Original Article (short paper)

Effects of concurrent training associated with N-acetylcysteine on bone density of spontaneously hypertensive rats

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Abstract – Aim: The present study aimed to analyze the effects of N-acetylcysteine supplementation associated with concurrent training on the bone mineral density of spontaneously hypertensive elderly rats. **Methods:** For the present study, 28 male spontaneously hypertensive rats, six months old, were distributed in the following groups: control (C, n=7); control + N-acetylcysteine (CNAC, n=7); concurrent training (T, n=7); and concurrent training+N-acetylcysteine (TNAC, n=7). The concurrent training was composed of aerobic training on a treadmill and resistance training in the same training session, three times a week. Animals of the NAC groups received a dose equivalent to 120 mg/kg/day orally for eight weeks. The animals in the trained groups underwent training for eight weeks. The animals were evaluated at the beginning and end of the experiment. After euthanasia, the tibias and femurs were submitted to bone densitometry analysis in an X-ray dual emission device. **Results:** Lower weight variation was observed in the trained animals and a reduction in pressure values in all groups, but without a statistical difference ($p > 0.05$). The animals in the T and TNAC groups presented a better performance in the physical tests ($p < 0.05$). In relation to bone, the NAC groups demonstrated a decrease in femoral bone density when compared to groups C and T. Finally, all experimental groups demonstrated an increase in tibial bone density, but with no statistical difference ($p > 0.05$). **Conclusion:** The animals in group T demonstrated better performance in the physical tests. In addition, the NAC caused a reduction in the bone mineral density of the femur.

Keywords: Hypertension, Oxidative Stress, Exercise, Bone Matrix.

Introduction

Metabolic disorders such as accelerated loss of bone mineral density, a high risk of osteoporosis, and frailty fractures are among various abnormalities in calcium metabolism, often associated with arterial hypertension (AH)[1-3]. Animal studies performed with spontaneously hypertensive rats (SHR) have observed an inverse relationship between blood pressure (BP) values and bone mass/quality[4-7].

Physical training (PT) represents one of the main forms of non-pharmacological treatment for hypertensive individuals[8]. Among several forms of PT, concurrent training (CT) can represent one form of treatment for systemic arterial hypertension. For Hickson[9], CT consists of the use of aerobic and resistance training in simultaneous and subsequent forms.

In the study of Nogueira[10], it was reported that PT is an effective model for reducing BP in elderly people. This process is the result of several factors associated with PT, such as training intensity/volume, number of sets and repetitions, recovery periods between sets and exercises, and the type of muscle contraction, causing small deformations in the bone architecture that stimulate osteogenesis.

These effects are beneficial to the rate of bone remodeling and can be observed in the metabolic and endocrine responses, through applied mechanical stimuli[11]. Among the benefits of PT, both aerobic and resistance, there is a reduction in the expression of pro-oxidant enzymes and an increase in the activity of antioxidant enzymes, which can be observed in different tissue types[12,13].

The functional alterations resulting from aging lead to a consequently sedentary lifestyle, after which the elderly population gradually begins to see physical exercise as a way to promote health [14,15]. Sedentarism combined with inadequate nutrition causes an imbalance between reactive oxygen species (ROS) and the body's antioxidants, which can lead to a condition denominated oxidative stress (OS)[16,17].

Lean et al[18], observed that the increase in OS due to the reduction in glutathione (the body's natural antioxidant) led to a substantial loss of bone mass, whereas in another study, antioxidant supplementation with the antioxidant n-acetylcysteine was shown to decrease bone loss in rats with OS induced by ethanol[19].

The association between AH, loss of bone mass, and OS has been studied in recent decades in both human and SHR models, due to a large number of pathological conditions that accompany aging in the hypertensive elderly population. To this end, the relevance of

the effects of N-acetylcysteine supplementation associated with CT on the OS balance and pathophysiological aspects of bone density needs to be investigated in an attempt to elucidate the numerous gaps in the literature.

Thus, the present study aimed to analyze the effects of N-acetylcysteine supplementation associated with concurrent training on the bone mineral density of spontaneously hypertensive elderly rats.

