Research Paper

The mitochondria-targeted antioxidant SkQ1 but not N-acetylcysteine reverses aging-related biomarkers in rats

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Abstract: **Abstract: Although antioxidants have been repeatedly tested in animal models and clinical studies, there is no evidence that antioxidants reduce already developed age-related decline. Recently we demonstrated that mitochondria-targeted antioxidant 10-(6'-plastoquinonyl) decyltriphenylphosphonium (SkQ1) delayed some manifestations of aging. Here we compared effects of SkQ1 and N-acetyl-L-cysteine (NAC) on age-dependent decline in blood levels of leukocytes, growth hormone (GH), insulin-like growth factor-1 (IGF-1), testosterone, dehydroepiandrosterone (DHEA) in Wistar and senescence-accelerated OXYS rats. When started late in life, supplementation with SkQ1 not only prevented age-related decline but also significantly reversed it. With NAC, all the observed effects were of the lower magnitude compared with SkQ1 (in spite of that dose of NAC was 16000 times higher). We suggest that supplementation with low doses of SkQ1 is a promising intervention to achieve a healthy ageing.**

INTRODUCTION

Aging is commonly defined as progressive deleterious alterations that lead to increased risk of disease and death with advancing age [1-3]. Several potential therapeutic approaches are now available to slow down the age-related functional decline of the organism [4-17] including treatment with antioxidants [1, 18, 19]. Indeed, generation of reactive oxygen species (ROS) by mitochondria is considered as one of mechanisms of aging [20-28]. However, current antioxidants are not selective to mitochondria and that might hamper their effectiveness [29]. Over the course of seven years we investigated retardation of aging with a mitochondria-targeted antioxidant SkQ1 [10-(6'-plastoquinonyl) decyltriphenylphosphonium]. SkQ1 is an antioxidant selectively targeted to mitochondria that protects mitochondria from oxidative damage and which has been shown to decrease mitochondrial damage in animal models of oxidative stress [29, 30]. We have shown that SkQ1 increased the median lifespan of organisms and also delayed, arrested, and in some cases even reversed development of many age-related pathological traits [29-34].

A complex change of the immune system occurs with aging. Immuno-senescence is defined as decreased cellular reactivity, and imbalance between inflammatory and anti-inflammatory networks, which results in low-grade, chronic, pro-inflammatory condition also known as "inflammaging" [35-39]. Aging affects all immune cells, and it leads to high susceptibility to infections and increased mortality observed in the elderly. Therefore age-related changes in immune cells could serve as a marker of health, biological age and longevity [40].

Here we evaluated the effect of the mitochondria-targeted antioxidant SkQ1 on markers of aging in the old OXYS rats, a unique animal model of accelerated senescence and age-related diseases, as well as normal Wistar rats. OXYS rats spontaneously develop several pathological phenotypes similar to human geriatric

disorders including cataract, retinopathy, osteoporosis, high blood pressure and behavioral alterations [41-50]. Most of these manifestations of accelerated aging develop in OXYS rats between 1 and 3 months of life. Recently we showed that SkQ1 at nanomolar concentrations is capable not only to prevent decline of the immune system and development of cataract and retinopathy but also can reverse already developed pathological changes in the lens and retina of OXYS rats as well as some age-related alterations in behavior [29, 51-53].

For comparison we used N-acetyl-L-cysteine (NAC) as a non-targeted antioxidant. NAC has been shown to prevent age-related cognitive defects and oxidative decline of mitochondrial functions in the brain [54, 55].

