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Brief Report

Effects of Life-Long Supplementation of Potassium Nitrate on Male Mice Longevity and Organs Pathology

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Abstract: Many short-term studies with dietary nitrate supplementation in humans and animal models reported positive effects on the cardiovascular system, exercise efficiency, and immune function. However, there has been long-standing concern related to cancer and adverse hormonal effects. We studied the long-term effects of different potassium nitrate (KNO_3) concentrations on laboratory mice longevity and structural changes in their organs. Four groups of male mice were treated with 0 mg (0%), 45 mg (1%), 90 mg (2%), and 140 mg (3%) KNO_3 in the drinking water. The groups were monitored for agility and health status daily. The lifespan of mice and organ pathological changes were analyzed. We found no detrimental effects of life-long supplementation of KNO_3 on the survival of mice in treatment groups. Nitrate supplementation was associated with a lower level of pathological changes ($p = 0.002$). We conclude that KNO_3 supplementation had no carcinogenic effect on mice and possibly prevented the organs from aging.

Keywords: nitrates; potassium nitrate; nitric oxide; mice; longevity; lifespan; organs pathology

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1. Introduction

Green leafy vegetables (lettuce, spinach, amaranth, etc.) and roots such as red beets are the main sources of dietary nitrates (NO_3^-) [1,2]. Nitrates are a naturally occurring compound in food [2,3] as well as a food additive and pharmaceuticals [1,4].

Nitrates had a bad reputation mostly due to their connection with nitrosamines and possible cancer risk [5]. Once in the human body, nitrates can bind to protein metabolic by-products in the gastrointestinal tract and can be converted to cancer-causing nitrosamines [6]. However, nitrosamines are mostly formed during heat treatment when the temperature reaches about 150 °C [7]. According to the European Food Safety Authority [8], epidemiological studies have not shown that nitrates in food increase the risk of oncological diseases. In the presence of excess nitrates, hemoglobin in the blood is converted to methemoglobin, which disrupts oxygen metabolism. However, it has been clinically established that oxygen transport is impaired only when methemoglobin concentrations exceed 10% total hemoglobin [9,10]. The suggestion that nitrates are harmful is rather debatable, considering that >80% of all nitrates that we receive come from vegetables, a product group that is known for its beneficial action on health [11].

Nitric oxide (NO) and nitrites, which are both the outcome of NO_3^- related products, induce vasodilatation by enchasing blood flow [12], thereby boosting the oxygen uptake and preventing oxidative processes in the working muscles [13]. Moreover, nitrates demonstrate the potential to increase the bioavailability of blood plasma, which is crucial for the exogenous pathway of nitrate/nitrite/NO and functions as a regulator of hypoxic

signals and NO-induced vasodilatation [14]. Nitrate/nitrite/NO has a mitochondrial and contractile efficacy on the muscle circulatory system [15,16] and may improve muscle blood flow circulation as well as the metabolic response to physical activity [17,18]. Supporting evidence was published concluding that the concentration of plasma nitrites alone is an independent factor of physical performance [5,12]. While recent research focuses mainly on so-called "organic nitrates", which come in the forms of juices, drinks, etc., the overall assessment of the "nitrate" topic remains incomplete.

Aging is related to an increased risk of cardiovascular diseases, type-II diabetes, metabolic syndrome, and cancer, because as the body ages, NO bioavailability decreases due to decreased eNOS activity and oxidative stress [19]. Aging, sedentary lifestyle, and poor nutritional habits play a significant role in developing obesity and type-II diabetes, followed by oxidative stress, impaired NO signaling, and cardiovascular diseases. Further understanding of pathophysiological mechanisms may help to create new strategies, e.g., using the therapeutic potential of increased NO bioavailability, for preventing and coping with these diseases [19]. A few studies have already confirmed the benefits of dietary nitrates to human health [20,21].

Thus, some research showed the harmfulness of nitrates to living organisms, some indicated their health benefits. However, the long-term effects of potassium nitrate and the pathology of organs were not assessed. Using an exploratory approach, in this study, we aimed to analyze the long-term effects of different potassium nitrate concentrations on laboratory mice longevity and structural changes in their organs, which, to our knowledge, has not been examined in relation to longevity in this species previously. We hypothesized that potassium nitrate may not be related to oncological factor formation, therefore exploring any potential impacts that may exist without preconceived notions about the direction of the effect.

2. Materials and Methods

All research involving animals was conducted according to the requirements of the European Commission directive and the permit (No. G2-172) from the Lithuanian ethics commission at the State Food and Veterinary Service Animal welfare department to perform procedures. The choice of the feeding dosages for this experiment was based on previous research [22,23].

