0.05; statistical power (1-β) = 0.8; effect size (d) = 0.7). Both control (CTR) and HD (R6/1) cohorts received either water (0.1 mL daily) or genistein at a dose of 150 mg/kg/day, administered in a total volume of 0.1 mL. Treatments were delivered via oral gavage using a 25 mm metal feeding needle. Experimenter blinding was ensured by physically performing all behavioral tests and conducting all biochemical analyses by members of the research team who did not know the experimental group the tested animals belonged to or the source of the biological material used for laboratory analyses. The results were unblinded only after all measurements were completed.

2.2 Genistein

Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H–1-benzopyran-4-one) was provided by the Pharmaceutical Research Institute, Warsaw, Poland (99% purity; #446-72-0), as a commercially available compound. DMSO-dissolved stock solutions were prepared and stored at –20 °C. For experiments, these solutions were diluted in water and administered orally to mice (as described in detail in

the preceding subsection). Note that in final preparations, DMSO concentrations were negligible (below 0.1%); thus, water was used in control experiments as a vehicle.

2.3 Motor Coordination and Balance Evaluation

Motor coordination and balance were evaluated using a rota-rod device (Yamato Instruments Corporation, Chuo-ku, Tokyo, Japan). Each mouse was initially positioned on a non-rotating rod and allowed to complete three successful acclimation trials. Subsequently, animals underwent three test sessions beginning with rotation at 3 rpm for 120 s in a clockwise direction, followed by gradual acceleration to 30 rpm over a 300 s interval, after which the maximum speed was maintained for an additional 120 s. The primary outcome measure was the duration for which each animal was able to remain balanced on the rotating rod.

2.4 Anxiety Behavior Assessment

Anxiety behavior testing was conducted using an elevated plus-maze positioned 50 cm above the floor. The apparatus consisted of four arms arranged in a cross configuration: two open arms (white; 50 cm × 10 cm) and two opposing enclosed arms of identical dimensions (black, with surrounding walls 40 cm in height). The arms were interconnected by a central white platform measuring 10 cm × 10 cm. Animal movements were monitored using a video camera linked to an automated tracking system (EthoVision XT, Noldus, The Netherlands). Each assessment included two sessions per animal, each lasting 5 min and separated by a 60-min interval (re-test condition). At the start of each trial, the mouse was positioned at the distal end of an open arm, oriented with its tail toward the central platform. Transfer latency was determined, defined as the time required to move from the open arm to a closed arm. Moreover, the duration spent in open versus closed arms was measured, and behavioral activity was scored in one-minute intervals.

2.5 Stress Sensitivity Estimation

Behavioral activity under stress conditions was assessed in an open-field apparatus consisting of a white wooden arena (100 × 100 × 60 cm) enclosed by surrounding walls. The floor surface was partitioned into 25 equal squares arranged in a 5 × 5 grid, allowing differentiation between central and peripheral zones. Animal movements were recorded using a camera system integrated with EthoVision XT 10 software (Noldus, Wageningen, The Netherlands). Each mouse was introduced individually into the arena, positioned facing a corner at the start of the trial, and monitored for a total duration of 15 min. The analyzed parameters included: (i) time spent within central versus peripheral regions, (ii) number of entries into each zone, (iii) total distance traveled in the respective areas, (iv) movement velocity, (v) exploratory behavior duration, and (vi) periods of immobility.

2.6 Spatial Memory Determination

Spatial memory was evaluated using a black circular water maze (150 cm in diameter, 60 cm in depth) filled with water (called the Morris water maze). Animal behavior was recorded with a camera linked to a processing system (EthoVision XT, Noldus, The Netherlands), which allowed virtual division of the pool into four quadrants. A high-contrast visual cue was positioned on the pool wall near a transparent circular platform (10 cm in diameter) located in a designated quadrant (CQ). Testing was conducted over four consecutive days. At the start of each trial, mice were placed in the pool facing the wall in the quadrant opposite the platform. During the initial training session (day 1), the platform was positioned 1 cm above the water surface. For the subsequent spatial memory assessment (days 2–4), the platform was submerged 1 cm below the water surface within the same quadrant (CQ) as used in the training session. Latency to reach the platform was measured.

2.7 Motor Coordination and Grip Strength Measurement

Motor coordination and grip strength were assessed using a wooden rod suspended 30 cm above the floor. Each mouse was placed on the rod, with its front paws secured and its head facing the experimenter. The duration that the animal remained balanced on the rod was recorded, starting when the mouse was positioned at the midpoint of the bar.

Hindlimb muscular strength was evaluated using a computerized grip-force meter (Model 47200, Ugo-Basile, Varese, Italy). The apparatus featured a T-shaped metal bar linked to a force transducer. Each mouse was gently held by the base of the tail and allowed to grasp the rod with its hind paws. Once the animal secured the grip, it was carefully pulled backward by the tail until it released the bar. The maximum force exerted by the hind paws was measured and recorded via the transducer connected to the rod.

2.8 Collection of Blood Samples, Anesthesia and Euthanasia

Blood samples were collected from mice anesthetized with a ketamine–xylazine mixture (ketamine 87.5 mg/kg and xylazine 12.5 mg/kg). The anesthetic solution was prepared from ketamine (50 mg/mL stock solution) and xylazine (100 mg/mL stock solution) and administered via intraperitoneal injection at a total volume of 0.1 mL per animal. Blood was then obtained from the venous plexus within the orbital cavity (retro-orbital sinus) behind the eyeball using an EDTA-coated capillary tube. To isolate plasma, collected blood was centrifuged at 400 ×g for 10 min at 4 °C, and the resulting plasma was stored at −80 °C until further analysis. Following the final blood sample collection (2nd measurement), the animals were euthanised using lethal dose of pentobarbital (120 mg/kg). Specifically, Euthasol (400 mg/mL, stock solution) was dissolved in 0.9% NaCl and administered via intraperitoneal injection (final volume of 0.1 mL).