Methods

Animals and experimental protocol

Twenty-eight spontaneously hypertensive rats (SHR) from the Central Animal Laboratory of the University of Campinas - UNICAMP, six months of age, were divided into four groups: control (C, n=7); control + N-acetylcysteine (CNAC, n=7); trained (T, n=7); trained + N-acetylcysteine (TNAC, n=7). This study complies with the Ethics Committee on Animal Use from State São Paulo University – UNESP (CEUA – 1162/2015).

N-acetylcysteine supplementation

The animals were housed in cages (4 animals/cage) and maintained in an environment with controlled temperature (25 ± 2°C) and photoperiod (12/12 hours reverse/dark cycles). The animals in the control + N-acetylcysteine (CNAC) and trained + N-acetylcysteine (TNAC) groups received NAC at a dose of 120 mg/kg/day, orally (combined with animal feed), for eight weeks[20]. The animals in the T and TNAC groups, in addition to supplementation, performed aerobic and resistance training three times a week, on alternate days, for eight weeks.

Aerobic Physical Capacity

Aerobic capacity was evaluated by the physical effort tolerance test, performed before and at the end of the training period. The test consisted of running on a treadmill, starting at a speed of 6 m/min, with increments of 3 m/min every three minutes until the animal reached exhaustion. The maximum running velocity was recorded and the total distance traveled calculated [21]. The animals were adapted to the test environment for one week prior to the evaluations, for 10 min/day.

Aerobic training protocol

Before and at the end of the training period, functional capacity was evaluated by the physical effort tolerance test. The test consisted of running on a treadmill, starting at a velocity of 6 m/min, with increments of 3 m/min every three minutes, until the animal reached exhaustion. The maximum running velocity and total distance traveled were calculated and recorded[21]. Prior to the test, the animals were adapted to the equipment for a period of one week for 10 min/day.

The aerobic training was performed on a treadmill, three times a week, on alternate days. Initially, the rats underwent an adaptation period, during which there was a gradual increase in the speed and time of exercise (Table 1). From the fifth week of training, the rats performed running sessions at 60% of the maximum capacity, equivalent to a velocity of 18 m/min, lasting 40 minutes, until the end of the experimental period.

Table 1: Adaptation to the aerobic training protocol

Week	Velocity (m/min)	Duration (min)
1st	5	10
2nd	7.5-10	20
3rd	12-14	30
4th	15-17	40

Legend: (m): meters; (min): minutes.

Determination of the Maximum Load for Resistance Exercise

Resistance exercise was performed on a ladder specially constructed for rats. Two days after adaptation to the protocol, each animal was evaluated to establish the maximum load capacity. The test consisted of ladder ascents with progressive increases in load[22-24]. Initially, 75% of the animal's body weight was added, increased by 15% until the load was reached at which the rat failed to completely ascend the ladder. The highest load with which the rat was able to climb the full length of the ladder was considered the maximum capacity of the animal.

Resistance training protocol

The resistance training was performed three times a week, with a gradual increase in intensity, adapted from a previously used protocol[23,24]. The training was carried out on a ladder specially constructed for rats (110 cm high, 18 cm wide, 2 cm grid, 80º slope). Initially, there was an adaptation period, without overload, during which, after reaching the top of the ladder, the animals remained resting for two minutes, before being stimulated to start the climb again. The procedure was repeated until the animals climbed the ladder three times without the need for stimulation (Table 2).

After the adaptation phase, the maximum load capacity of each animal was evaluated. The test consisted of ladder ascents with progressive load increases[22,24], starting at 75% of the body weight of the animal and adding 15% of the body weight until reaching the intensity at which the animal was not able to fully ascend the ladder. The greatest overload with which the animal was able to completely climb the ladder was adopted as the maximum capacity.

From the second week, the training sessions consisted of four climbs with overloads corresponding to 50%, 75%, 90%, and 100% of the maximum capacity of each rat, with a two-minute recovery period between each ascent. The training was maintained until the end of the experimental period.