2.1. Experimental Animals

Mice (*Mus musculus*) from Balb/C line (n = 21) were chosen for the experiments; weight: 29.1 $\pm$ 4.67 (mean $\pm$ SD) grams. Mice of 12–16 weeks of age were chosen to exclude possible effects of KNO_3 supplementation on the mice development and growth. In order to eliminate possible sex-specific variation in longevity [24], we chose the male mice. The control group consisted of 6 male mice. Three experimental groups consisted of 5 male mice each. Mice were ad libitum fed food designed for rodents. Animals were kept in similar conditions in purposely equipped cages for each group, separately; with a 12:12 hour day/night cycle. The noise level did not exceed 85 dB.

2.2. Feeding Assay

The animals were given the tested substance with water from water bottles, and the water was always available. The water bottles were filled with 150 mL of water and 1%, 2%, and 3% of KNO_3, respectively to groups 1st, 2nd, and 3rd. The tested substance does not change the taste of the water; and the water supply was not interrupted. The dose of the tested substance was given to animal groups, respectively to 1st group 45 mg KNO_3 per mouse, 2nd group 90 mg KNO_3 per mouse, 3rd group 140 mg KNO_3 per mouse.

The animals were checked daily. Agility and health status were evaluated. Once a month, the animals were evaluated by two investigators using blind testing, with investigators not knowing differences between groups. The general health of the animals was evaluated according to Burkholder et al. [25] and an assessment table was created (Table 1).

Table 1. Mice agility and general health status evaluation criteria.

Score	Animal Status	Agility	Coat	Body Posture	Health Status	Appetite
5	Excellent	Moves fast and a lot, climbs the cage, burrows in the beddings, curious	White, soft, shiny	Characteristic to species, movements are comfortable, body physiologically bent	No changes	Good, feeds constantly, drinks after eating
4	Very good	Moves fast and a lot, burrows in the beddings, doesn't climb the cage mesh and walls, curious	White, sometimes matted, looks like wet	Characteristic to species, movements comfortable, body physiologically bent	No changes	Good, feeds constantly, drinks after eating
3	Good	Movements characteristic to species but agility is reduced, avoids interacting with other animals	The coat „wet ", „matted ", „sticky"	Characteristic to species, the back curved, legs under the body	No changes	Appetite is reduced, shows less interest in food and water
2	Satisfactory	Moves less, burrows in the beddings, spends time laying down, lethargic	Coat yellowish, matted, looks wet	The body snuggled down, the spine bent in a hump, all feet under the body	No changes	The appetite is bad, no evident interest in food or water
1	Bad	Almost no movement, laying snuggled down on the bottom, the head down in the beddings, no interest in surrounding	Sticking, yellowish, with bald spots	The body snuggled down, the spine bent in a hump, all feet under the body	Possible discharge from the eyes, nose, and signs of diarrhoea, in some cases sniffing and coughing. Must additionally indicate when evaluating.	The appetite is bad, no evident interest in food or water. On the touch feels skinny, the ribs sticking out.

2.3. Preparation

The animals that died during the investigation were dissected and postmortem findings were registered as soon as possible after death using the simplified necropsy technique [26]. Kidney, liver, and lungs were histologically examined and structural changes in the organs were evaluated based on gross pathology findings [27]. Pulmonary hyperemia was found when assessing the color and size of the lungs. Lung edema and hyperemia were assessed. Then, diffuse redness and fluid effusion of the organ was seen. Kidney and liver failure were named. Then, color, size, and shape changes of the organ were found. Postmortem tissue histology was used to detect any possible early tumor formations.

2.4. Statistical Analysis

A log-rank test was run to determine if there were differences in the survival for different groups of KNO_3 treatment. Ordinal regression was used to reveal the relationship between groups and pathological changes. Pathological changes were defined as a dependent ordinal variable with four categories arranged in ascending order (from minimum

pathologies to maximum pathologies), and the treatment group was defined as an independent variable with four categories. The tests were two-tailed, where $p < 0.05$ was considered statistically significant and results are shown as mean ± SE. All statistical analyses were performed using IBM SPSS Statistics 26 (IBM Corporation, Armonk, NY, USA).

3. Results

The average survival time (±SE) was longest in 3% KNO_3 mice group (241 ± 34 days) and followed by 2% KNO_3 group (227 ± 52 days), with one mouse surviving for 408 days. While shorter average survival times were recorded in the control (0% KNO_3) and 1% KNO_3 treatment groups: 213 ± 17 and 159 ± 27 days, respectively (Figure 1A). The shortest survival time was registered in the 1% KNO_3 and in the 2% KNO_3 treatment groups (both 126 days). However, the differences in survival of different KNO_3 treatment groups were non-significant (log-rank test: $\chi^2(3) = 6.334$, $p = 0.096$).

