

Knockdown and Overexpression of Nmnat in the Heart Improve Cardiac Function When Accompanied by Early Repeated Exercise in Aging *Drosophila*

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Abstract

Aim: To determine whether early exercise in the form of instinctive upward walking can mitigate the negative effects of cardiac aging in *Drosophila* with cardiac-specific Nmnat knockdown and overexpression.

Methods: Flies were given exercise periods of 2 h per day, 5 days a week for 3 weeks starting when they were 2 days old. The heart model was used in conjunction with M-mode echocardiography traces to analyze cardiac function. Exercise capacity was assessed using a climbing index, and the survival rate was calculated with respect to the age of each subject upon death.

Results: Cardiac-specific Nmnat knockdown severely compromised cardiac function with age by increased incidence of arrhythmias and decreased fractional with extreme dilation. The repeated exercise regimen was found to mitigate the deterioration of systolic function and rhythm caused by knockdown of myocardial Nmnat. Unexpectedly, hearts overexpressing Nmnat exhibited similar cardiac function to those of flies with normal expression after exercise training, and provided notable benefits with respect to lifespan and age-related locomotor decline.

Conclusions: Nmnat plays a critical role in maintaining cardiac function and that beginning a repeated exercise regimen later in life may improve cardiac health. It may provide a basis for further research in mammals.

Keywords: Aging; Cardiac rhythm; Cardiac systolic; Nicotinamide mononucleotide adenylyltransferase; Regular exercise

Introduction

Cardiac aging is the slow, progressive decline of heart function with age. It is a major risk factor for metabolic alterations and the development of cardiovascular disease, and it can worsen the quality of life of the elderly considerably. Regular exercise is broadly accepted as one way of reducing the incidence of cardiovascular disease and slowing down age-related declines in functional mobility in both rodents and humans [1]. In this regard, it is worth focusing on understanding the genetic and molecular mechanisms by which exercise affects the cardiac aging process. Recently, adult *Drosophila* hearts have come to be considered very suitable for investigations of cardiac function and aging [2]. In training paradigms, the short lifespan and genetic power of *Drosophila* model systems are highly advantageous. They can be controlled using a mechanized platform or slewing gear [3,4]. Trained flies can be used to examine several physiological effects, including alterations in energy metabolism reminiscent of those recorded in vertebrate models [3].

Nicotinamide mononucleotide adenylyltransferase (Nmnat) is the central enzyme in the NAD synthesis pathway, which is present in all organisms. It catalyzes both de novo and salvage pathways of NAD synthesis [5]. NAD⁺ plays an important role in maintaining the

intracellular redox state and controlling cell survival [6]. Exercise-training caused a marked increase in the NAD⁺/NADH ratio [7]. Recent yeast studies have indicated that NAD⁺ might extend the lifespan when associated with caloric restriction. This might help our understanding of the mechanisms involved in controlling age-related metabolic diseases in human [8]. One recent study reported that Nmnat2, one of the isoforms of Nmnat found in rats, prevents the incidence of cardiac hypertrophy [9]. *Drosophila* contains a single *Nmnat* gene, whose overexpression is associated with a delay in axonal degeneration [10]. However, surprisingly little is known about the effects of *Drosophila* Nmnat on cardiac function, which is known to respond to regular exercise.

Here, the role of Nmnat was investigated in a *Drosophila* heart model. First, the issue of whether cardiac-specific expression of Nmnat drastically compromises heart function was investigated. Second, Nmnat expression threshold in adult myocardial cells are required for regular exercise to induce changes to cardiac performance, here defined as climbing ability during aging, and for exercise training to affect lifespan.

Materials and Methods

Fly stocks and husbandry

P{KK101988}VIE-260B and $\gamma[1] w[*]; P\{w[+mC]=UAS-Nmnat.HA\}$ 2 flies for the *Nmnat* (CG13645) gene were obtained from the Vienna *Drosophila* Resource Center and Bloomington *Drosophila* Stock Center, respectively. W1118 and the cardiac-tissue specific Hand-Gal4 were a generous gift from Xiushan Wu of Center for Heart Development. Hand-Gal4 driver flies were crossed with the W1118, UAS-nmnat-RNAi line and UAS-nmnat overexpression line. Approximately 700 female F-1 progeny were collected from each cross and incubated at 25 and 50% humidity with a 12 h light/dark cycle. Flies were transferred to fresh food composed of 10% sucrose, 10% yeast, and 2% agar in water every third day and mortality was recorded after each transfer.