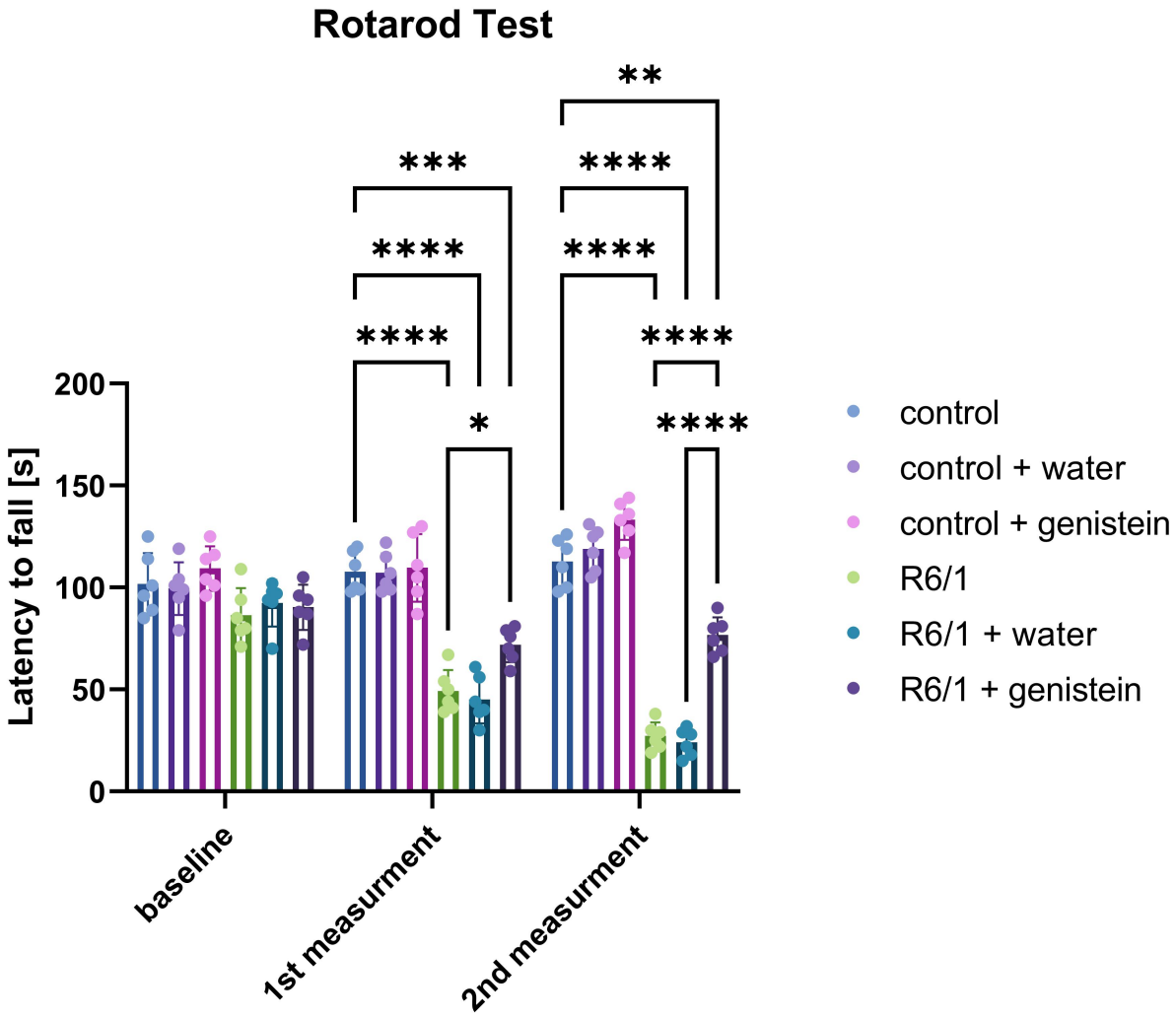

Fig. 1. Improvement of motor coordination and balance in Huntington's disease (HD) female mice by genistein. HD female mice (the R6/1 model) or control animals (the C57BL/6J line) were either untreated, treated with water (+ water), or treated with genistein (at the final dose of 150 mg/kg/day, starting at the age of 16 weeks; + genistein). Rota-rod test was performed with mice at the age of 16, 22 and 30 weeks (marked as baseline, 1st measurement, and 2nd measurement, respectively). Results are shown as mean values from measurements performed with 6 mice in each group with error bars indicating standard deviation (SD), and all measured values are indicated by dots. Statistically significant differences between indicated groups (in two-way ANOVA (Analysis of Variance) with Tukey's post hoc test) are shown as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

2.9 Determination of Levels of Hormones and Biochemical Parameters

Levels of hormones and biochemical parameters were determined using commercially available ELISA kits and EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). To estimate levels of creatine kinase, adrenocorticotropic hormone (ACTH), estradiol, corticosterone, and reactive oxygen species (ROS), the following products (purchased from MyBioSource Inc., San Diego, CA, USA) were employed: #MBS9301010, #MBS2024580, #MBS701244, #MBS494312, and #MBS2803510, respectively.

2.10 Statistical Tests

Experimental data are presented as mean values ± standard deviation (SD). No outliers were excluded from statistical analyses, which were conducted using SPSS 21.0 software (SPSS Inc., Armonk, NY, USA). The Kolmogorov-Smirnov test was applied to assess normality of variable distributions, while the Levene test evaluated homogeneity of variances. For datasets that deviated from normality, non-parametric analyses using the Kruskal-Wallis test followed by Dunn's post hoc comparisons were performed. For normally distributed data, two-way Analysis of Variance (ANOVA) with Tukey's post hoc test was applied. Statistical significance was defined as $p < 0.05$.

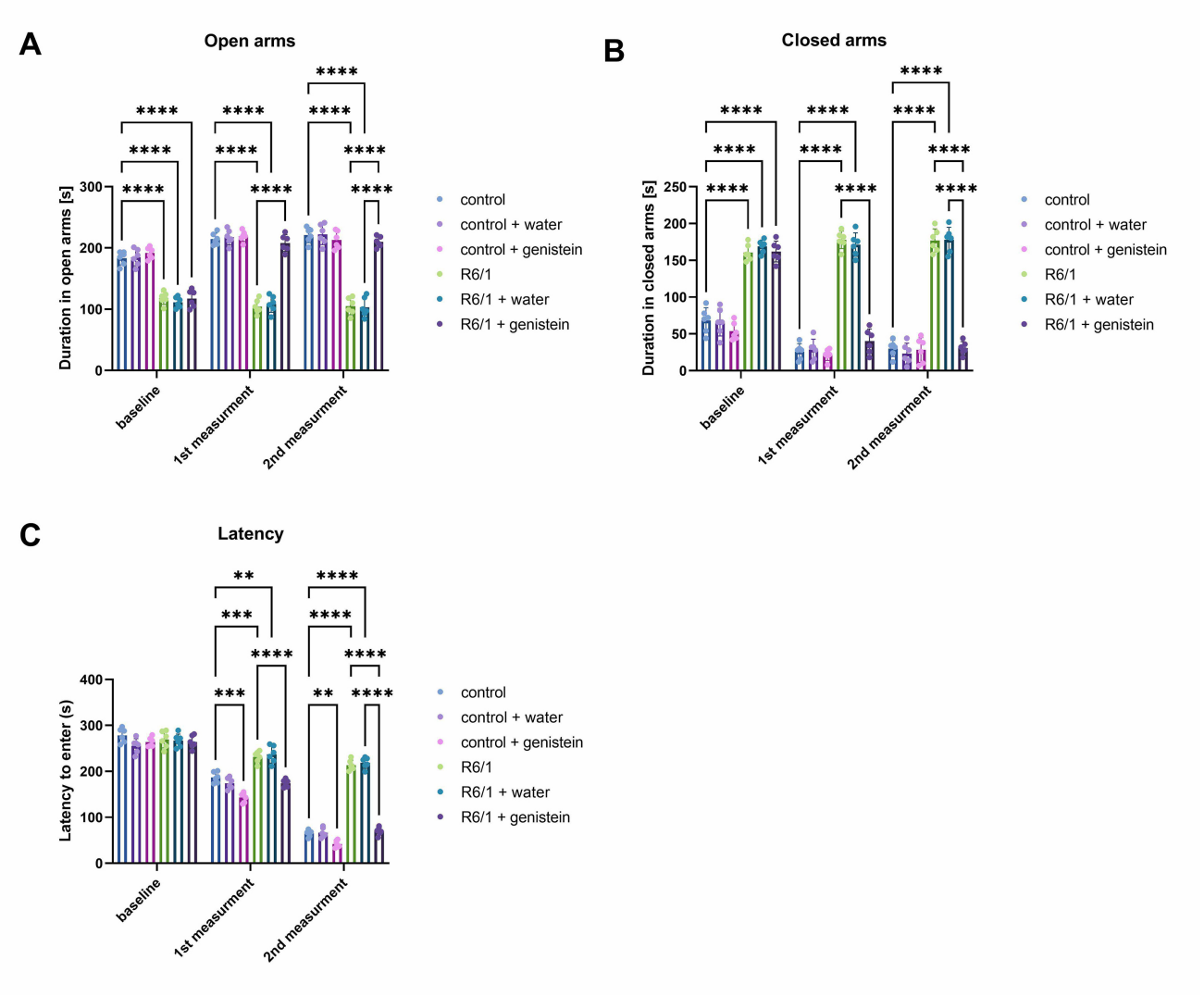

Fig. 2. Correction of anxiety-related disturbances in HD female mice by genistein. HD female mice (the R6/1 model) or control animals (the C57BL/6J line) were either untreated, treated with water (+ water), or treated with genistein (at the final dose of 150 mg/kg/day, starting at the age of 16 weeks; + genistein). Elevated maze test was performed with mice at the age of 16, 22 and 30 weeks (marked as baseline, 1st measurement, and 2nd measurement, respectively). Following parameters were analyzed: (A) time spent in open arms; (B) time spent in closed arms; (C) latency time. Results are shown as mean values from measurements performed with 6 mice in each group with error bars indicating SD, and all measured values are indicated by dots. Statistically significant differences between indicated groups (in two-way ANOVA with Tukey's post hoc test (A,B) or Kruskal-Wallis with Dunn's post hoc test (C)) are shown as follows: **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

2.11 Availability of Data and Materials

All data are presented in the manuscript. Datasheets with raw results are available from the corresponding author on reasonable requests from professional researchers.

3. Results

In this study, female R6/1 mice, a transgenic model of Huntington's disease (HD), and age-matched control C57BL/6J mice were examined. R6/1 animals carry the first exon of the human *HTT* gene with 116–120 CAG repeats integrated into their genome. This model has been extensively characterized, demonstrating that a combination of behavioral assessments and blood-based analyses closely recapitulates HD-related symptoms and provides a reliable means to monitor disease progression [17]. In female R6/1 mice, initial HD-like symptoms typically emerge at approximately 14 weeks of age. Accordingly, in the present experiments assessing the therapeutic effects of genistein, supplementation (150 mg/kg/day) commenced at 16 weeks, while control animals received water; data collected at this point are referred to as "baseline". Oral administration of genistein or water continued daily throughout the study. Subsequent evaluations were conducted at 22 weeks ("1st

