

The effect of glycine, N-acetylcysteine, and L-theanine on Klotho, sirtuin 1, and interleukin-1 β in the blood plasma of domestic cats

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Abstract. The study's relevance lies in the growing interest in influencing age-related processes in pets by adjusting their diets, including the use of varying amino acid contents and ratios. The main aim was to evaluate the impact of feeding domestic cats amino acid supplements containing glycine, N-acetylcysteine (NAC), and L-theanine (L-The) on the levels of Klotho protein, interleukin-1-beta (IL-1 β), and sirtuin-1 (SIRT1) in blood plasma. Twenty-one adult cats were divided into three groups, each receiving food with one of these supplements: glycine (Gly), NAC, or L-The. After six weeks, the cats' general condition and body weight were recorded, and plasma levels of SIRT1, Klotho, and IL-1 β were measured by Western blot. The results were compared to baseline (Wilcoxon test). NAC significantly lowered IL-1 β levels and showed a trend toward higher SIRT1. L-The increase Klotho levels. In the Gly group, no statistically significant changes were observed, although there was a trend toward elevated SIRT1 and IL-1 β . These findings suggest certain beneficial effects of these amino acid-enriched diets on markers linked to aging. However, given the short trial duration, these data remain preliminary. They require further in-depth studies with extended intake periods, careful dose adjustments, and comprehensive combination evaluations in future research.

1 Introduction

The number of companion animals worldwide is increasing each year, reflecting growing awareness of responsible pet ownership. Companion animals significantly influence the psychoemotional state of their owners by helping reduce stress, improve mood, and increase physical activity [1]. Conversely, the death and loss of a pet bring grief and potential psychoemotional disorders. Therefore, researchers are increasingly focusing on studies related to aging in animals and on strategies to extend their lifespan, a direction that can also offer insights into human aging.

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Aging is characterized by a series of changes occurring at the molecular, cellular, tissue, and systemic levels. Early signs of aging are observed at the molecular and cellular levels and include features such as genomic and epigenomic instability, telomere shortening, mitochondrial dysfunction, inflammation, cellular senescence, and disrupted nutrient sensing [2]. With advancing age, these changes lead to imbalances in homeostasis, impacting overall functional status and underlying the development of age-related diseases. These mechanisms are highly conserved in evolution and are observed across different types of organisms, from unicellular eukaryotes to mammals, including domestic cats and dogs.

Diet and specific dietary components can play a key role in modulating the aging-induced processes and hallmarks of aging. It is known that various dietary restrictions, including calorie restriction, can increase lifespan in several organisms, including dogs [3, 4]. The role of macronutrients in processes related to lifespan extension is also significant, with proteins and their monomers—amino acids—being of particular interest. Restriction of certain sulfur-containing branched-chain amino acids, such as methionine, has been shown to promote increased longevity [5]. Conversely, dietary enrichment with other amino acids can improve age-related traits and potentially prolong lifespan in model organisms [6]. For example, increased glycine (Gly) intake in the diet extended the lifespan of rats [7] and mice [8]. The proposed mechanisms of Gly's geroprotective action include the mitigation of methionine excess—thereby inducing autophagy—and the enhancement of glutathione metabolism, an antioxidant system known to decline with age [9]. Another amino acid of interest is L-theanine (L-The). Studies have shown that it can prolong the lifespan of model organisms [10] and regulate various aging mechanisms, including advanced glycation end products, oxidative stress, and inflammation [11]. N-acetylcysteine (NAC) is also noteworthy in the context of aging; supplementation with NAC extends lifespan in mice [12] and *Drosophila* [13]. The mechanisms underlying NAC's effects are likely related to its ability to increase glutathione synthesis and modulate pro-inflammatory signaling pathways such as NF- κ B [14]. Studies in humans have indicated that NAC exerts geroprotective effects by reducing levels of pro-inflammatory cytokines (IL-6) and acute phase response, as well as markers of cellular senescence (p16 and β -galactosidase) [15].

Although the aforementioned amino acids have been partially studied in domestic animals, specifically domestic cats, there has been no clear demonstration of whether these amino acids, when used as dietary supplements, can regulate signaling pathways and biomarkers associated with aging and longevity. The present work is a pilot study exploring the effects of amino acid supplementation as a part of ongoing efforts to investigate their combined and synergistic actions.

The aim of the study was to investigate the influence of food enriched with amino acids (glycine, N-acetylcysteine or L-theanine) on some blood plasma markers associated with aging and geroprotection in the domestic cats.