RESULTS

Body weight

The body weight of 19-month-old rats was measured before the treatment with antioxidants. Rats were randomly assigned to control and experimental groups. OXYS rats are characterized by lower body weight in comparison with Wistar rats. Accordingly, before treatment at the age of 19 months the body weight was dependent only on the genotype ($F_{1.82}$ = 472.7, $p < 0.000$) and was lower in OXYS rats (Table 1). At the start of treatment with antioxidants experimental groups did not differ in weight ($p = 0.34$ for Wistar, $p = 0.52$ for OXYS). At the end of the 4-month treatment with NAC and SkQ1 the body weight remained lower in OXYS rats ($F_{1.82} = 245.6$, $p < 0.000$) and it was not affected by the antioxidants ($F_{2.82} = 1.6$, $p = 0.2$). A paired dependent comparison showed that body weight of OXYS rats treated with NAC at the age of 23 months was even lower than their weight at the age of 19 months ($p < 0.015$). OXYS rats treated with SkQ1 showed tendency to lose weight, whereas the body weight of control OXYS rats was not changed by the age of 23 months.

The white blood cell analyses

In agreement with earlier findings [56], we observed an age-dependent decrease in the number of lymphocytes and an increase in neutrophils (Tabl. 2). The counts of neutrophils in the peripheral blood of Wistar rats at the age of 19 months were higher than those in 3 months old rats ($p < 0.05$, comparison of group mean values); and number of neutrophils in 23 months old animals was higher than that in 19 months old rats ($p < 0.009$, paired dependent comparisons of the same animals). At the same time the counts of lymphocytes in the blood of Wistar rats decreased ($p < 0.009$ for 19 months old and $p < 0.04$ for 23 months old). In OXYS rats, these parameters also varied with age, but the increase in the counts of neutrophils and decrease in lymphocytes were statistically significant only at the age of 19 months compared with the 3-month-old animals ($p < 0.05$). Treatment of Wistar rats with NAC almost completely prevented the drop in lymphocytes/neutrophils ratio, while 4 months treatment with SkQ1 not only prevented its decrease, but actually increased this ratio almost to the level of young rats. In OXYS rats, neither aging from 19 to 23 months nor antioxidants significantly affected the white blood cell counts.

Table 1. Body weight (g) of control and SkQ1-or NAC-treated Wistar and OXYS rats at the age of 19 and 23 months

Strain	Wistar			OXYS		
Treatment	0	SkQ1	NAC	0	SkQ1	NAC
19 months	612±18	630±18	637±17	404±9*	424±14	411±6
23 months	560±27	642±20	604±27	393±8*	390±8	385±9^

*– statistically significant differences between the strains of the same age; ^ - statistically significant differences between 19- and 23-month-old rats (paired-dependent comparisons of the same animals).

Table 2. White blood cell counts in Wistar and OXYS rats at the age of 3, 19 and 23 months. Effects of 4 months treatment with SkQ1 or NAC. The results are given as % of the total number of white blood cells

Strain	Age, month	Treatment	Lymphocytes	Neutrophils	Eosinophils	Monocytes	Lymphocytes/Neutrophils ratio
Wistar	3	0	74.1±5.8	20.4±0.21	2.13±0.25	4.10±0.25	3.63±0.41
	19	0	63.0±1.32^	29.4±1.27^	3.36±0.29^	3.43±0.20	2.46±0.14^
	23	0	54.5±4.42^	38.6±4.47^	4.50±1.26	2.38±0.34^	1.77±0.29^
		SkQ1	66.9±3.19#	25.9±2.92#	4.00±0.83	3.22±0.28	2.92±0.39#
		NAC	62.4±3.72	31.7±3.79	2.93±0.66	3.00±0.33	2.26±0.34
OXYS	3	0	73.1±6.1	19.36±1.6	2.22±0.21	5.27±0.52	3.80±0.35
	19	0	66.0±0.91^	28.0±0.82^	2.77±0.20	3.0±0.21^	2.54±0.10^
	23	0	64.3±1.78*	30.3±1.91	2.53±0.45*	2.87±0.31	2.28±0.19
		SkQ1	57.1±3.17	38.4±3.05#	1.83±0.53#	2.65±0.31	1.76±0.22
		NAC	58.3±2.14	36.6±2.23	2.23±0.28	2.86±0.18	1.77±0.15

*– statistically significant difference between the strains of the same age; # – significant effect of the drug within the strain; ^ – significant age-related differences from the previous age within the strain.

