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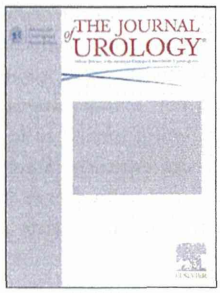

Long-term caloric restriction in rats may prevent age-related impairment of *in vitro* bladder function

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Title:

Long-term caloric restriction in rats may prevent age-related impairment of *in vitro* bladder function

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Abstract

Purpose: Bladder function is often impaired with aging. In other organs, caloric restriction (CR) has been shown to have a prophylactic effect on biological changes associated with aging. We aimed to test the hypothesis that long-term CR can prevent age-related impairment of bladder function in the rat.

Materials and Methods: Fischer 344 male rats were divided into three groups: young (6 months-old) fed *ad libitum* with normal food (Y, N = 16), old (25-28 months-old) fed *ad libitum* with normal food (O+AL, N = 15), and old (25-28 months-old) that had been fed with normal food three days a week since 6 weeks-old (O+CR, N = 16). Frequency volume (FV) measurements, *in vitro* organ bath functional studies using full-thickness of longitudinal detrusor strips, evaluation of muscarinic- and purinergic-receptor mRNA expressions, and histological examination with Masson-trichrome staining of the bladder tissues were performed.

Results: In the FV measurements, no significant differences were found among the three groups. The O+AL group showed weaker contractile responses to carbachol and electrical field stimulation (especially in the cholinergic component), lower expression of M_3-receptor mRNA and higher collagen deposition compared with the Y group. These age-related changes in the bladder were milder in the O+CR group than the

O+AL group.

Conclusions: This study suggests that in the rat, long-term CR has a preventive effect against age-related functional and morphological changes of the bladder, including the impairment of detrusor contractility that may be related to decreased expression of M_3 receptors and to fibrosis of the bladder wall.

Introduction

Detrusor hyperactivity with impaired contractile function (DHIC), as well as impaired bladder emptying, are commonly observed among elderly. [1, 2] Regarding an age-related impairment of detrusor contractility, previous histological examinations indicated an age-related reduction of the density of the smooth muscle and increased collagen deposition, [3] which may lead to contractile impairment. [4] In addition, another *in vitro* study using human detrusor smooth muscle revealed decreased cholinergic neurotransmission and compensatory increased purinergic transmission with aging. [5] However, this age-related impairment of detrusor contractility is not always found clinically and is still controversial. [6]

An appropriate study to clarify the pathophysiology of this impairment is needed, but to perform such an investigation in humans faces many limitations. In the elderly, there are large indirect interactions from comorbidities such as hypertension, diabetes mellitus, hyperlipidemia, and cardiovascular diseases, and it is difficult to separate the influence of such disorders from that of age. The influence of at least some of such co-morbidities can be avoided in animal models. However, studies in animals have given contradictory findings concerning age-related functional changes. For example, previous *in vitro* functional studies showed that age-related detrusor

contractility was decreased [7] or unchanged. [8]

Currently, CR is receiving much attention, and its anti-aging effects on various biological functions including prophylactic effects against age-related oxidative stress, fibrosis, and chronic inflammation in rodents [9, 10] and in humans [11, 12] have been emphasized. **In addition, intermittent fasting has been widely accepted as a version of CR, [13] and previous rodent studies showed that intermittent fasting extends life span and increases resistance to major age-related diseases, such as type 2 diabetes, cardiovascular disease or cancers.[13, 14]** However, to the best of our knowledge, there is no report on prophylactic effects of CR **associated with intermittent fasting** on the age-related functional or morphological changes that occur in the urinary bladder.

In the present study, we tested the hypothesis that CR can protect against age-related impairments of bladder function. This was done by comparing results of FV measurements, *in vitro* contractile and relaxant studies of detrusor strips, histological examinations, and blood chemical analyses in young rats and in old animals without or with long-term CR treatment.

Materials and methods

Animals and experimental groups

Male Fischer 344 rats (Charles River Laboratories International, Inc. Yokohama, Japan) were divided into three groups: Y (6 months-old, *ad libitum* fed with normal food, N = 16), O + AL (25-28 months-old, *ad libitum* fed with normal food, N = 15) and O + CR (25-28 months-old, that had been intermittently fed three days (Monday, Wednesday, and Friday) a week since 6 weeks-old, N = 16). The O + CR groups showed approximately 40% caloric restriction compared with the O + AL groups. The rats were maintained under standard laboratory conditions with a 12:12 h light (9:00-21:00): dark (21:00-9:00) cycle, and free access to food pellets and tap water. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Tokyo and Tokyo Metropolitan Hospital and Institute of Gerontology, and conformed to NIH guidelines for the care and use of experimental animals.