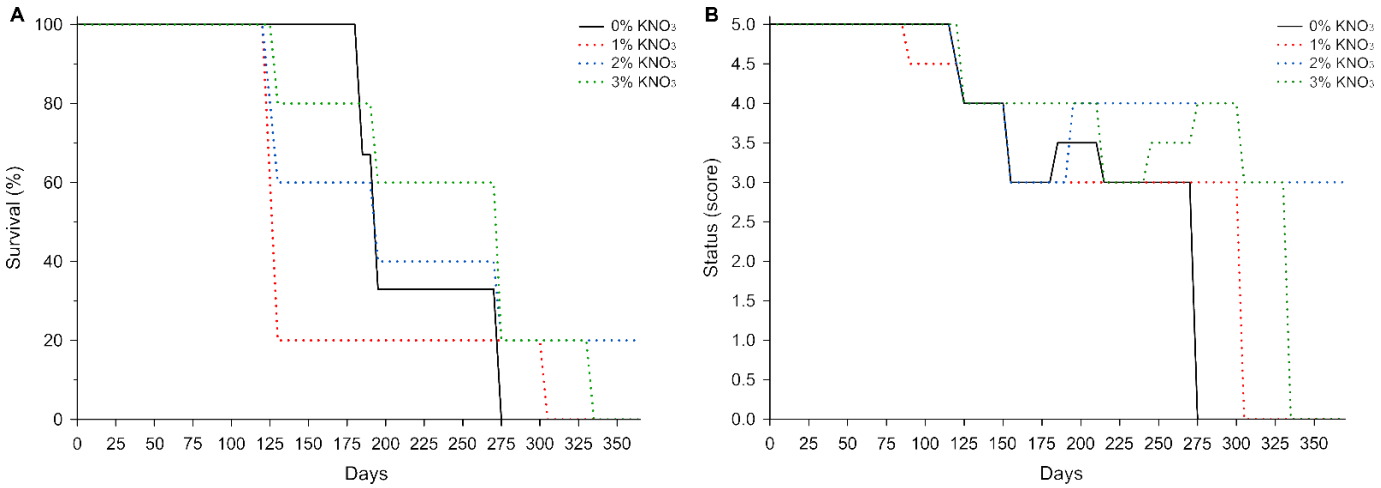

Figure 1. Survival (**A**) and status scores (**B**) of mice in groups with different KNO_3 concentrations.

Ordinal regression revealed a significant relationship of treatment groups with the pathological changes ($\chi^2(3) = 15.11$, $p = 0.002$). The regression model explained 55.4% (Nagelkerke R^2) of variance of the pathological changes. The experimental treatment groups had significantly fewer pathological changes in comparison to the control group (1% KNO_3 group: Wald $\chi^2(1) = 4.096$, $p = 0.043$; 2% and 3% KNO_3 groups, both: Wald $\chi^2(1) = 8.336$, $p = 0.004$). Moreover, mice from the control group and 1% KNO_3 group were in the worst condition. Mice that were fed 2% and 3% KNO_3 were able to maintain a good condition the longest (Figure 1B).

The most common pathology in all groups was pulmonary hyperemia and edema (Table 2). Mice that were fed 2% and 3% KNO_3 solution revealed only pulmonary hyperemia and edema, while in other groups more morphologic changes in organs were detected (Table 2). One more frequent pathology that was detected during the autopsy was pulmonary hyperemia, edema, and hepatic insufficiency; it was registered in groups that were fed 0% and 1% KNO_3 (Table 2). Pulmonary hyperemia, edema, as well as hepatic and kidney insufficiency were registered only in the control group (Table 2). Post-mortem tissue histology did not show any early-stage lesions of cancerogenic origin.

Table 2. Organ pathologies registered during *post-mortem* examination and mean survival in groups with different KNO_3 concentrations. Mean number of pathologies is shown in the last row.