Serum GH levels

The level of GH naturally was decreased with age in rats of both strains albeit more profoundly in OXYS rats than in Wistar rats ($p < 0.0001$ for age of 3 months, $p < 0.009$ for 19 months and $p < 0.008$ for 23 months). Before the treatment with antioxidants GH levels depended only on the genotype ($F_{1.42} = 60.96$, $p < 0.000$) and at the age of 19 months OXYS rats had lower levels of GH (Table 3). At the start of supplementation with antioxidants the experimental groups did not differ in the levels of GH ($p = 0.49$ for Wistar, $p = 0.52$ for OXYS).

After 4-month of treatment with NAC or SkQ1 GH levels remained lower in OXYS rats ($F_{1.42} = 49.6$, $p < 0.000$) and were affected by antioxidants ($F_{2.42} = 8.8$, $p < 0.0008$). In both Wistar and OXYS rats treated with SkQ1, GH levels were significantly higher than in control groups of the corresponding strain ($p < 0.004$ and $p < 0.02$, respectively). In Wistar rats treated with NAC - no difference was found. Yet a paired dependent comparison showed that GH levels in Wistar rats treated with SkQ1 and NAC were higher at the age of 23 months than at the age of 19 months ($p < 0.009$ and $p < 0.046$, respectively). A paired dependent comparison showed a small but significant increase in GH levels from the age of 19 months to 23 months in OXYS rats treated with SkQ1 ($p < 0.000$) or NAC ($p < 0.046$). In control OXYS rats GH levels decreased significantly ($p < 0.02$) by the age of 23 months. In addition, SkQ1 treated OXYS rats and control Wistar rats of the same age (23 months) did not differ in GH levels**.**

Serum IGF-I levels

At the age of 3 months, serum IGF-1 levels were maximal in Wistar and OXYS rats and there was no difference in IGF-1 levels between the strains. IGF-1 levels decreased in rats with age, more profoundly in OXYS rats than in Wistar rats ($p < 0.002$ for the age of 19 months and $p < 0.048$ for 23 months). Before treatment with antioxidants IGF-1 levels were dependent on the genotype ($F_{1.42} = 12.3$, $p < 0.002$) and at the age of 19 months IGF-1 was lower in OXYS rats than in Wistar rats (Table 3). At the start of treatment with antioxidants experimental groups did not differ in the levels of IGF-1 ($p = 0.87$ for Wistar and $p = 0.81$ for OXYS rats).

After 4 months treatment with antioxidants IGF-1 levels were dependent on the genotype, ($F_{1.42} = 8.1$, $p < 0.008$) and were affected by antioxidants ($F_{2.42} = 19.4$, $p < 0.00$). In Wistar rats treated with either SkQ1 or NAC, IGF-1 levels were significantly higher ($p < 0.0002$ and $p < 0.014$, respectively) than in the control group. The paired dependent comparison showed that IGF-1 levels in 23-month-old Wistar rats treated with either NAC or SkQ1 were even higher than they have been at the age of 19 months ($p = 0.032$), while in the control rats IGF-1 was significantly lower ($p < 0.001$).

Table 3. Levels of GH, IGF-1, DHEA-S and testosterone (ng/ml) in serum of intact Wistar and OXYS rats at the age of 3, 19 and 23 months and of rats treated with SkQ1 or NAC from the age of 19 months to 23 months