2 Materials and methods

This study was approved by the Ethics Committee of Izhevsk State Medical Academy (Protocol № 766/1 dated November 20, 2023), in accordance with current local and national regulations, rules, and guidelines. All procedures adhered to principles of humane treatment of animals. Cats were not subjected to any invasive or stressful procedures other than the routine collection of blood samples, performed by standard methods without anesthesia. The animals were housed in groups of 3–4 cats per enclosure under the supervision of a veterinarian; every cat had its individual box. A total of 21 adult domestic cats (*Felis catus*) of mixed breed with an average body weight of 3.31 ± 0.72 kg were included in this study. The cats were randomly divided into three groups of seven animals each. The first group received Gly at a dosage of 3.0 g/kg of feed, the second group received NAC at 4.0 g/kg of

feed, and the third group received L-The at 1.0 g/kg of feed. The feeding period lasted 6 weeks, during which the animals continued to consume their usual commercial dry food (produced by order of Spring Ltd. by Pets Food, Grodno, Republic of Belarus), which meets AAFCO (Association of American Feed Control Officials) requirements and Russian feed standards for non-productive animals (GOST R 55453-2022). The daily feed portion was individually calculated for each animal based on body weight and overall health status to avoid overfeeding or underfeeding, and was provided twice a day. The cats had free access to drinking water, and the primary diet remained unchanged throughout the study.

Blood samples were collected from the peripheral veins of each cat before and after the feeding period (end point). Plasma was obtained using EDTA tubes, centrifuged at 3000 g for 10 minutes. The separated plasma was stored at $-80\text{ }^{\circ}\text{C}$ until further analysis.

Levels of SIRT1, Klotho, and IL-1 β in blood plasma were measured by Western blotting. Plasma samples were mixed with 2 \times Laemmli sample buffer and heated at $95\text{ }^{\circ}\text{C}$ for 5 minutes. Proteins were separated by SDS-PAGE in 10–12% polyacrylamide gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes at 100 V for 2 hours, and transfer quality was verified by Ponceau S staining. Membranes were then blocked with 5% bovine serum albumin solution for 1 hour at room temperature. Next, the membranes were incubated overnight at $4\text{ }^{\circ}\text{C}$ with primary antibodies (rabbit polyclonal antibodies against SIRT1, Klotho, and IL-1 β , 1:1000, Affinity Biosciences). After several washes with TBST (Tris-Buffered Saline + 0.1% Tween 20), membranes were incubated with HRP-conjugated secondary antibodies for 1.5 hours at room temperature. Chromogenic detection was performed with DAB substrate, and band intensities were quantified using ImageJ software (NIH, USA). Signal normalization was done against total protein (Ponceau S staining).

Data were processed using Python 3.12 (Python Software Foundation) and R (R Core Team, Austria). Post-feeding values were calculated as the ratio of post- to pre-intervention levels, forming an individual coefficient of change. The Wilcoxon signed-rank test was used to compare biomarker levels within each group before and after supplementation, with statistical significance set at $p < 0.05$. Body weight and body condition score (BCS) data are presented as mean \pm SEM. Western blot results for the markers are visualized using separate bar graphs, where the height of each bar indicates the median value of the corresponding marker, and vertical error bars denote the interquartile range (25th to 75th percentile).

3 Results

Table 1 presents body weight and BCS data before and after the 6-week feeding period. As expected, there were no significant differences in these indicators, owing to the continued consumption of the animals' standard diet.

Table 1. The body weight and BCS before and after feeding.

Group	Gly		NAC		L-The	
	Before	After	Before	After	Before	After
Body mass, kg	2.82 \pm 0.17	2.93 \pm 0.14	3.34 \pm 0.22	3.32 \pm 0.24	3.92 \pm 0.30	4.71 \pm 0.29
BCS	3.71 \pm 0.18	3.86 \pm 0.14	4.43 \pm 0.30	4.43 \pm 0.30	4.71 \pm 0.29	4.71 \pm 0.29

Figure 1 shows the impact of amino acid supplementation on Klotho, IL-1 β , and SIRT1. Antibodies against Klotho detected a distinct band of about 60 kDa, corresponding to the soluble form of Klotho (sKlotho). A comparison of results before and after the feeding period in the Gly and NAC groups revealed no appreciable changes in Klotho content. By contrast, 6-week feeding with L-theanine (L-The) led to a small but significant increase in sKlotho ($p < 0.05$).

Changes in the pro-inflammatory cytokine IL-1 β were most pronounced in the NAC group, showing a significant decrease associated with food enriched with this amino acid ($p < 0.05$). No statistically significant variations were observed in IL-1 β levels in the Gly or The groups.

No statistically significant changes in SIRT1 were detected in any group. In the L-The group, SIRT1 levels remained near baseline, whereas the Gly group showed a trend toward increased SIRT1 without statistical significance, and the NAC group exhibited results close to significance ($p = 0.052$). Although NAC and Gly both produced a visible rise in SIRT1, these effects did not reach statistical significance under the conditions of this study.