Exercise training device and protocols

Flies were exercised using an exercise device, which takes advantage of the flies' instinctive negative geotaxis characteristic to iteratively encourage climbing. Vials housing 20 flies each were loaded vertically into a wooden box that could be revolved around a horizontal axis [4]. The box was equipped with a motor and rotated repeatedly on a cyclical mode of "counterclockwise 180°-rest 15 s-clockwise 180°-rest 15 s" regulated by an intelligent controller. Thus, each 180° rotation caused the box to flip 180°, which caused flies to climb. These 2 day-old flies were exercised for 2 h five times per week for 3 weeks.

Semi-intact *Drosophila* heart preparation and image analysis

The hearts were exposed and analyzed using the methods described by Rolf Bodmer and Karen Ocorr [11-13]. Recording of semi-intact *Drosophila* heart contractions were recorded using HC Image software (Hamamatsu) with a high-speed EM-CCD camera (Hamamatsu) at rates of 100-200 frames/s. These videos were analyzed to quantify heart periods, arrhythmia indexes, systolic and diastolic diameters, and fractional shortening using Semi-automatic Optical Heartbeat Analysis software, which was kindly provided by Rolf Bodmer and Karen Ocorr. The videos were also used to generate M-mode records [11-13].

Negative geotaxis assay

A RING assay was performed on 100 flies from each group were used to assess age-specific mobility [14]. Flies were transferred into five empty narrow vials 18 cm in length which served as fly movement areas. These were loaded into a wood frame structure. The tubes were quickly tapped five times at intervals of 24 s to drive all the flies to the bottom of the tube, and the overall movement process of the flies was recorded using a digital camera (SONY, HDR-PJ580E). Five photographs per replicate were taken after flies had been allowed to climb for 5 s. For every photograph, each vial was divided into nine parts of equal height. Flies that reached the highest part of the grid were given a score of 9, flies in the next highest grid a score of 8, and so on. Flies that remained at the bottom of the vial were given a score of 0. The "climbing index" was the total points of each fly in that vial.

qRT-PCR

Total RNA was extracted from the hearts of 50 flies per group, each 7 days old using Trizol (Invitrogen). After treatment with DNaseI (Fermentas), first-strand cDNA was synthesized using SuperScript™

III Reverse Transcriptase System (Invitrogen). Quantitative PCR was carried out by staining with SYBR Green I using an ABI 7900HT (PE Bio systems). The expression of Rp49 was used for normalization. Primer sequences are listed below.

Nmnat-1 forward: 5'-CTCCGACCGAATGCGACTCT-3'

Nmnat-1 reverse: 5'-GGGCAAGTGGTGGGATTG-3'

rp49 forward: 5'-CTAAGCTGTCGCACAAATGG-3'

rp49 reverse: 5'-AACTTCTTGAATCCGGTGGG-3'

Statistical analysis

Data sets were subjected to Gaussian distributions using the D'Agostino and Pearson omnibus normality test. Data sets that passed this test were given a one-way ANOVA followed by a Tukey's multiple comparison post-hoc test. Data sets that did not show a normal distribution were estimated using the Dunn's multiple comparison post-hoc test when the Kruskal-Wallis test was significant (Tang et al.) [2]. Lifespan data were run through log rank tests and presented as survival curves. In all cases, P values below 0.05 were considered statistically significant. Statistical tests were performed using a GraphPad Prism (v.6.02).

Results

Nmnat gene expression in fly hearts

To corroborate the effect of Nmnat knockdown (KD) and overexpression (OE) in the cardiac muscle of *Drosophila*, Nmnat mRNA expression was measured in the hearts of unmodified control flies using qRT-PCR, which showed that unlike the Nmnat control seen at 1 week, Nmnat KD levels in the adult hearts were approximately 50% lower than in Nmnat controls at 1 week and OE RNA levels were 42% higher (Figure 1).