Table 2: Adaptation to the resistance training protocol

Day	Load (% body weight)	Number of ascents
1st	0	3
2nd	15	3
3rd	30	3

Blood Pressure Measurement

Systolic blood pressure (SBP) was measured in the tail of the animal before and at the end of the experimental period. For the measurement of SBP, the plethysmography method was used.[25] The rats were placed in a wooden box (50x40 cm), lined with pine shavings, at 40ºC for 5 minutes in order to cause vasodilation of the caudal artery. For the measurement of the SBP, an electro-sphygmomanometer *NarcoBio-System*® was used, model 709-0610 (*International Biomedical Inc*., USA). The cuff was positioned around the tail of the animal and connected to a pressure transducer (*Gould*, OH, USA). The cuff was inflated to a pressure of 200 mmHg and subsequently deflated. The arterial pulsations were recorded in a computerized data acquisition system (AcqKnowledge® MP100, Biopac Systems Inc., Santa Barbara, CA, USA).

Collection of Material

The animals were submitted to 12 hours of fasting before euthanasia, performed through anesthetization with sodium pentobarbital (50 mg/kg, intraperitoneally), followed by decapitation, 24 hours after the final training session[26].The tibia and femur were removed from the right limb and placed in a container with a physiological solution (NaCl 0.9%) and frozen in a freezer at -20°C.

Bone densitometry

The tibia and femur were submitted to densitometry analysis in an X-ray dual emission densitometer (DXA), DPX - Alpha, LUNAR. For analysis, the bones were submerged in a plastic container containing 2 cm of water to simulate soft tissue (in vivo). The middle third of the tibia was delimited by the device, and this area covered by the DXA (5 cm x 4 cm). The densitometer laser was set above the center of the bone to begin image capture.

After the image capture, the bones were analyzed using a manual analysis tool. The desired area for analysis was delimited in the region of the middle third of the tibial diaphysis and contoured to obtain the values of bone mineral content and bone mineral density[26].

Statistical analysis

After data collection, the Shapiro-Wilk test was performed to verify normality. The multiple ANOVA test with verification by the Tukey post-hoc was used to compare the means, followed by Tukey's post-test for comparison between groups. For the comparison of the initial and final periods, we used two way ANOVA variance analyses with repeated measures. Statistical conclusions were discussed at a significance level of 5% ($p<0.05$), using the software with statistical package SPSS 22.0.

Results

From the results obtained, it was observed that all animal groups showed an increase in body weight after eight weeks ($p<0.05$). In addition, although without a statistically significant difference, it was possible to observe smaller variations in the animals of the T group and higher variations in the TNAC group ($p>0.05$) (Table3).

The present study verified the physical capacity of different animal groups. It was observed that the T and TNAC groups demonstrated greater performance for the variables distance, velocity, and maximum load in comparison with the C and CNAC groups ($p<0.05$). In addition, the T group demonstrated higher performance for the maximal load variable after the eight week period when compared to the TNAC group (Table 4).

It was observed that all animal groups presented decreased blood pressure values after eight weeks in the intergroup comparison in the pre and post moments ($p <0.05$). However, although the NAC supplemented animals (CNAC and TNAC) demonstrated greater reductions in this variable, there was no statistically significant difference in the comparison between groups ($p> 0.05$). In addition, something similar occurred with the variation (delta) in this variable at the end of the experiment ($p> 0.05$).

After analyses of the femurs, it was possible to verify that the rats that received NAC demonstrated a decrease in bone mineral density (Figure 1). In this case, it was observed that the C and T groups presented superior bone mineral density in relation to the CNAC and TNAC groups ($p <0.05$).

Regarding analyses of the tibias, the results found did not demonstrate significant alterations. However, it can be observed that the animals which performed the concurrent training protocol presented greater mineral density in relation to the other groups of animals (Figure 2).

Table 3. Alterations in body weight in different periods.

	C	CNAC	T	TNAC
Initial Body Weight	348,66	343,76	355,38	344,42
Final Body Weight	368,93a	370,31a	365,80	374,05a
Δ	20,3	26,5	10,4	29,6

Analysis of variance ANOVA (two way) with repeated measures and post test of Bonferroni with significance of 5% ($p <0.05$).
a: Difference between initial and final body weight (C): Control Group; (CNAC): N-acetylcysteine Control and Supplementation Group; (T): Physical Training Group (TNAC): Physical Training and N-Acetylcysteine Supplementation Group.

Table 4. Physical aerobic capacity and maximum load for resistance training.