Strain	Age, month	Treatment	GH	IGF-1	Testosterone	DHEA
Wistar	3	0	8.14±0.2	535±62	1.42±0.3	-
	19	0	5.28±0.1+	483±7	0.61±0.04+	0.66±0.01
	23	0	4.98±0.2+	448±12+	0.60±0.07	0.63±0.02+
		SkQ1	5.50±0.1 #^	500 ± 9#	0.61±0.05	0.66±0.01
		NAC	5.37±0.2^	486±10#	0.61±0.03	0.66±0.03
OXYS	3	0	6.24±0.3*	498±64	1.99±0.31	-
	19	0	4.57±0.1 *+	444±7*+	0.64±0.06+	0.58±0.01*
	23	0	4.27±0.1*+	399±9*+	0.56±0.02	0.55±0.03*+
		SkQ1	4.80±0.1#^	499±9 #^	0.58±0.02	0.60±0.02^
		NAC	4.58±0.08^	449±13#	0.57±0.02	0.59±0.02

*– statistically-significant difference between the strains of the same age; # – significant effect of the drug within the strain; +– significant age-related differences from the previous age within the strain, ^– statistically-significant difference between the levels before and after treatment with SkQ1 or NAC (paired-dependent comparisons of the same animals).

From the age of 19 to 23 months IGF-1 levels in control group of OXYS rats also decreased significantly ($p < 0.001$). In OXYS rats treated with either SkQ1 or NAC, IGF-1 levels were significantly higher than in the control group ($p < 0.013$ and $p < 0.031$, respectively). The paired dependent comparison showed that in rats treated with NAC, at the age of 23 months IGF-1 levels remained similar to the 19-month-old animals, whereas, in the rats treated with SkQ1 its level increased significantly ($p < 0.000$). In addition, in 23-month-old OXYS rats treated with NAC, IGF-1 levels were similar to those in Wistar control rats of the same age, but in OXYS rats treated with SkQ1, IGF-1 levels were similar to IGF-1 levels of young Wistar rats.

Serum testosterone and DHEA levels

ANOVA analyses showed that the level of testosterone in Wistar and OXYS rats was maximal at the age of 3 months and decreased by the age of 19 months in both strains ($F_{2.62} = 74.7$, $p < 0.00$) and remained unchanged by the age of 23 months. Treatment with NAC and SkQ1 had no effect on the testosterone level (Table 3).
We did not measure DHEA in three-month old animals. At the age of 19 months, DHEA level was slightly lower in OXYS rats ($F_{1.42} = 19.2$, $p < 0.0001$). In 23 months old Wistar and OXYS rats, level of DHEA differs only slightly from that in 19 months old animals (Table 3).

DISCUSSION

Our results indicate that when started late in life, treatment with SkQ1 not only prevented age-related decline, but also partially reversed it. Effects of NAC were of the lower magnitude compared to SkQ1, despite the higher dose of NAC used.

One reason for body weight loss in old age is sarcopenia - a gradual decline in muscle mass. After 4 months treatment with antioxidants the body weight of OXYS and Wistar rats decreased only slightly. In SkQ1 treated group weight was very similar to weight of control rats in both rat strains. These findings are consistent with unpublished data of L.E. Bakeeva and V.B. Saprunova, who found that SkQ1 reduced the age-related decline in muscle mass in OXYS and Wistar rats. Another reason for body weight reduction in old age is osteoporosis, which results in increased risk of fractures. Feeding SkQ1 prevented loss of mineral content associated with senile osteoporosis in OXYS rats [34]. In our study the paired dependent comparison showed that body weight decreased significantly ($p < 0.015$) only in OXYS rats treated with NAC.

White blood cells (WBC) counts could serve as a marker of health, biological age and longevity. In humans, lymphocytes increase early in life until age of 16-21 years [56]. Some studies indicate that number of