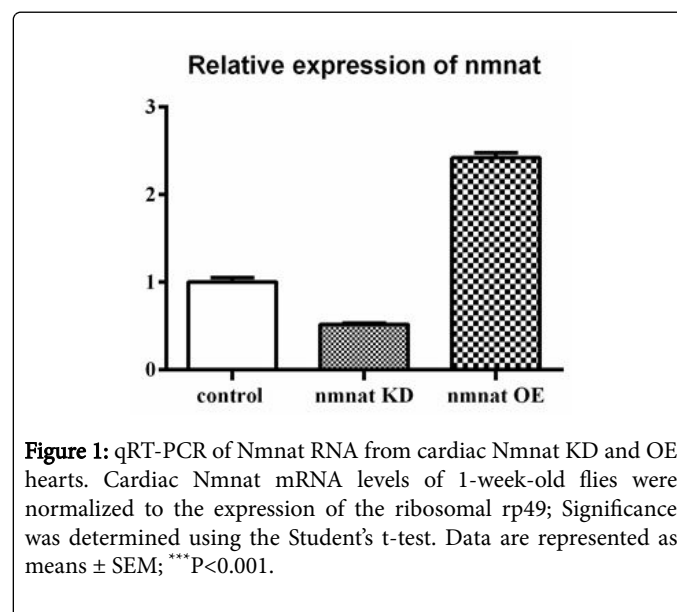


Figure 1: qRT-PCR of Nmnat RNA from cardiac Nmnat KD and OE hearts. Cardiac Nmnat mRNA levels of 1-week-old flies were normalized to the expression of the ribosomal rp49; Significance was determined using the Student's t-test. Data are represented as means \pm SEM; ***P<0.001.

Silencing of *Nmnat* in the heart and its impact on cardiac function

A recent study reported that Nmnat2 protein expression and enzyme activity in rats prevents cardiac hypertrophy [9]. However, the impact of the Nmnat on cardiac function in *Drosophila* remains unknown. To determine the role of Nmnat in maintaining normal heart function, we used Hand-Gal4 to perform heart specific Nmnat knockdown. As shown in Figure 2, the arrhythmicity index of cardiac Nmnat KD at 5 weeks of age was considerably higher than in age-matched controls (Hand-Gal4/+C), which was more pronounced than that observed at 3 weeks of age. Nmnat KD caused a reduction in contractility in 3- and 5-week-old flies ($P < 0.001$). This decrease was caused by an accelerated increase in systolic diameter relative to the increase in diastolic diameter ($P < 0.001$). Note that cardiac Nmnat KD flies in the experiment experienced cardiac dysfunction similar to that of the Nmnat KD flies obtained from the Bloomington stock center in a previous study. Increasing amounts of evidence of the effects of exercise in age-related heart disorders in flies have been reported. An established protocol was used here to determine whether Nmnat KD flies are capable of responding to exercise. Indeed, Nmnat KD flies demonstrate a significant degree of improvement in heart contractility after 3 weeks of an exercise regimen consisting of upward climbing ($P < 0.01$), and this improved efficiency was found to persist after 2 weeks of rest (week 5). However, regular exercise according to the program did not significantly decrease incidence of arrhythmias. In contrast, heart contractility displayed more obvious improvements than rhythm parameters in response to exercise (Figure 2).