		C	CNAC	T	TNAC
Distance (meters)	Initial	432,33 ± 154,58	381,69 ± 137,36	424,69 ± 118,85	414,35± 106,76
	Final	391,41±97,32*	391,84±45,73*	893,23±119,66*#	842,21±106,65*#
	Δ	-40,91	10,15	468,53a,b	427,85a,b
Velocity (m/min)	Initial	29,0 ± 6,17	27,0 ± 5,19	29,30 ± 4,09	28,07 ± 4,79
	Final	29,25±1,35*	29,15±1,81*	43,84±3,36*#	42,28±2,49*#
	Δ	0,25	2,15	14,54a,b	14,21a,b
MaximumLoad (grams)	Initial	306,08±59,61	306,84±57,95	367,23±57,01	307,57±52,11
	Final	328,33 ± 59,61*	305,15 ± 38,28*	689,0 ± 88,13*#+	566,85 ± 87,93*#¥
	Δ	22,25	-1,69	321,76a,b	259,28a,b

(a,b): Statistically significant difference in comparison of groups C and CNAC from ANOVA one way variance analysis with Tukey post test at a significance of 5% ($p < 0.05$). (*): Difference between pre and post moments for intergroup variables. (#): Significant difference between C and CNAC with T and TNAC at the 8-week moment. (+): Difference of T from C, CNAC, and TNAC. (¥): TNAC different from C, CNAC, and T at eight weeks after analysis of variances ANOVA two way with repeated measures with Bonferroni post test at significance of 5% ($p<0.05$). (C): Control Group; (CNAC): N-acetylcysteine Control and Supplementation Group; (T): Physical Training Group (TNAC): Physical Training and N-Acetylcysteine Supplementation Group.

Table 5. Systolic Blood Pressure (SBP) at the beginning and end of the experiment.

	C	CNAC	T	TNAC
SBPinitial (mmHg)	347,90±21,32	246,67±19,96	247,95±20,96	247,19±23,81
SBP final (mmHg)	224,53±22,25a	205,07±15,37a	223,47±28,79a	212,85±14,07a
Δ	-23,37	-41,59	-24,47	-34,33

Analysis of variance ANOVA (two way) with repeated measures and post test of Bonferroni with significance of 5% ($p < 0.05$). a: Difference between initial and final body weight. (C): Control Group; (CNAC): N-acetylcysteine Control and Supplementation Group; (T): Physical Training Group (TNAC): Physical Training and N-Acetylcysteine Supplementation Group.

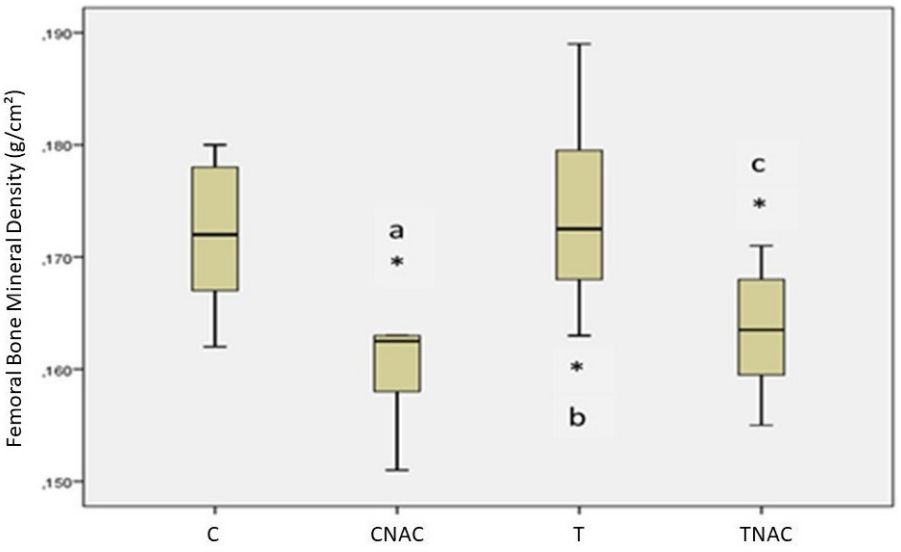

Figure1: Analysis of femoral bone mineral density in the four groups of animals. (a): CNAC different from C and T, (B): T different from CNAC and TNAC, (C) TNAC different from C and T. ANOVA analysis of variance test with significance level of 5% ($p < 0.05$).C: Control; CNAC: Control with N-acetylcysteine supplementation; T: Concurrent Training; TNAC: Concurrent Training with N-acetylcysteine supplementation.