lymphocyte decreases with age [57-59]. In line with these findings, we observed that the lymphocyte/neutrophil ratio was high in the 3-month-old Wistar and OXYS rats and decreased with age: the counts of lymphocytes were decreased and neutrophils were increased. Lymphocytes are important effector cells and therefore their activation is essential for immune responses [57]. Diminished lymphocyte production and function are major contributors to disease in elderly [56]. 4 months treatment with NAC almost completely prevented the decrease in ratio between lymphocytes and neutrophils in 23-month-old Wistar rats compared with 19-month-old ones. SkQ1 increased the lymphocyte/neutrophil ratio, and thereby partially reversed decline of this parameter, which was observed at the age of 19 months. Our present results in Wistar rats are in line with the previous reports that NAC [60, 61] and SkQ1 [32,58] prevent the age-linked decrease in lymphocyte level in mice. However, both antioxidants did not affect the blood lymphocyte/neutrophil ratio in OXYS rats. The age-related decrease in lymphocytes is a consequence of involution of thymus, the major organ of lymphocyte maturation. The OXYS rats exhibit accelerated involution of the thymus and SkQ1 reduces age-related thymic involution in both OXYS and normal Wistar rats [52]. It is possible that lack of SkQ1 and NAC effects on the WBC count in old OXYS rats is associated with impairment in bone marrow hematopoiesis. We have previously reported the age-associated changes in the functional status of hematopoietic stem cells in OXYS rats [62]. It can be assumed that in OXYS rats the lymphocyte/neutrophil ratio was already stabilized at low level in the 19 month-old rats so that antioxidants could not have a favorable effect. Antioxidants had no effect on the levels of eosinophils and monocytes in both rat strains.

Circulating GH levels are at the highest during the neonatal period; they decrease during childhood, peak again during puberty and fall dramatically in the elderly [63]. A reduction in GH level in older humans and rodents correlates with a decline in serum levels of an anabolic mediator IGF-1 [64]. The present study confirmed an age-dependent GH and IGF-1 decrease in rats of both strains. In addition, we showed that the serum GH level in all studied groups of OXYS rats was lower than in age-matched Wistar rats. Interstrain differences were highest in the three-month old animals (23%), while at the age of 19 and 23 months difference was 13% and 14%, respectively. There were no interstrain differences in the blood levels of IGF-1 in the 3-month-old animals but at the ages of 19 and 23 months they were slightly (but statistically significant) reduced in OXYS rats (by 9 and 11%, respectively).

Our study also showed for the first time that NAC supplementation from the age 19 to 23 months fully prevented the GH and IGF-I decline in both Wistar and OXYS rats. SkQ1 not only stopped the decline in hormone levels between the ages of 19 and 23 months, but it also increased the levels of GH and IGF-I above the levels of those found in 19 month-old animals (Table 3). It is well known that GH/IGF-I plays an important role in brain aging [65, 66]. Age-related reduction in the activities of somatotropic axis may influence brain function in the elderly [67]. Recently we have shown that SkQ1 treatment of the middle-aged (12 month) Wistar and OXYS rats had beneficial effects on the locomotor and exploratory activity. SkQ1 also decreased anxiety compared to age-matched controls as well as significantly improved visual ability of the OXYS rats, which suffered from retinopathy and cataract [49]. In the present study, we observed a positive effect of SkQ1 and NAC on the behavior of rats of both strains (data are not shown). In the last series of experiments, we studied effect of SkQ1 on the levels of growth hormone and IGF-1 in 3-month-old rats (data are not shown). We found that SkQ1 caused small (about 25%) but statistically valid increase in the blood hormone level. In addition, SkQ1 prevented the development of retinopathy and cataract and had beneficial effects on behavior, learning ability and memory of OXYS rats. We suggest that the recovery of GH - IGF-I in old age to the levels of those in young age can have a positive impact on the function of the aging brain and the immune system.

At the age between 3 and 19 months, the testosterone levels fell and then remained unchanged between 19 and 23 months in rats of both strains. It was not surprising that there was no interstrain difference in the testosterone level even in old animals. As was previously shown in our group, OXYS males demonstrate an early decrease in sexual motivation; however a decrease in hormonal component of sexual behavior was not detected in aged OXYS males [68]. Recently we showed that SkQ1 is effective not only in preventing but also in reducing already developed age-related decline in male sexual behavior [69]. In the present study, we did not evaluate the sexual behavior, and neither SkQ1 nor NAC treatments affected testosterone levels in period between 19 and 23 months (Table 3). Noteworthy, NAC partially inhibits the mTOR (Target of Rapamycin) pathway [70]. Given the involvement of mTOR in cellular and organismal aging as well as age-related diseases [71-78], slight inhibition of mTOR may contribute to the therapeutic effects of this non-selective antioxidant.