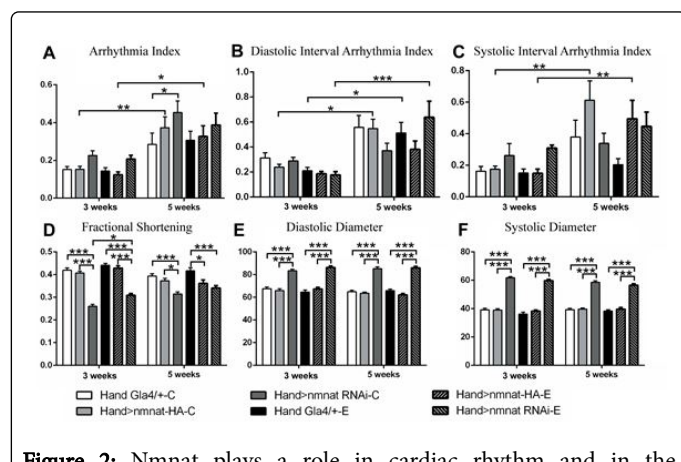


Figure 2: Nmnat plays a role in cardiac rhythm and in the contractility of late middle *Drosophila*; A: The small increases in cardiac arrhythmias were observed in Nmnat KD hearts in 5-week-old flies; The age-dependent increase in AI, DIAI, and SIAI become more significant as Nmnat OE flies aged (A, B, and C); D: Knockdown of Nmnat in the heart caused a significant reduction in fractional shortening due to (F) an accelerated increase of systolic diameter relative to (E) the greater diastolic diameter relative to background controls at 3 weeks of age; However, at 5 weeks of age (after 2 weeks of rest) the reverse was true; Exercise-trained Nmnat KD flies displayed more pronounced fractional shortening than their unexercised siblings at 3 weeks of age; Both exercise-trained control flies and Nmnat OE flies were analyzed and were not found to be significantly different from unexercised siblings; Data are represented as means \pm SEM; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Overexpression of Nmnat in the heart and its impact on cardiac function

It has been reported that Wallerian degeneration slow (WldS) mice over-expresses a chimeric protein containing the NAD synthase Nmnat and mitigate injury-induced axonal from degeneration [15-17]. However, the effect of Nmnat overexpression has not been analyzed on cardiac contractility and rhythm. To determine whether overexpression of Nmnat in *Drosophila* has any effect on cardiac function, we decided to express Nmnat in *Drosophila* using the UAS-Gal4 system as previously reported [18]. As shown in Figure 2A, the arrhythmia index increased with age in both undisturbed and exercised Nmnat overexpression females ($P < 0.05$), which was primarily due to increased variability of the systolic intervals ($P < 0.01$; Figures 2B and 2C). Nmnat OE undisturbed flies display increased fractional shortening compared to Nmnat KD siblings at 3 weeks of age and 5 weeks of age ($P < 0.001$; Figure 2D), which was not significantly different from that of Hand/+ flies ($P > 0.05$; Figure 2D). These findings suggest that arrhythmias become more common with age in both Hand/+ and Nmnat KD flies but much more rapidly in Nmnat OE flies and that exercise-training has a small effect in Nmnat OE in adult hearts.

Negative geotaxis

Negative geotaxis is a standard measure of mobility across ages in *Drosophila* [19]. Age-specific declines in climbing ability are susceptible to genetic background [14]. In young flies, exercise training is associated with significantly less arrhythmia than undisturbed flies [3,20]. To determine whether exercise training in young flies could improve mobility in flies expressing different levels of Nmnat at different ages, longitudinal measurements were performed weekly to record the changes in the climbing index of each group.

As expected, the three genotypes revealed a tendency to decline of negative geotaxis speed with age (Figures 3A-3C). Cardiac Nmnat OE flies in weeks 1 and 2 displayed significantly higher climbing speed than age-matched Nmnat KD and control flies (Figures 3A-3C). However, unexercised Nmnat OE flies showed considerably less climbing ability than age-match unexercised control flies in weeks 5 and 6 (Figures 3A and 3C). In any case, cardiac Nmnat KD reduced climbing ability in both unexercised and exercised flies more severely than in Nmnat OE and control flies. (Figures 3A-3C). Climbing ability was better in exercised Nmnat OE flies than age-matched undisturbed flies for all weeks after initiation of the exercise program (Figure 3C). However, neither Nmnat KD nor OE flies showed any significant difference between exercise and unexercised flies from 4 weeks to 7 weeks of age (Figures 3B and 3C).

Longevity

Regular exercise is known to extend mean lifespan and improve quality of life in a number of species [21]. Heart-specific Nmnat knockdown decreased the median survival time of the flies by 33%, and the curves of both Nmnat KD and OE flies were significantly shorter than the control survival curve ($P < 0.001$; Figure 4A), indicating a decreased lifespan in flies expressing Nmnat at low or high levels. To determine whether exercise altered the survival of flies due to change in Nmnat expression levels, flies were given a standard three-week regular training protocol. Among exercised flies, those with a wild-type background again experienced the greatest protection from death, ($P < 0.001$; Figure 4B), and the median survival of Nmnat KD

flies was 61 days compared to 55 days for the undisturbed control flies ($P < 0.001$; Figure 4), indicating that Nmnat delays the aging process when accompanied by exercise.