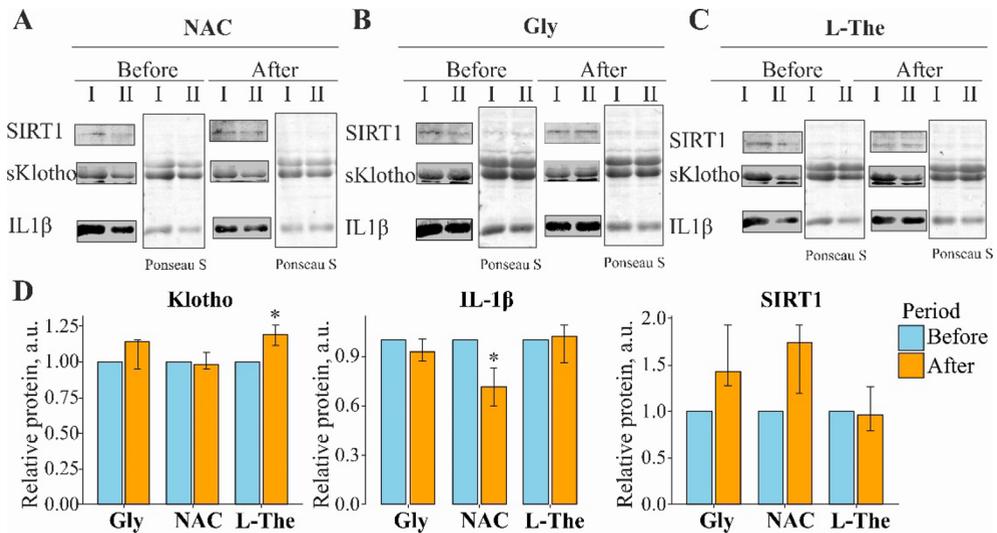


Fig. 1. Western blot analysis before and after feeding. Western blot images for NAC (A), Gly (B), and L-The (C). (D) Relative amounts of Klotho, IL-1 β , and SIRT1 in blood plasma before and after feeding, normalized to total protein. * – statistically significant differences in within-group comparisons ($p < 0.05$).

4 Discussion

This pilot study demonstrated how a 6-week feeding regimen supplemented with Gly, NAC, or L-The could affect sKlotho, SIRT1, and IL-1 β —proteins involved in age-associated changes (Klotho, IL-1 β) and lifespan (SIRT1). Although these markers are well-studied in model organisms and humans, they are less explored in companion animals, including cats. Given the high degree of evolutionary structural conservativeness of each factor explored, it can be assumed that their functions are highly similar in domestic cats.

Concentration of sKlotho decreases with age, and it is often regarded as an indicator of aging and metabolic health [16]. In humans and laboratory animals, elevated Klotho levels have been associated with improved renal function, reduced inflammation, and increased longevity [17–19]. Studies of Klotho in domestic cats have been conducted in the context of hypertrophic cardiomyopathy, but no associations were found [20]. Here, we have shown

that, among the three tested amino acids, only L-The significantly influences Klotho and elevates its levels. One study reported that L-The enhanced cognitive function in Klotho-deficient model animals and increased Klotho expression by modulating the JAK2/STAT3 pathway [21]. These data support our finding that L-The may affect Klotho expression.

IL-1 β is a pro-inflammatory cytokine that significantly increases with age in domestic cats [22], reflecting the concept of age-related chronic inflammation (inflammaging). In this study, only the NAC group exhibited a significant reduction in IL-1 β , likely through the inhibition of NF- κ B signaling, ultimately reducing pro-inflammatory cytokine synthesis [14]. This effect of NAC has been documented in various studies. The other two amino acids showed no significant effect on IL-1 β levels, which might be partly due to the relatively young animals used in our study and their lack of pronounced inflammatory responses at baseline.

Interestingly, both Gly and NAC showed a trend toward increased SIRT1, although the changes did not reach statistical significance. We assume that this result may be due to the short duration of using the enriched diet or a small number of experimental animals. The results in the NAC group ($p=0.052$) were on the borderline of statistical significance, however in this group, as previously indicated, a significant decrease in IL-1 β was found. SIRT1 is often referred to as an "anti-aging" protein because of its involvement in the regulation of signs of aging and its correlation with an increase in the lifespan of model organisms. [23]. SIRT1 can also influence inflammatory processes by downregulation of NF- κ B [23]. Probably, it can explain the decrease in IL-1 β .

5 Conclusion

In this pilot study involving a small cohort of domestic cats, a 6-week feeding supplemented with N-acetylcysteine, glycine, and L-theanine was accompanied by changes in several markers linked to aging and longevity (Klotho, IL-1 β , and SIRT1). Although NAC supplementation decreased IL-1 β and showed a trend toward increased SIRT1, and L-theanine increased Klotho, it is premature to draw definitive conclusions regarding geroprotective effects because of the limited number of animals and the relatively short duration of the experiment. Therefore, these findings should be considered as preliminary. Future research may require extended feeding periods, adjusted dosages, and larger sample sizes, as well as an evaluation of the combined administration of these supplements, to more thoroughly investigate their impact on age-related processes in companion animals.

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