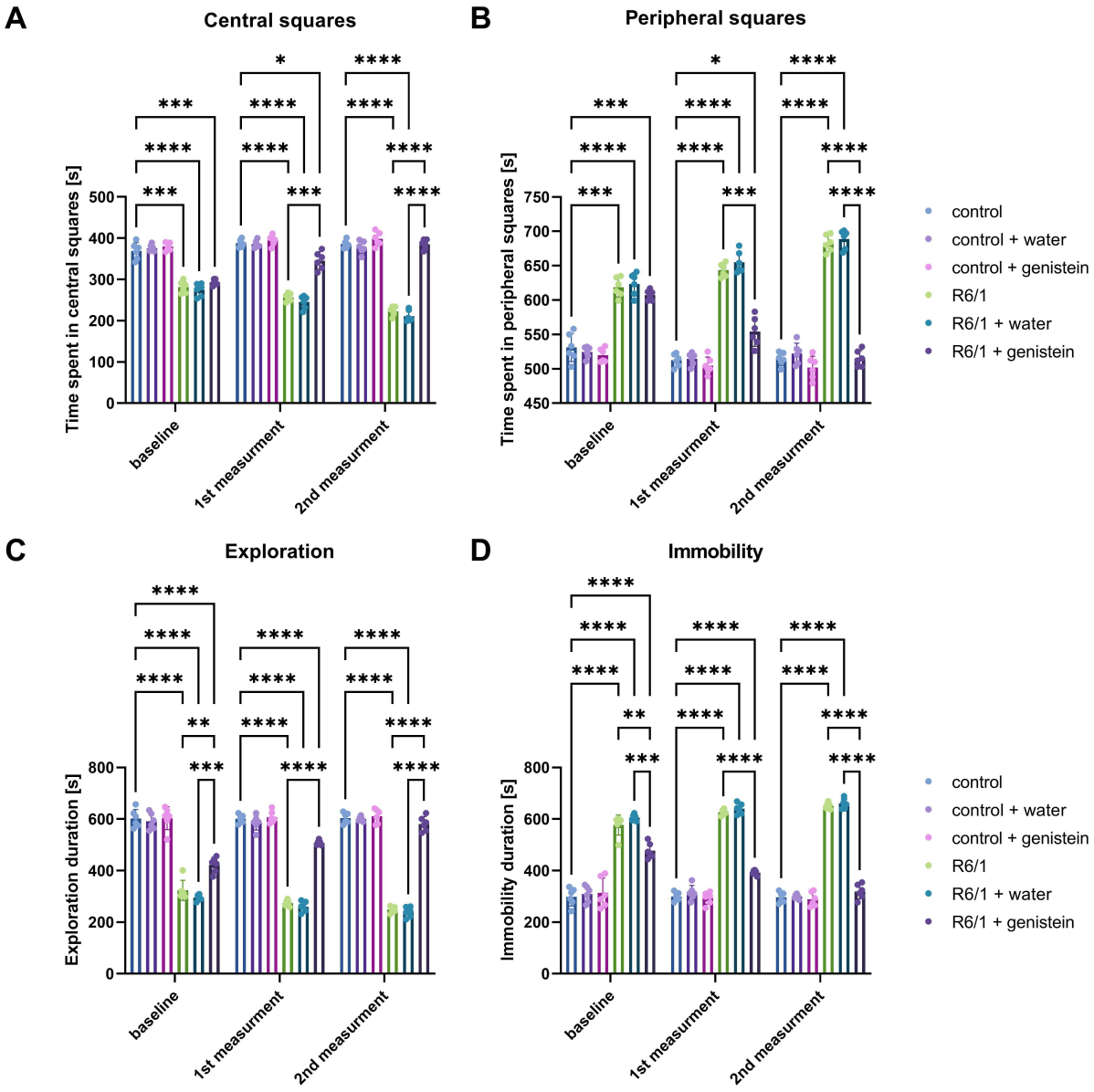

Fig. 3. Stress responsiveness in HD female mice is reduced by genistein. HD female mice (the R6/1 model) or control animals (the C57BL/6J line) were either untreated, treated with water (+ water), or treated with genistein (at the final dose of 150 mg/kg/day, starting at the age of 16 weeks; + genistein). Open field test was performed with mice at the age of 16, 22 and 30 weeks (marked as baseline, 1st measurement, and 2nd measurement, respectively). Following parameters were analyzed: (A) time spend in central squares; (B) time spend in peripheral squares; (C) exploration time; (D) immobility time. Results are shown as mean values from measurements performed with 6 mice in each group with error bars indicating SD, and all measured values are indicated by dots. Statistically significant differences between indicated groups (in Kruskal-Wallis with Dunn's post hoc test (A,B) or two-way ANOVA with Tukey's post hoc test (C,D)) are shown as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

measurement") and 30 weeks ("2nd measurement"). These time points were slightly adjusted from those in previous studies in R6/1 males [15] to better capture potential sex-specific effects. To assess any putative effects of the administration procedure, additional controls of untreated R6/1 and C57BL/6J female mice were included.

Motor coordination deficits are among the most prominent symptoms of HD, including in the R6/1 mouse model. Accordingly, motor performance was evaluated using the Rota-rod test. At baseline, no statistically significant differences in the ability to maintain balance on the rod were observed between female HD mice and controls (Fig. 1). Marked motor impairment became apparent in

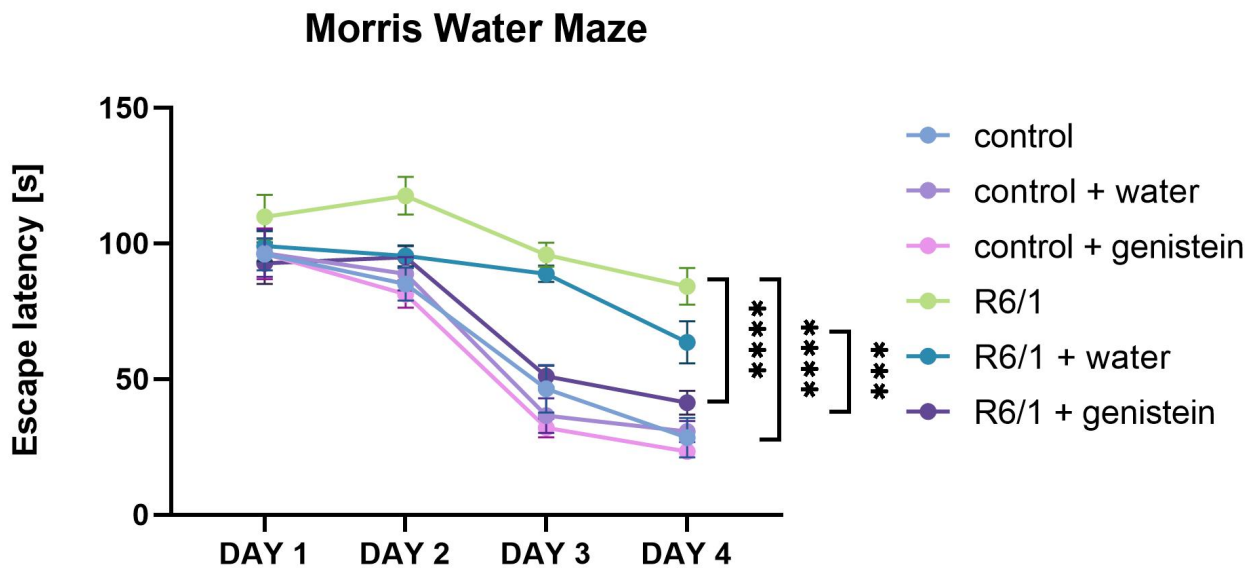

Fig. 4. Spatial memory deficits are corrected by genistein in HD female mice. HD female mice (the R6/1 model) or control animals (the C57BL/6J line) were either untreated, treated with water (+ water), or treated with genistein (at the final dose of 150 mg/kg/day, starting at the age of 16 weeks; + genistein). Morris water maze test was performed with mice at the age of 30 weeks. Results are shown as mean values from measurements performed with 6 mice in each group with error bars indicating SD. Statistically significant differences between indicated groups (in Kruskal-Wallis with Dunn's post hoc test) are shown as follows: ***, $p < 0.001$; ****, $p < 0.0001$.

untreated and water-treated R6/1 mice at the 1st and 2nd measurements (22 and 30 weeks of age, respectively) compared with control animals. In contrast, R6/1 females receiving genistein were able to remain on the rotating rod significantly longer than untreated HD mice, although their performance did not fully reach the level of healthy controls (Fig. 1). These findings suggest that early post-symptomatic administration of genistein can partially ameliorate the motor deficits characteristic of HD in female R6/1 mice.

Anxiety-related disturbances represent another feature of HD, occurring also in the R6/1 mouse model [17]. To evaluate these behaviors, the elevated-plus maze test was employed. In this assay, open arms represent anxiety-provoking, aversive conditions, while closed arms provide a perceived safe environment; the natural reluctance of mice to explore the open arms is quantified as transfer latency from open to closed arms. At baseline, no significant differences were observed between all experimental groups. However, at the 1st measurement, untreated and water-treated R6/1 females displayed significantly longer transfer latencies compared with control mice and R6/1 animals treated with genistein, whereas the latter two groups did not differ significantly. By the 2nd measurement, R6/1 mice receiving genistein exhibited transfer latencies comparable to controls and significantly shorter than those in untreated or water-treated HD females (Fig. 2). Analysis of the time spent in open versus closed arms revealed that R6/1 mice differed from C57BL/6J controls already at baseline, and this difference persisted throughout the study. Importantly, genistein treatment fully normalized these behavioral parameters at both the 1st and 2nd measurements (Fig. 2). These results indicate that oral administration of genistein, initiated after the onset of HD symptoms, effectively corrected anxiety-related behavioral disturbances as measured in the elevated-plus maze.