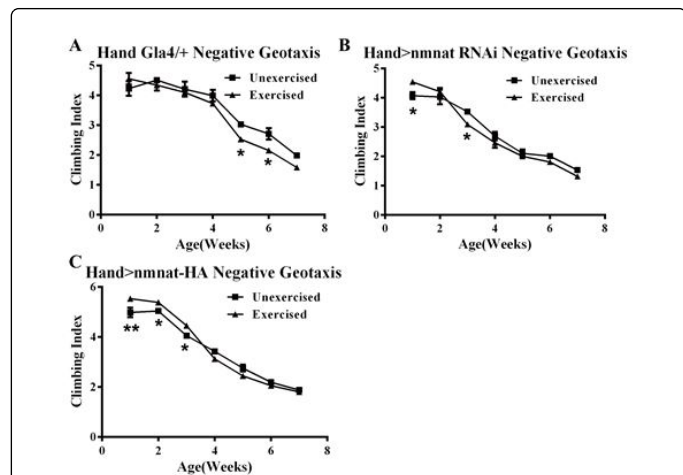


Figure 3: A: Exercise and Nmnat overexpression in the heart can improve negative geotaxis during treatment; RING assays across ages in Hand-Gal4/+; B: Hand>nmnat RNAi; and C: Hand>nmnat-HA flies; Significance was determined using a one-way analysis of variance (ANOVA) followed by an LSD test between controlled and exercised flies; Data are represented as means \pm SEM; * $P < 0.05$; ** $P < 0.01$.

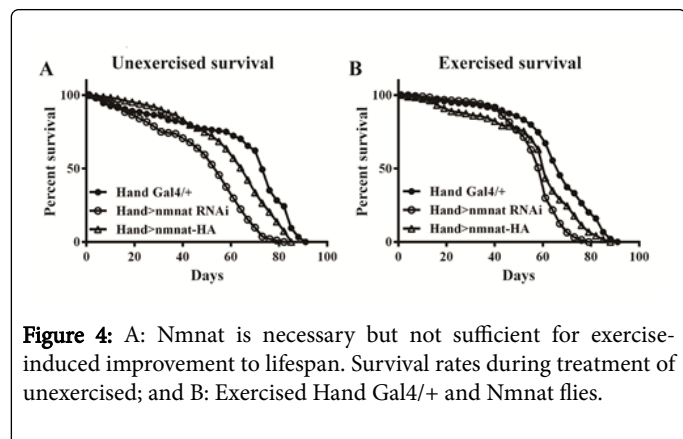


Figure 4: A: Nmnat is necessary but not sufficient for exercise-induced improvement to lifespan. Survival rates during treatment of unexercised; and B: Exercised Hand Gal4/+ and Nmnat flies.

Discussion

Exercise-training is beneficial to heart contractility and lifespan in wild-type flies

Given that regular exercise improves cardiomyocyte contractility, which optimizes cardiac performance in mammals [22]. The effects of cardiac function on several parameters were measured during three weeks of training in young flies. Cammarato et al. showed that wild-type *Drosophila* hearts exhibited a steady decrease in diastolic dimensions, which was significantly greater than that of systolic dimensions [23]. This highlights age-related deterioration in contractile performance as indicated by the significant rate of fractional shortening. Exercised flies had somewhat higher heart contractility than unexercised wild-type flies, and they displayed less

pronounced age-associated functional declines. The contractile function of exercised flies began to exceed that of unexercised controls slightly after the exercise program was initiated. Age-specific increase in cardiac rhythmicity were even more sensitive to exercise training, as the rhythmicity of exercised flies increased rate similar to that of unexercised flies. These senescence-dependent changes in heart rhythm, are considered indicative of impaired myocardial relaxation and are reminiscent of the atrial fibrillation in elderly humans [24].

Studies confirmed that Sir2 can extend lifespan and stress resistance by increasing NAD⁺ levels [25]. Similarly, Nmnat KD is associated with substantial lifespan reduction in aging flies by decreasing the level of NAD⁺, which reduces the activity of Sir2. Exercise is known to affect longevity in mammals [21,26]. In a previous study, a repeated exercise regimen was found to have no effect on the lifespan of aging flies [4]. What is more, continuous flying exercise throughout flies' lives has been found to be related to reduced lifespan [27]. In the current set of experiments, positive effects on longevity may allow flies to live to greater age, but maximum lifespan has shown no difference between unexercised and exercised wild-type flies. The survival of flies was recorded during the aging process, and improvement persisted after a period of exercise, suggesting that upward climbing has long-term effects.