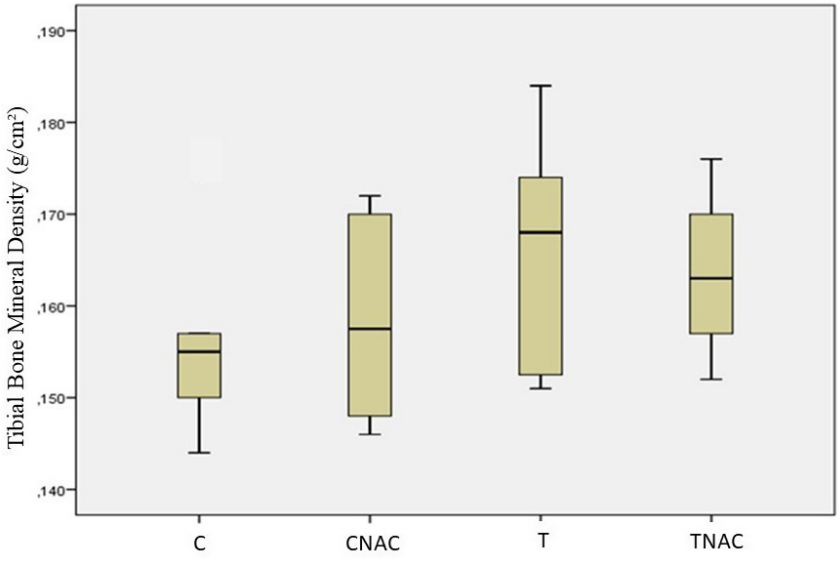

Figure2:Analysis of tibial bone mineral density in the four groups of animals. C: Control; CNAC: Control with N-acetylcysteine supplementation; T: Concurrent Training; TNAC: Concurrent Training with N-acetylcysteine supplementation. ANOVA Analysis of variance test, with significance of 5% ($p > 0.05$).

Discussion

The present study analyzed the bone mineral density of spontaneously hypertensive rats (SHR), submitted to a concurrent training protocol and consumption of the antioxidant n-acetylcysteine (NAC). It was possible to verify that the group which performed isolated training presented superior results to the supplementation group, both in the femur, for which a significant difference ($p < 0.05$) was observed, and in the tibia, for which the difference was not significant ($p > 0.05$).

In addition, the T group presented lower body weight (delta) variation, however, with no statistically significant difference ($p > 0.05$). Another point to be observed is that the animals in the T group presented a higher increase in the variables of physical performance (distance, velocity, and maximum load) when compared to the TNAC group ($p < 0.05$).

In relation to bone mineral density, these findings corroborate Ozaki[26], Cadore[27], and Aguiar[11], who demonstrate that physical training, due to the possible adaptations precipitated in the bone tissue, can be considered as a means of intervention capable of increasing bone mineral density.

It should be mentioned that in this case specifically, we used concurrent training, which is composed of two distinct forms of aerobic and strength training.

In humans, Cadore, Brentano,Kruel[27], argue that strength training can have positive effects on the bone density increase in young and old individuals, both male and female. In animals, the Ozaki study [26], indicates that physical exercise can be used as a therapeutic means to increase the bone mass of adult and elderly rats, even after the immobilization process.

The beneficial effects of training on bone mineral density of rats can also be observed in the study by Castoldi[28], in which aerobic, anaerobic (strength), and concurrent training were found to significantly increase the bone density of the tibia of the animals. The study was divided into two periods, four and eight weeks. In the four-week period, aerobic training did not demonstrate any significance when compared to anaerobic or concurrent training. However, over the course of eight weeks, both training protocols induced effects and were able to increase bone density. In addition, anaerobic training stood out, demonstrating a greater increase in bone mineral density in the two periods.