Stress responsiveness in both control and R6/1 mice, with or without genistein treatment, was assessed using the open-field test. This assay evaluates stress-related behavior by exposing animals to an open, brightly lit arena, which is inherently aversive to rodents. The primary parameter measured was the time spent in the central squares during a 15-minute session, with assessments conducted at baseline (16 weeks, corresponding to the start of genistein treatment) and at 22 and 30 weeks of age (1st and 2nd measurements, respectively). As shown in Fig. 3, genistein treatment improved behavioral responses in R6/1 females, which otherwise displayed altered stress sensitivity. By 30 weeks of age, genistein-treated HD females performed similarly to wild-type controls in this test. These findings were further supported by analyses of exploratory activity and immobility duration (Fig. 3). Collectively, the results indicate that genistein administration effectively normalized stress-related behavioral disturbances in female R6/1 mice.

Spatial memory deficits, previously reported in the R6/1 mouse model of HD [17], were evaluated using the

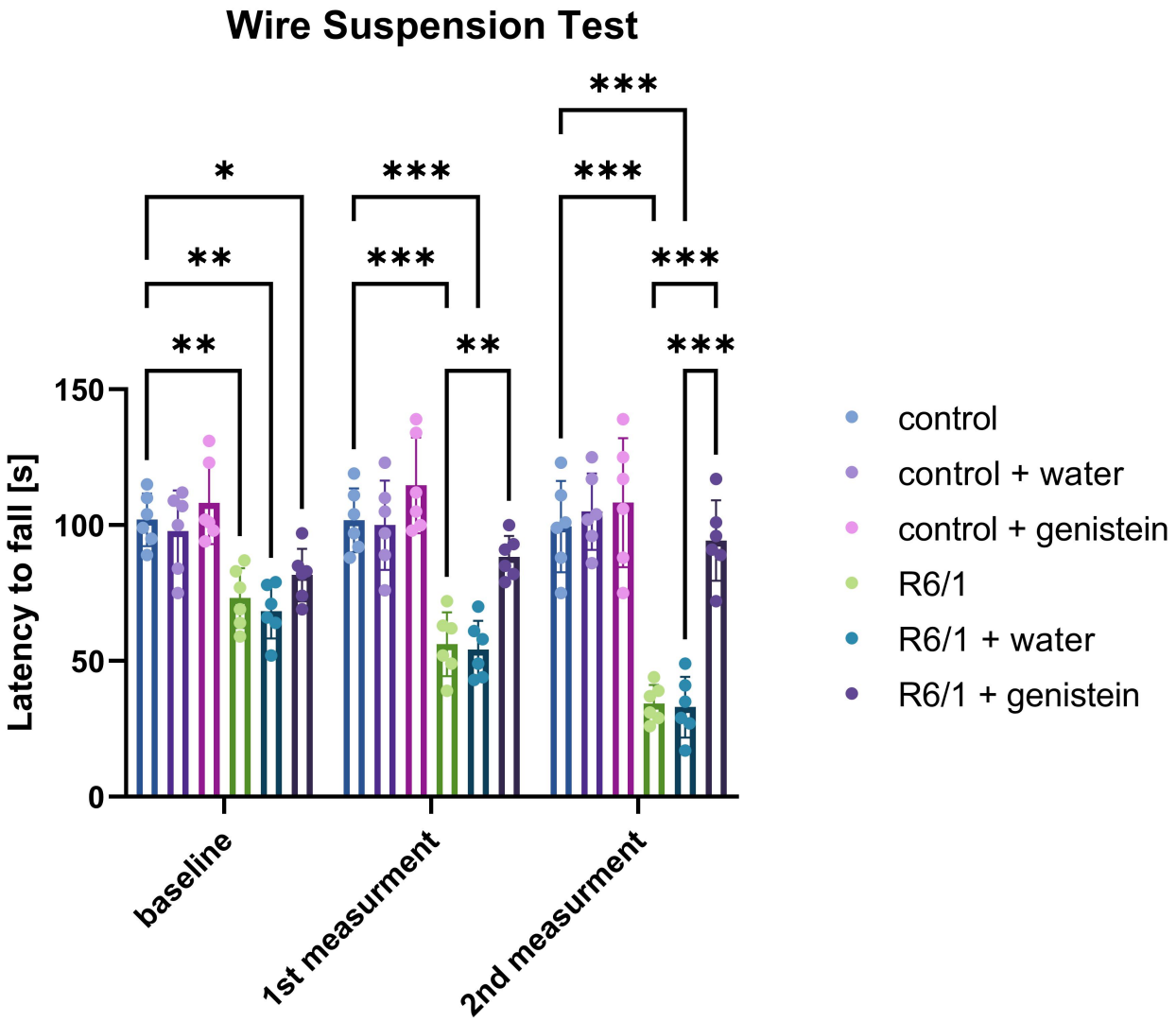

Fig. 5. Genistein improves neuromuscular functions in HD female mice. HD female mice (the R6/1 model) or control animals (the C57BL/6J line) were either untreated, treated with water (+ water), or treated with genistein (at the final dose of 150 mg/kg/day, starting at the age of 16 weeks; + genistein). Wire suspension test was performed with mice at the age of 16, 22 and 30 weeks (marked as baseline, 1st measurement, and 2nd measurement, respectively). Results are shown as mean values from measurements performed with 6 mice in each group with error bars indicating SD, and all measured values are indicated by dots. Statistically significant differences between indicated groups (in two-way ANOVA with Tukey's post hoc test) are shown as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Morris water maze. Latency to reach the hidden platform was recorded across consecutive days to assess the animals' ability to learn and recall the platform's location. Data collected at 30 weeks of age are presented in Fig. 4. Water-treated and untreated R6/1 mice exhibited significantly longer latencies during the learning and memory phases compared with control animals, whereas genistein administration reduced these impairments. One should consider the potential influence of motor dysfunction on the results obtained in this test, assuming that deficits in platform finding platforms might be partially due to slower movement in HD animals. However, previous studies demonstrated that there is no significant difference in the speed of swimming between wild-type and R6/1 mice at about 6 months of age [15,17]. Therefore, results reported here indicate that genistein treatment partially ameliorates spatial memory deficits in female HD mice.

Given that HD affects neuromuscular function, assessment of muscle strength was critical for evaluating symptom severity in genistein-treated, water-treated, and untreated R6/1 females, as well as in corresponding wild-type controls. Using the wire-handling test, the duration that mice remained on the rod was recorded. Genistein treatment significantly improved muscle strength, with the most