Organ Pathology	Survival Days: Mean ± SD (Number of Cases)			
	0% KNO_3	**1% KNO_3**	**2% KNO_3**	**3% KNO_3**
Pulmonary hyperemia	-	267 (1)	340 ± 96 (2)	301 ± 38 (2)
Pulmonary edema and hyperemia	267 (1)	137 ± 0 (2)	152 ± 35 (3)	202 ± 70 (3)
Pulmonary edema and hyperemia, liver failure	224 ± 59 (2)	126 ± 0 (2)	-	-
Pulmonary edema, hyperemia, liver and kidney failure	189 ± 6 (3)	-	-	-
Number of organ pathologies: mean ± SD (number of cases)	3.3 ± 0.8 (6)	2.2 ± 0.8 (5)	1.6 ± 0.6 (5)	1.6 ± 0.6 (5)

4. Discussion

Dietary non-organic nitrates have a bad reputation mostly due to their supposed connection with creating nitrosamines and possible cancer risk [28,29]. In this study, we found that life-long supplementation of dietary nitrates had no evident adverse effects in mice. The treatment groups that were fed with 2-3% KNO_3 solution showed somewhat longer life span and developed significantly fewer structural pathologies than the mice from 0–1% KNO_3 treatment groups. Similarly, the treatment groups (those fed with 1–3% KNO_3) had significantly fewer pathological changes in comparison to the control group. These results are in line with previous studies [30,31] as well as recent long-term studies on male mice diet supplementation with $NaNO_3$ by Hezel et al. [32] and rats by Carvalho et al. [33], adding to growing evidence considering the safety and possible beneficial effects of dietary nitrates. However, the effects on both sexes still need to be addressed.

As for the possible mechanisms resulting in longer lifespan of mice, we suggest a viewpoint to nitrate-related compounds that has been applied in recent research where lower levels of the oxidation (using hydrogen peroxide) stimulated a scavenging enzyme that helped slow down the aging of the yeast cells [34]. As shown by our previous experiment with fruit flies [22], this could be explained by the previously hypothesized inverted "U shape" curve effect for this given compound, assuming that too small a concentration has very little or no effect and too high a concentration is/could be harmful. This hypothesis is supported by other studies showing the importance of quantity, where the low doses of nitrite, but not higher ones, had protective effects in in vivo and in vitro models of vascular dysfunction [35,36], myocardial ischemic injury, and liver ischemia-reperfusion injury [37,38].

The newest research has shown that usual nitrate and nitric oxide production in the bodies can prevent cardiovascular and metabolic diseases that shorten the lifespan [39–43], as well as help to maintain the capacity of mitochondria and, in unison, the whole organism [15,44,45]. Scientists suggest that by the mid-21st century 20–25% of the population in developed countries will consist of people over 65 years old [46–48]. Hence, the number of concomitant conditions that accompany aging (e.g., sarcopenia, cardiovascular, neurodegenerative diseases, type II diabetes, and cancer) will increase together with increasing lifespan [49]. There is a demand for innovative and reliable methods that would increase longevity and prevent chronic conditions [50]. This is why it is important to find natural components that would allow for improving body functioning even while aging. Nitric oxide holds an important position among all health-enhancing supplements. Our results suggest that nitric oxide, along with nitrate supplementation, may delay pathological changes within the body and prolong life with no chronic conditions.

Due to a lack of a standardized approach in rodent aging and toxicological studies, we examined the organs most likely to be affected: lungs, liver, kidneys, and spleen, but excluded endocrine and reproductive as well as cardiovascular systems, because they tend

to be affected by the timing of actual death more than the internal organs. The data obtained from this study suggest the two potential benefits of potassium nitrate supplementation: (i) increase in lifespan, and (ii) a delay in the onset of age-related organ pathology. Initially, our team posited a null hypothesis on the role for potassium nitrate in the development of cancerogenic formations in matured male mice. However, our data did not support this conjecture. Instead, a trend towards longer overall survival and a reduction in age-related organ pathologies was observed in the study subjects. Further research is required to gain a more comprehensive understanding of the underlying mechanisms driving these effects.

5. Conclusions

The results of the investigation revealed no detrimental effects of life-long supplementation of KNO_3 on the survival of mice, inducing only minimal structural changes in organs. Moreover, based on ordinal regression analysis, significant ($p = 0.002$) changes were observed between control and experimental groups (1% KNO_3–3% KNO_3) which demonstrated fewer pathologies. Based on the results of this investigation, we conclude that 2% and 3% KNO_3 supplement had no carcinogenic effect on mice and possibly prevented the organs from aging.

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Ethical Approval and Consent to Participate: All experimental procedures involving animals conformed to the European Community guiding principles and approved by the Ethics Committee of the State Food and Veterinary Service of the Republic of Lithuania (permission No. G2-172). Animals used for this study were housed and cared at the Lithuanian University of Health Sciences, Biological Research Centre. Animals were housed under conditions specified in the EU requirements, during the study.

Consent for Publication: Authors give their consent for the publication of identifiable details, which can include photograph(s) and/or videos and/or case history and/or details within the text ("Material") to be published in the above Article.

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