Exercise-training does improve cardiac function in aging Nmnat knockdown flies

For senescent hearts, alterations in myocardial energy patterns and calcium homeostasis may play an important role in contractile performance and cardiac rhythm during aging [28,29]. The data collected here indicate that Nmnat is essential to preventing the decline in cardiac performance in senescent flies. The mechanism by which cardiac function becomes impaired in Nmnat KD flies might involve decreases in the level of NAD⁺, which interacts with age-induced declines in NAD⁺ and could accelerate cardiac deterioration. These changes regulate energetics and calcium homeostasis in elderly flies.

Recently, researchers have proven that pathologic cardiac dilation is associated with depletion of cellular NAD, and NAD treatment can block the agonist-triggered cardiac hypertrophic response [30]. Enzymes crucial to NAD biosynthetic pathways have drawn attention for these reasons. It has been reported that regular exercise can increase the concentration of NAD⁺ in cells by altering enzyme activity, which enhances the processing of myocardial oxygen and anaerobic metabolism. The results of the current work showed that elevated in heart contractility by training in cardiac Nmnat KD flies may explain the optimal stimulation that stems from the exercise programs, render the characteristics of dilated cardiomyopathy less pronounced, and enhance myocardial compensatory ability. Together, these data provide strong evidence that highly active flies have significantly better heart contractility after 3 weeks of exercise in the form of upward climbing, but this regimen has no discernible effect on the rate of arrhythmia. Previous findings have suggested that Nmnat2 protein expression and enzyme activity in rats prevents the incidence of cardiac hypertrophy. Certainly, whether exercise increases the level of NAD⁺ through similar or different signaling pathways requires further investigation.

Negative geotaxis associated with flies overexpressing *Nmnat*

The relationship between negative geotaxis, exercise-training, and functional aging is a complex one. To better assess the role of *Nmnat* on training, negative geotaxis testing was conducted at weekly intervals for weeks 1-7 of the experiment on flies that had been given an exercise regimen and on undisturbed controls. As expected, climbing ability declined with age in all *Nmnat* background flies. The current interpretation is that the age-related decreases in climbing speed are the major contributor to senescence of negative geotaxis [31]. The loss of *Nmnat* function reduced climbing ability in both unexercised and exercised flies, but the effects were more pronounced in exercised than unexercised flies. Negative geotaxis juvenility was ameliorated in flies overexpressing *Nmnat*, but not in *Nmnat* knockdown flies. This supports the conclusion that improved reflex locomotor function is a common feature in both unexercised and exercised *Nmnat* background flies. Senescent flies showed similar results. However, climbing ability was reduced in older *Nmnat* OE flies than in their age-matched *Nmnat* control flies, which showed that aging is the major factor associated with accelerated loss of muscle activity by decreasing the level of NAD^+ [32]. The lack of improvement in negative geotaxis senescence in exercised *Nmnat* overexpression flies, despite the greater heart contractility than in *Nmnat* KD flies, suggests that exercise training does not play a significant role in the amelioration of negative geotaxis senescence associated with *Nmnat* expression in the myocardium. By comparison with exercise treatment, the levels of *Nmnat* expression in young *Drosophila* were found to play a more important role in determining climbing speed.

Conclusion

In summary, knockdown and overexpression studies were here performed to analyze *Nmnat* cardiac function in adult *Drosophila*. Results demonstrated that myocardial *Nmnat* gene expression is critical to maintaining cardiac systolic function and rhythm, and proper levels of *Nmnat* expression showed significantly better climbing performance in young flies. The current study suggests that 2 h of exercise daily for three weeks does mitigate the deterioration of systolic function and rhythm caused by knockdown of myocardial *Nmnat*, also providing acute benefits to age-related locomotor decline and lifespan. This raises the possibility that exercise-training modulating *Nmnat* activity may be utilized to maintain physical fitness as part of healthy aging.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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