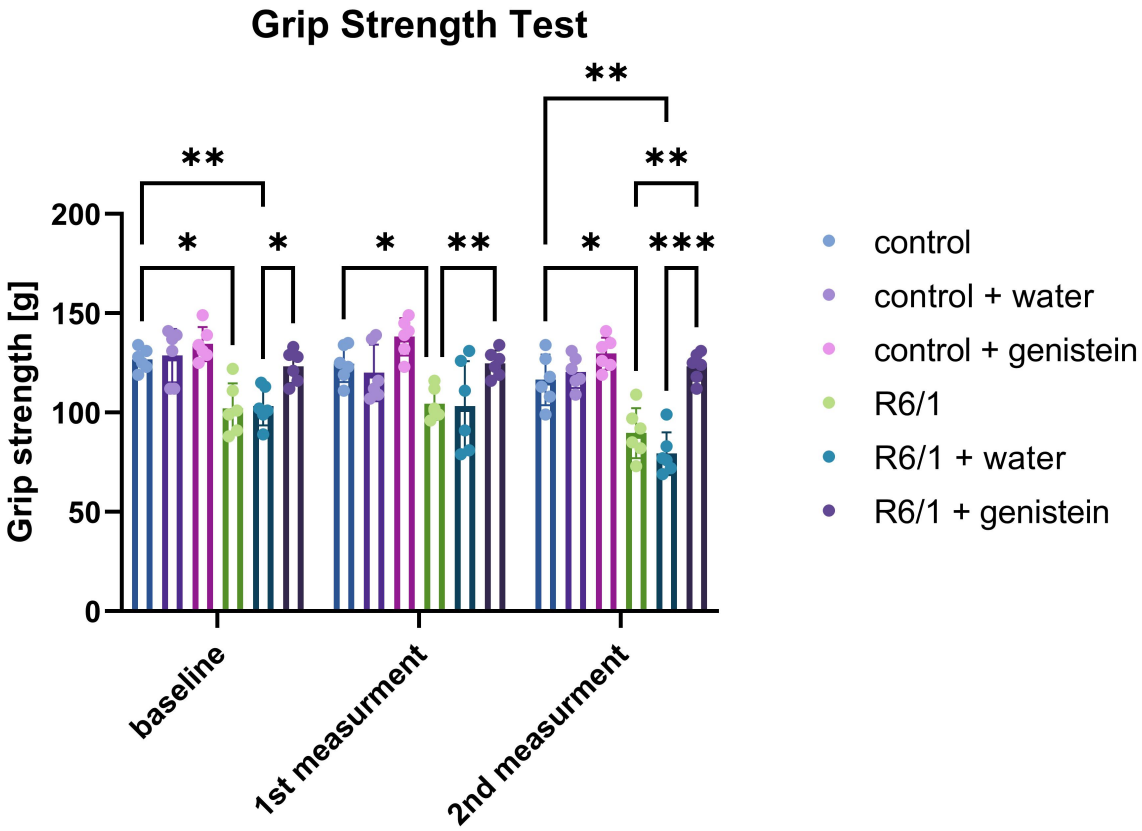

Fig. 6. Muscle strength is improved in HD female mice after genistein treatment. HD female mice (the R6/1 model) or control animals (the C57BL/6J line) were either untreated, treated with water (+ water), or treated with genistein (at the final dose of 150 mg/kg/day, starting at the age of 16 weeks; + genistein). Grip strength test was performed with mice at the age of 16, 22 and 30 weeks (marked as baseline, 1st measurement, and 2nd measurement, respectively). Results are shown as mean values from measurements performed with 6 mice in each group with error bars indicating SD, and all measured values are indicated by dots. Statistically significant differences between indicated groups (in two-way ANOVA with Tukey's post hoc test) are shown as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

pronounced effect observed at the 2nd measurement, when performance of R6/1 females approached that of wild-type animals (Fig. 5). Comparable results were obtained using a grip-force meter to quantify hindlimb grip strength, further confirming the beneficial effect of genistein (Fig. 6).

Creatine kinase, an indicator of skeletal muscle damage, was measured in blood to assess muscle integrity. Elevated enzyme levels reflect muscle deterioration. As shown in Fig. 7, water-treated and untreated R6/1 mice exhibited significantly higher creatine kinase levels compared with controls. In contrast, genistein administration effectively protected muscle tissue in HD females, as evidenced by significantly reduced enzyme levels. Consistent findings were observed for reactive oxygen species (ROS), where genistein treatment normalized ROS levels in female R6/1 mice (Fig. 8). Together, these results support the protective effects of genistein, especially on muscle health, in female HD animals.

Since levels of hormones are parameters that significantly distinguish males and females biochemically, and because hormonal abnormalities have been previously reported in HD mice [15,17], the concentrations of selected hormones (based on results from those reports) were determined in plasma samples derived from female R6/1 mice. Levels of adrenocorticotropic hormone (ACTH) were significantly increased in water-treated and untreated R6/1 females relative to wild-type mice, while treatment with genistein improved (week 22) and then normalized (week 30) this parameter (Fig. 9). Corticosterone levels were also significantly improved in HD female animals after genistein administration (Fig. 10). These results indicate improvement in global regulatory processes at the organismal level. Importantly, estradiol levels, otherwise considerably less abundant in R6/1 females relative to wild-type females, were evidently normalized by treatment with genistein (Fig. 11). As expected, phytoestrogenic effects were also visible in wild-type animals treated with this isoflavone (Fig. 11).

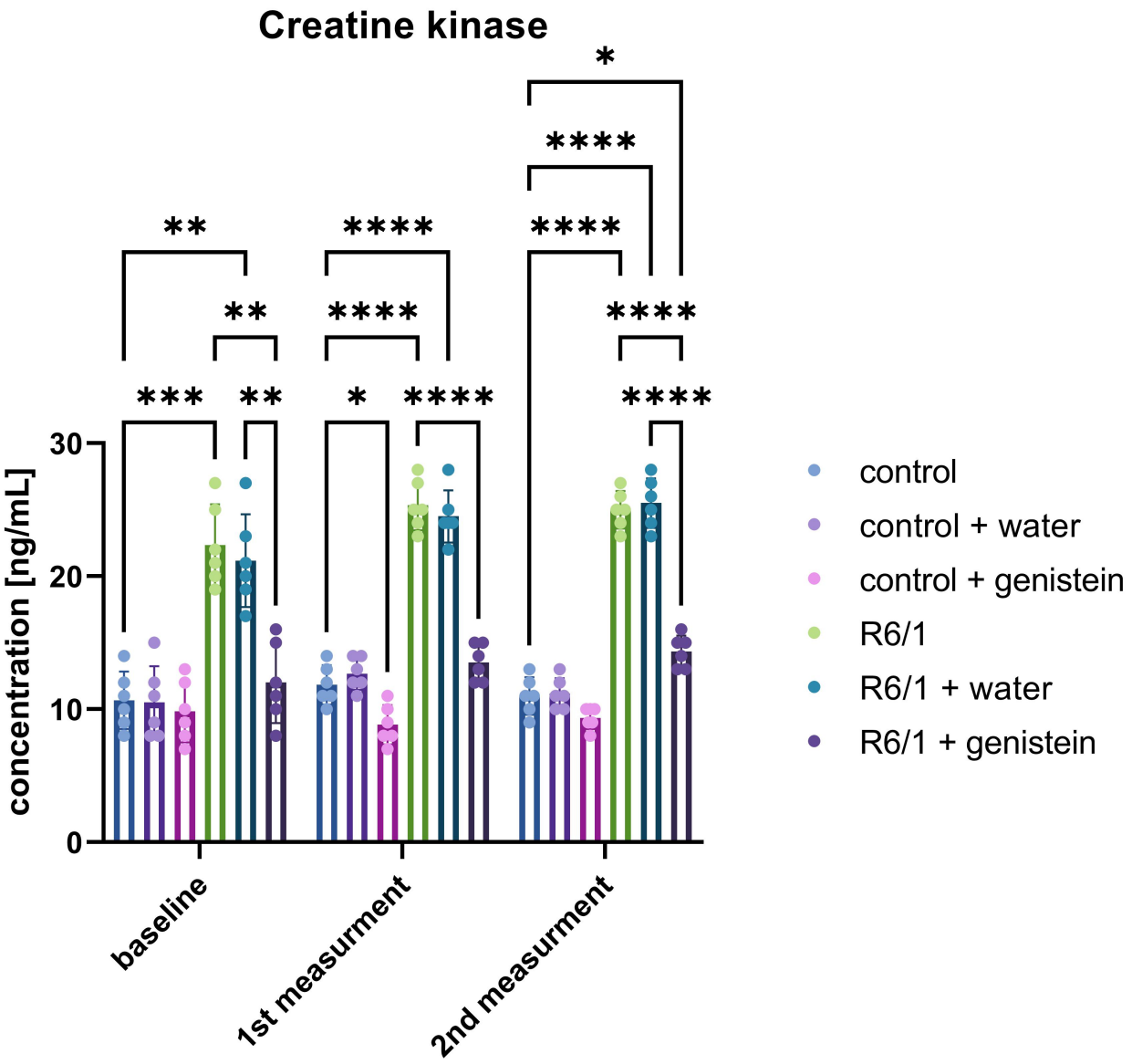

Fig. 7. Genistein reduces creatine kinase levels in plasma of HD female mice. HD female mice (the R6/1 model) or control animals (the C57BL/6J line) were either untreated, treated with water (+ water), or treated with genistein (at the final dose of 150 mg/kg/day, starting at the age of 16 weeks; + genistein). Estimation of creatine kinase levels was performed with mice at the age of 16, 22 and 30 weeks (marked as baseline, 1st measurement, and 2nd measurement, respectively). Results are shown as mean values from measurements performed with 6 mice in each group with error bars indicating SD, and all measured values are indicated by dots. Statistically significant differences between indicated groups (in two-way ANOVA with Tukey's post hoc test) are shown as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

4. Discussion

HD is a severe neurodegenerative and neuromuscular disorder for which no effective therapy currently exists [2]. This highlights the urgent need to develop new approaches capable of mitigating disease progression. Among various strategies, stimulation of autophagy has emerged as a promising avenue, as it can promote the degradation of already-formed mutant huntingtin aggregates or prevent their accumulation [6,7,8,9,10]. Recent studies suggested that the delayed onset of HD symptoms in patients may be due to the requirement for extensive somatic expansion of CAG repeats (approximately 150 copies) in exon 1 of the *HTT* gene. This process occurs over decades in humans [3,4]. This implies that toxic aggregates of mutant huntingtin accumulate predominantly in neurons in adulthood, and that interventions targeting their clearance, such as autophagy activation, could be particularly effective.