DHEA and its sulfate-bound form (DHEAS) are important precursors of sex steroid hormones. Structure of DHEA is similar to testosterone and levels of both hormones reach their maximal levels in puberty and decrease dramatically with age [79]. Given its multiple metabolic effects, this decline in DHEA levels has been thought to play a role in the aging process [80]. In this study, we can assume that the DHEA levels are maximal in 3-month-old Wistar and OXYS rats. At the age between 19 and 23 months, DHEA levels decreased and SkQ1 increased DHEA only slightly (by 5%).

Deficiencies in multiple hormones are a biomarker of health status in older persons [79].

Here we conclude that SkQ1 not only prevented age-associated hormonal alterations but partially reversed them. These results suggest that supplementation with low doses of SkQ1, even in chronologically and biologically aged subjects seem to be a promising strategy to maintain health and retard the aging process.

MATERIALS AND METHODS

Animals and diet. Male senescence-accelerated OXYS and age-matched male Wistar rats were obtained from the Breeding Experimental Animal Laboratory of the Institute of Cytology and Genetics (ICG), Siberian Division of the Russian Academy of Sciences (Novosibirsk, Russia). All the experiments on rats were carried out according to Animal Care Regulations of ICG Institute of Cytology and Genetics, Novosibirsk. The OXYS rat strain was established based on Wistar rat strain at the Institute of Cytology and Genetics as described earlier [51, 52] and registered in the Rat Genome Database (http://rgd.mcw.edu/). At the age of 4 weeks, the pups were taken away from their mothers and housed in groups of five animals per cage (57×36×20 cm) and kept under standard laboratory conditions (at 22±2^{o}C, 60% relative humidity, and natural light), provided with a standard rodent feed, PK-120-1, Ltd. (Laboratorsnab, Russia), and given water ad libitum.

Starting from the age of 19 months OXYS and Wistar rats were randomly assigned to three groups (n = 17-24): control diet, diet supplemented with 250 nmol SkQ1 (synthesized as described earlier [33]) or 650 mg NAC (MP Biomedicals, LLC, France) per kg of body weight per day. The weight was measured before the start of treatment and at the end of experiment.

Hormone levels and the white blood cell counts. The levels of GH, IGF-1, testosterone and DHEA-S in the blood serum were analyzed before and after treatment with SkQ1 or NAC in OXYS and Wistar rats (two times total for each rat) and compared with intact 3-month-old rats (n=10) of the same strains. GH, DHEA were measured by ELISA (Creative Diagnostics, USA). IGF-1 was measured by ELISA (ALPCO Diagnostics, Salem, NH). Testosterone was measured by ELISA (JSC Vector-Best, Russia). The assays were run using the manufacturer's instructions. For WBC counts peripheral blood was drawn from the tail vein. Blood smears were fixed in methanol and subsequently stained with Wright-Giemsa.

Statistical analysis. The data were analyzed using repeated measures ANOVA and nonparametric tests with the statistical package Statistica 6.0. Two-way ANOVA was used to evaluate the differences between rat strains (genotypes) and effects of treatment (antioxidants). To validate the effect of the diets on parameters, the genotype and antioxidants were chosen as independent variables. A Newman–Keuls *post hoc* test was applied to significant main effects and interactions in order to estimate the differences between particular sets of means. One-way ANOVA was used for individual group comparisons. Data are represented as mean ± S.E.M. Comparisons between means were analyzed with one-way or repeated measures analysis of variance (ANOVA). Results were considered statistically significant if p value was less than 0.05.

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Conflict of Interest Statement

The authors of this manuscript have no conflict of interests to declare.

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