Regarding NAC, despite being considered an antioxidant, it can also be considered as an antidote to poisoning, especially in the liver[29,30]. Other authors highlight its anti-inflammatory properties, including as a means of use against diseases [31,32].

However, the NAC seems to have negatively influenced the physical performance of the animals analyzed. This may be an indication that despite being considered as an antioxidant, this substance somehow seems to interfere with the increase in physical capacity.

Regarding blood pressure, NAC appears to have facilitated a hypotensive effect in supplemented animals (CNAC and TNAC). However, although there was a reduction, it was not statistically significantly different in comparison to the other groups of animals ($p > 0.05$).

When related to Systemic Arterial Hypertension (SAH), NAC has been widely used, mainly for its antioxidant action, as it has demonstrated anti-inflammatory effects and protection after myocardial perfusion [32,33]. In this way, NAC could aid in the prevention and treatment of hypertension.

Regarding the responses in the bone tissue, especially in the femur, it was observed that the NAC groups demonstrated a reduction in the bone mineral density of the animals analyzed. Therefore, this substance should be used with caution, since it can impair tissue mineralization.

In the bone mineral density of the tibia, the NAC groups, as well as the PT, demonstrated an increase in this variable. However, in the latter case, there was no statistically significant difference ($p > 0.05$).

The difference between the decrease or lack of change in the bone mineral density of the femur and tibia, respectively, can be explained by the biomechanics of the animal. In this case, the negative effect observed in the femur did not occur in the tibia bone. It is possible that the overload and effect of the force contrary to the movement occurred differently in the two bones analyzed.

Thus, one region may have been more affected than another during climbing and running on the treadmill, which resulted in a decrease in bone mineral density of the femur in the group of animals supplemented with NAC. However, the effects of NAC on bone tissue have been poorly investigated, revealing a shortage of studies in the literature.

From the few studies found, the use of NAC together with antibiotic load in bone cement did not promote a significant effect on tissue mechanical resistance[34]. In an "in vitro" study, it was observed that the NAC protected the tissue against bacterial biofilm formation caused by staphylococci[35].

However, no studies were found that observed the effects of NAC on the bone tissue of hypertensive rats. In this case, the findings of the present study may provide subsidies for future studies since it can be considered as a pioneer in this type of investigation.

In addition, it is worth noting that bone mineral density has been shown to be reduced in the breed of animal used (SHR). The findings in the present study may raise a question about the use of NAC supplementation in relation to bone tissue since the majority of hypertensive individuals are classified as elderly and frequently use this substance.

In this case, research on the subject could contribute to the correct use of NAC supplementation, as, in this respect, not only must the benefits of supplementation be considered, but also the risks of eventual demineralization.

On the other hand, when the blood pressure variable was observed, the CNAC and TNAC groups demonstrated a greater reduction when comparing the pre and post eight weeks with the different groups of animals (-41.59 and -34.33 mmHg), respectively. However, no statistically significant difference was observed in this case ($p > 0.05$).

From this finding, we can highlight the hypotensive effect on blood pressure since previous studies have demonstrated that NAC can decrease blood pressure values [32,33]. However, further studies are needed to confirm this idea.

Finally, the T group demonstrated a lower increase in the body weight variable, although without a statistical difference ($p>0.05$) and a greater increase in variables obtained in the effort test ($p<0.05$). Studies demonstrate that physical exercise is one method to decrease body weight and increase physical capacities[22,23,28].

This process is the result of the increase in energetic demand caused by muscle contraction process[22]. Thus, the training protocol utilized in the present study demonstrates the influence on physical capacity.

Thus, the present study collaborates with the literature in investigating the effects of NAC supplementation on the bone tissue of spontaneously hypertensive rats (SHR). However, some limitations should be considered, such as the physical protocol (form, intensity, and volume) and dosage of supplementation used. Moreover, in the present study, normotensive animals were not used as a form of analysis. Therefore, future studies that seek to investigate the use of NAC under different conditions may contribute to the results found to date.

Conclusion

It is possible to conclude that NAC promoted a decrease in femoral bone mineral density. In addition, although increased in the animals of the T and TNAC groups, this variable demonstrated no statistically significant difference in the tibia. Finally, the animals trained in isolation presented better performances in the physical tests.

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