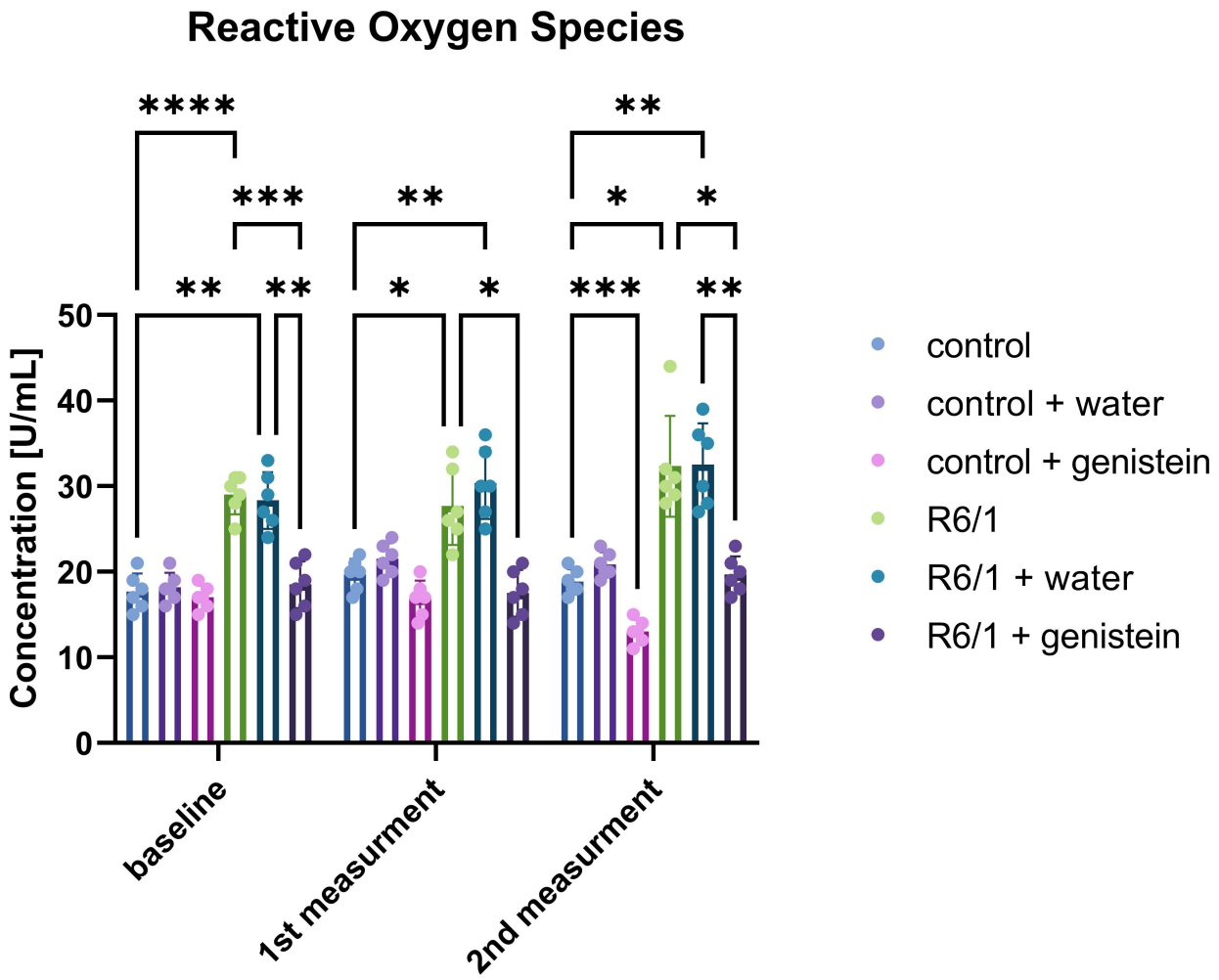

Fig. 8. Genistein reduces oxidative stress in HD female mice. HD female mice (the R6/1 model) or control animals (the C57BL/6J line) were either untreated, treated with water (+ water), or treated with genistein (at the final dose of 150 mg/kg/day, starting at the age of 16 weeks; + genistein). Concentrations of ROS were measured at the age of 16, 22 and 30 weeks (marked as baseline, 1st measurement, and 2nd measurement, respectively). Results are shown as mean values from measurements performed with 6 mice in each group with error bars indicating SD, and all measured values are indicated by dots. Statistically significant differences between indicated groups (in two-way ANOVA with Tukey's post hoc test) are shown as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

In this context, genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) has been shown to effectively induce autophagy via a FOXO3-dependent pathway, resulting in efficient elimination of huntingtin aggregates [11,12,15]. This compound is orally bioavailable, can cross the blood-brain barrier, and has been demonstrated to be safe in humans even at high doses (up to 150 mg/kg/day) over prolonged periods (more than 12 months) [14,15], supporting its potential as a therapeutic agent for HD. Previous studies in R6/1 mice, however, focused exclusively on post-symptomatic males [15], a choice made to simplify interpretation of the experimental results due to hormonal fluctuations and earlier symptom onset in females [17]. Given that HD affects males and females equally in humans [1,2], it is essential to investigate whether genistein is also effective in females when administered shortly after the first disease manifestations. The present study addresses this gap.

Our results demonstrated that genistein administered post-symptomatically can significantly ameliorate HD-related impairments in female R6/1 mice. Behavioral improvements were observed across multiple domains, including motor coordination, anxiety, stress sensitivity, spatial memory, and general locomotor activity. In addition, genistein positively influenced neuromuscular parameters, such as muscle strength, and reduced biochemical markers of muscle damage and oxidative stress. Hormonal levels, which are often disrupted in HD, were also normalized or significantly improved following genistein treatment. Importantly, the effects of genistein were more pronounced

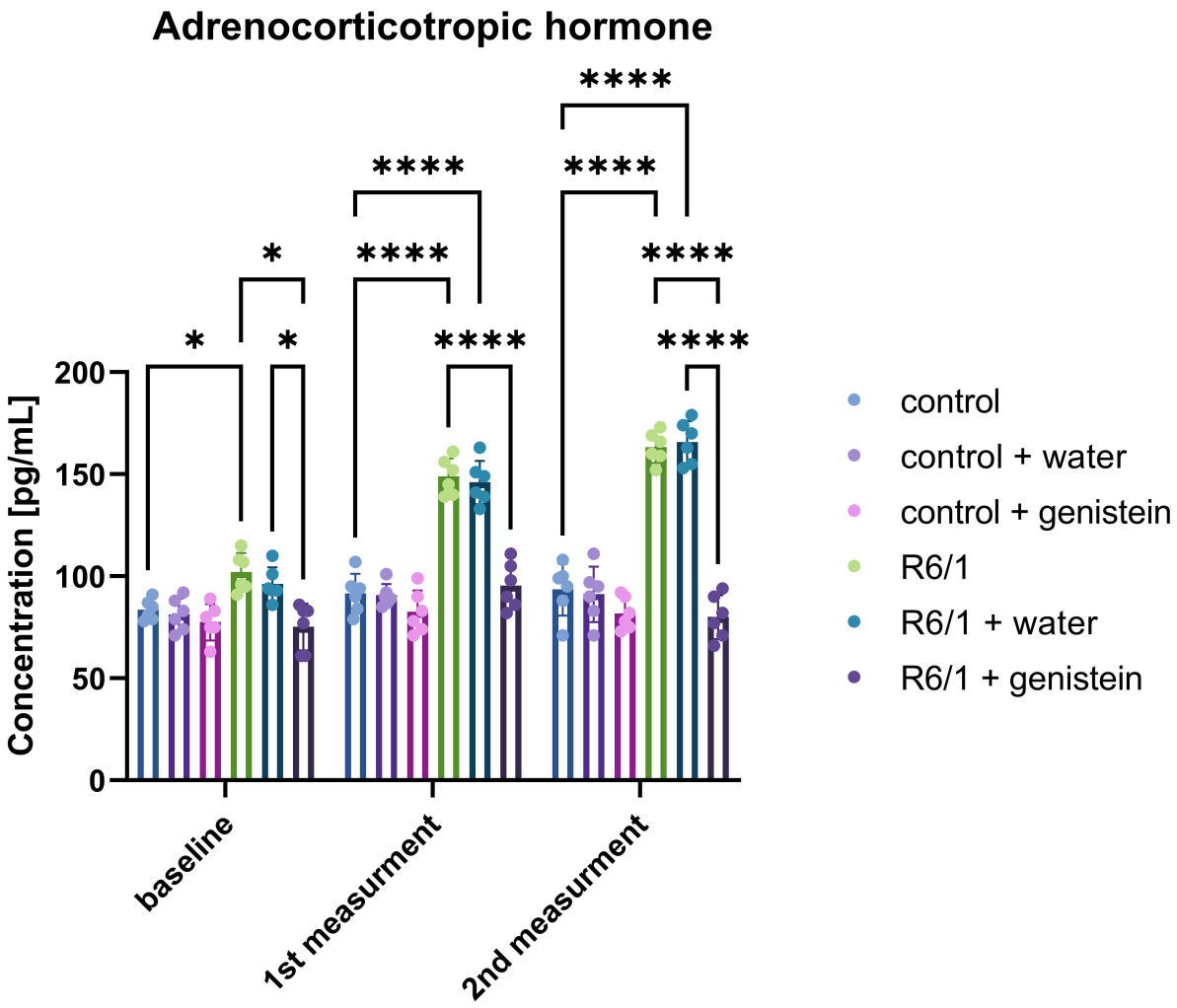

Fig. 9. Genistein normalizes adrenocorticotropic hormone level in HD female mice. HD female mice (the R6/1 model) or control animals (the C57BL/6J line) were either untreated, treated with water (+ water), or treated with genistein (at the final dose of 150 mg/kg/day, starting at the age of 16 weeks; + genistein). Levels of adrenocorticotropic hormone were determined at the age of 16, 22 and 30 weeks (marked as baseline, 1st measurement, and 2nd measurement, respectively). Results are shown as mean values from measurements performed with 6 mice in each group with error bars indicating SD, and all measured values are indicated by dots. Statistically significant differences between indicated groups (in two-way ANOVA with Tukey's post hoc test) are shown as follows: *, $p < 0.05$; ****, $p < 0.0001$.

when HD animals were treated longer; namely, results obtained at the second measurement (at the age of 30 weeks) were more similar to those of wild-type animals and revealed more pronounced differences relative to untreated R6/1 mice than those obtained at the first measurement (at the age of 22 weeks). Considering that the treatment was started at 16 weeks of age, when the HD symptoms already existed, these results demonstrate not only the efficacy of genistein, but also its long-term and continuing effects of the treatment.

In a previous study, the effects of genistein in improving various deficits in HD appeared more pronounced in males of the R6/1 mouse model [15]; nevertheless, females still exhibited substantial improvements. For example, while genistein fully restored performance in males in the Rota-rod and open-field tests [15], female R6/1 mice showed partial, but highly significant, enhancement in the same tasks (this report). Conversely, the estrous cycle in females could modulate the effects of the treatment on some behaviors and levels of hormones, thus such a comparison should be made carefully. Definitely, a direct comparison of efficacy between sexes would require testing both males and females side-by-side within the same experimental setup, optimally with synchronization of the estrous cycle in females.

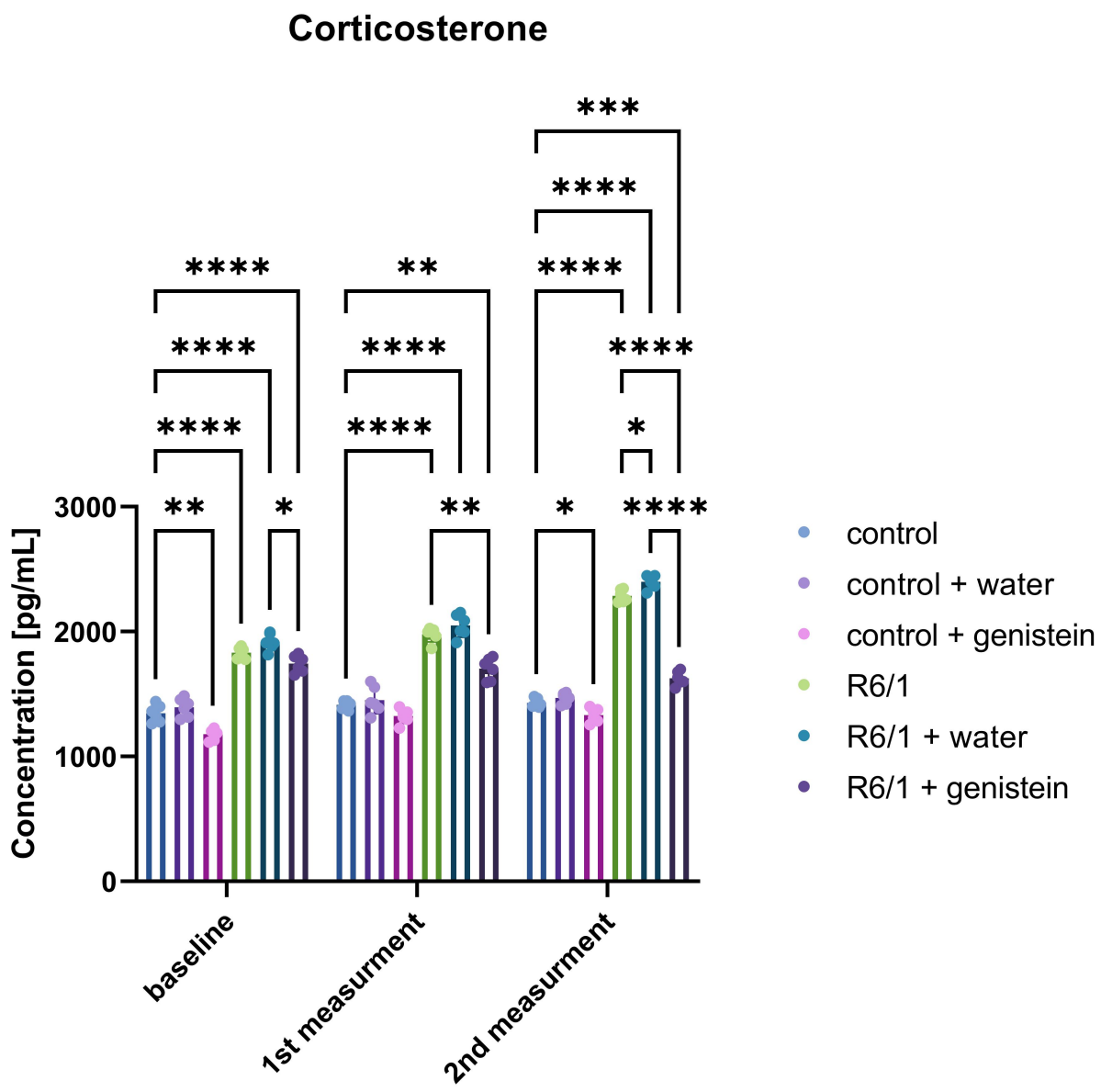

Fig. 10. Improvement of corticosterone levels in HD female mice after genistein treatment. HD female mice (the R6/1 model) or control animals (the C57BL/6J line) were either untreated, treated with water (+ water), or treated with genistein (at the final dose of 150 mg/kg/day, starting at the age of 16 weeks; + genistein). Corticosterone concentration was determined at the age of 16, 22 and 30 weeks (marked as baseline, 1st measurement, and 2nd measurement, respectively). Results are shown as mean values from measurements performed with 6 mice in each group with error bars indicating SD, and all measured values are indicated by dots. Statistically significant differences between indicated groups (in two-way ANOVA with Tukey's post hoc test) are shown as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Previous studies strongly suggested that the major mechanism of genistein-mediated improvement of cellular, biochemical, and behavioral parameters in cellular and animal models of HD is related to stimulation of the autophagy process [11,12,15]. However, genistein has also phytoestrogenic properties [18]. Indeed, as expected, the phytoestrogenic effects of genistein were also evident in this study, being particularly evident in control (wild-type) animals (Fig. 11). Therefore, one might assume that the observed effects of genistein on HD females are related not only to autophagy activation, but also to stimulation of estrogen receptors by this isoflavone. If this is true, the molecular mechanisms underlying genistein-mediated improvement of HD symptoms might be more complex than simply affecting a single cellular process.

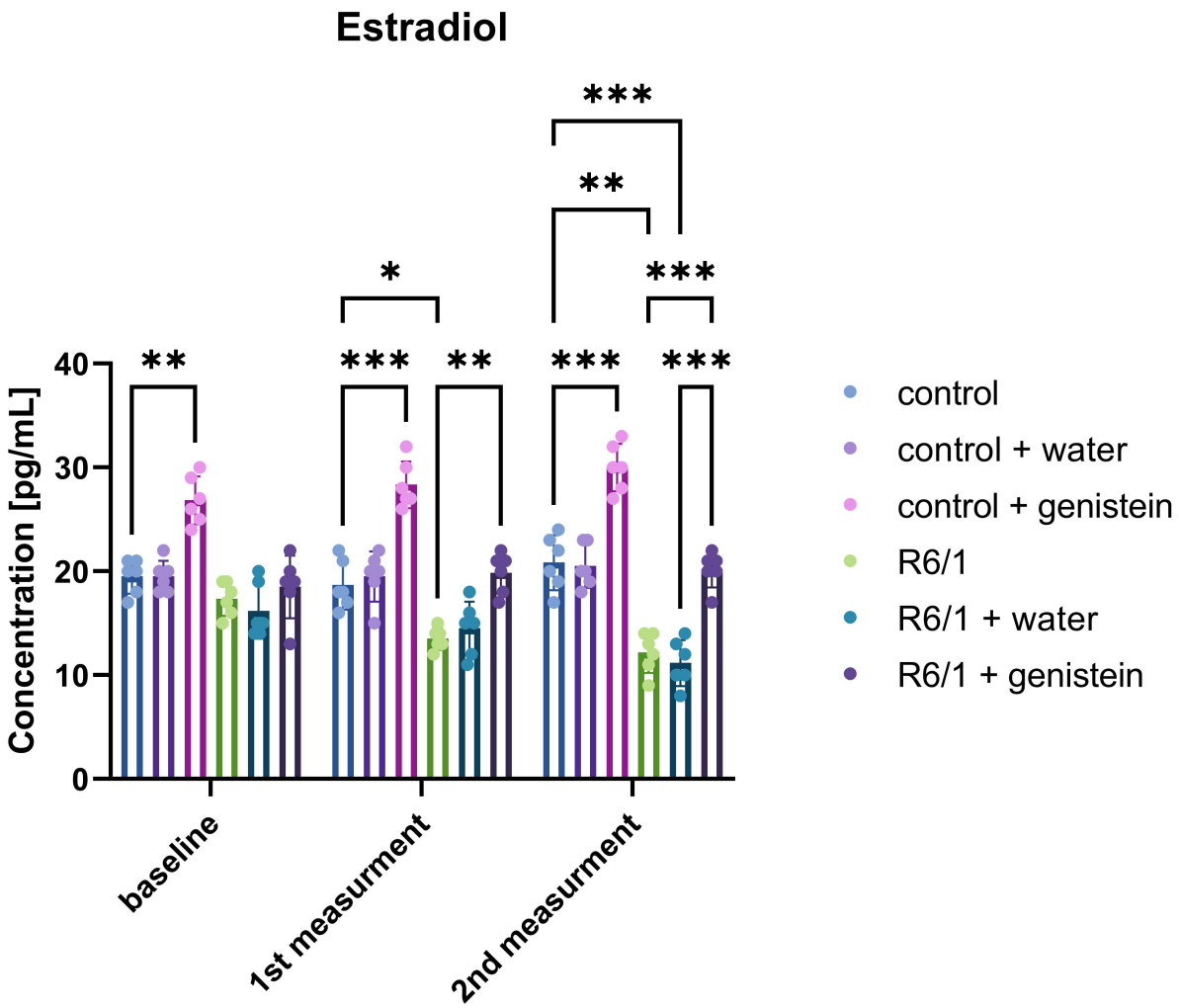

Fig. 11. Normalization of estradiol levels in HD female mice after genistein treatment. HD female mice (the R6/1 model) or control animals (the C57BL/6J line) were either untreated, treated with water (+ water), or treated with genistein (at the final dose of 150 mg/kg/day, starting at the age of 16 weeks; + genistein). Levels of estradiol were measured at the age of 16, 22 and 30 weeks (marked as baseline, 1st measurement, and 2nd measurement, respectively). Results are shown as mean values from measurements performed with 6 mice in each group with error bars indicating SD, and all measured values are indicated by dots. Statistically significant differences between indicated groups (in two-way ANOVA with Tukey's post hoc test) are shown as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

These findings reinforce the potential of genistein as a therapeutic candidate for HD, highlighting its efficacy in both male and female R6/1 mice. They provide a strong rationale for further preclinical and clinical investigations to determine whether similar benefits can be achieved in humans. While interspecies differences in anatomy and physiology limit direct translation from mouse models to patients, genistein has also demonstrated efficacy in human cell cultures *in vitro* [11,12], supporting the principle that this isoflavone may counteract HD pathology across species. It is worth mentioning that other potential anti-HD drugs were previously tested in the R6/1 mouse model. However, little or no effects of cannabinoid agonists and an endocannabinoid metabolism inhibitor could be observed [19]. More promising results were obtained when sildenafil, acting as an inhibitor of phosphodiesterase 5, was used to increase cGMP levels in R6/1 mice; this was accompanied by improved memory in these animals, as assessed in behavioral tests (new object recognition and passive avoidance tests) [20]. Irrespective of the efficacy of the tested compounds, these studies, along with this report, confirm that the R6/1 mouse line can be a proper model for testing various potential anti-HD drugs.

The dose of genistein equal to 150 mg/kg/day, administered orally, was used in this study. The choice of this dose was based on previous reports which demonstrated

that due to effective but limited (efficiency of about 10%) penetrance of the blood-brain-barrier, orally-administered genistein reveals its biological activities in murine models of neurodegenerative diseases at the dose of 15 mg/kg/day in visceral organs [21], however, about 10-times higher dose is required for the brain treatment [15,22]. Importantly, similar doses of genistein were employed in humans, demonstrating in clinical studies that such amounts of genistein are safe for patients [14] and reveal biochemical effects [23] in another neurodegenerative disease (mucopolysaccharidosis type III).

In summary, this study demonstrated that genistein, when administered shortly after symptom onset, effectively mitigated behavioral, neuromuscular, and biochemical deficits in female HD mice. In line with previous studies in males [15], these results suggest that genistein may be a potential candidate for HD therapy. Such a suggestion can be corroborated by recent analyses indicating that polyphenols (such as genistein) might be beneficial in the treatment of neurodegenerative diseases [24,25].

5. Limitations

This work includes some limitations. The most important one is that this study was focused on female mice, and it is obvious that the female estrous cycle can influence anxiety-related behaviors and hormone levels. Because of the importance of the time-course of the experiments (the requirement of performing tests at specific ages of mice to obtain comparable results), the estrous cycle was not synchronized. The variability of the cycle was also not incorporated as a covariate in the statistical analysis, due to the limited number of animals. Therefore, one should consider that the presented results may be affected by the variability in the stages of the estrous cycle in the investigated animals, possibly limiting the efficacy of genistein in correcting both behavioral and biochemical parameters.

Very recent reports indicated that the R6/1 mouse model of HD also reveals neuro-inflammatory disorders and depression-like behaviors [26,27]. These parameters were not tested in this study; thus, one might consider performing complementary research in the future to assess also the effects of genistein on neuroinflammation and depression-like symptoms in this animal HD model.

The molecular mechanism of genistein-mediated improvement in the R6/1 mouse model of HD, based on FOXO3-dependent activation of autophagy, was previously demonstrated only in males [15]. Here, we did not measure the effects of FOXO3 or estimate levels of autophagy markers, such as LC3-II or p62. Therefore, the autophagy-related mechanism of action of genistein in female R6/1 mice is likely, but still somewhat speculative in the absence of such measurements. The same concerns histopathological and immunohistochemical analyses, which were performed in the previous study with R6/1 males [15], but not in this study.

Finally, the R6/1 mouse line is an established and useful model of HD [16,17]. However, it cannot fully reflect the human disease. Differences in anatomy and physiology between mice and humans are obvious [28], hence, one should consider that results obtained with murine models of human diseases, including HD, cannot be simply extrapolated to patients suffering from such diseases. Furthermore, R6/1 mice are transgenic animals bearing exon 1 of the human *HTT* gene with 116–120 CAG repeats [16]. Therefore, this genetic defect, despite mimicking the human disease and mimicking its symptoms, is still different from those occurring in HD patients, suggesting additional caution when interpreting the results of experiments in light of their translational potential.

6. Conclusions

Post-symptomatic genistein administration (at the dose of 150 mg/kg/day) significantly improved behavioral, neuromuscular, and biochemical deficits in female HD mice. Together with previously reported evidence of the efficacy of this compound in male HD animals, these findings support further preclinical and translational studies toward testing genistein as a potential anti-HD drug.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding authors on reasonable request.

Author Contributions

Conceptualization, KP and GW; methodology, LG, ER, DM, MP; formal analysis, LG, ER, DM, MP, KP and GW; investigation, LG, ER, MP; resources, KP and GW; data curation, LG, ER, DM, MP; writing—original draft preparation, LG, and GW; writing—review and editing, LG, ER, DM, MP, KP and GW; visualization, LG, ER; supervision, KP and GW; project administration, KP and GW; funding acquisition, KP and GW. All authors have read and agreed to the published version of the manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Experiments with animals were approved by the Local Ethics Committee for the Care and Use of Laboratory Animals in Bydgoszcz (approval no. 48/2021). The study was carried out in accordance with the ARRIVE guidelines. All procedures complied with the Polish Act on the Protection of Animals Used for Scientific or Educational Purposes (Journal of Laws, February 26, 2015) and adhered to European Commission guidelines regarding animal welfare in scientific research.

Acknowledgment

The authors thank Joanna Bart for administrative support.

Funding

This work was supported by the National Science Center, Poland (project grants no. 2017/25/N/NZ2/00812 to K.P. and 2020/37/B/NZ2/01050 to G.W.). E.R. is a holder of the START scholarship from Foundation for Polish Science.

Conflicts of Interest

The authors declare no conflicts of interest. Given his role as the Guest Editor, Grzegorz Węgrzyn had no involvement in the peer-review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Antoni Camins.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL51780.

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