FV measurements

The rat was placed without any restraint in a metabolic cage (MCM/TOA-UF001-006, Mitsubishi Chemical Medience, Tokyo, Japan). This cage has a specially designed net enabling precise measurement of voided urine volume. [15] After

24-h adaptation, voided volume, voiding frequency and water intake volume were recorded using a PowerLab® (AD Instruments, Sydney, Australia) data acquisition system continuously for 24 h starting at 9:00 with a 12:12 h light (9:00-21:00): dark (21:00-9:00) cycle.

In Vitro Functional Studies of Detrusor Strips

This investigation was performed as described in a previous report. [16] In brief, the bladder body was harvested after the rat was sacrificed with an overdose of pentobarbital sodium. Subsequently, longitudinal full-thickness bladder strips were transferred to 5-ml organ baths. After a 2-h equilibration period with a stable tension of 10 mN for the contractile and 5 mN for the relaxant experiments, the investigations were started. The strip was first exposed to a high K^+ (62 mM KCl) Krebs solution, and then the contractile responses to the following stimuli were examined: 1) CCh (10^{-8} M to 10^{-3} M), 2) ATP (10^{-6} M to 10^{-2} M), or 3) EFS (pulse-width: 0.8 ms, 50 V, pulse duration: 5 s, stimulation interval: 1 m, at 2, 5, 10 and 20 Hz). After baseline measurements, contractions were again evoked by EFS after 10^{-6} M atropine exposure, purinoceptor desensitization by repeated administrations of 10^{-5} M mATP, and finally 10^{-6} M TTX. In separate specimens, relaxant responses to isoproterenol (10^{-10} M to 10^{-4}

M) were evaluated under a mechanical increase in tension of 5 mN. Papaverine (10^{-3} M) was applied afterwards as a reference drug to induce relaxation.

Real-time RT-PCR analysis

The bladder body was dissected out and immediately placed in the RNA*later* RNA Stabilization Reagent (QIAGEN, Venlo, Netherland). Total RNA extracted from tissues using the miRNeasy Mini Kit (QIAGEN) was reverse transcribed to cDNA by the SuperScript VILO Master Mix (Life technologies, Carlsbad, CA, USA) according to the manufacturer's procedure. For relative quantification of mRNA expression, real-time PCR was performed using the Power SYBR Green PCR Master Mix and gene specific primers on the StepOnePlus Real-Time PCR System (Life Technologies). Primer sequences used are shown in Table 1. **The primer sequences of M1, M3 and P2X1 receptors were originally designed. Regarding M2 receptor, we used a commercially available primer (Takara Bio Inc., Shiga, Japan). Relative expression levels of each receptor were calculated from Ct values using the standard curve methods and Gapdh gene as an internal control for normalization.**

Histological examinations

Isolated bladder specimens were fixed in 4% paraformaldehyde-PBS, then

embedded in paraffin and cut into 3-μm sections. Masson-trichrome staining was performed to analyze fibrosis in both the detrusor muscle layer and in the whole bladder wall. [7] The collagen-deposition was determined in three randomly selected sections. [17] The images were analyzed using Adobe and Image J software (http://rsb.info.nih.gov/ij/). [18]

Heart rate and blood pressure measurements

The rat was placed in a restraint cage and heart rate and blood pressure were measured by tail-cuff plethysmography (BP-98A-L; Softron, Tokyo, Japan). The measurements were performed three times in each rat and the values were averaged.

Blood chemical analysis

Rats were anaesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg). The peritoneal cavity was opened through a mid-line abdominal incision, and whole blood (approximately 4 ml) was harvested from the inferior vena cava. The blood chemistry data were analyzed by SRL, Inc. (Tokyo, Japan) using routine enzymatic assays, and the serum parameters shown in Table 3C were measured.

Drugs

CCh, ATP, atropine, and TTX were purchased from Wako Chemical Co. (Tokyo, Japan). mATP and isoproterenol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Papaverine was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The Krebs solution was of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, NaHCO3 25.0, KH_2PO_4 1.2, glucose 11 (pH 7.4).

Statistical Analysis

All data are expressed as the mean ± SEM values. Results were analyzed using **Kruskal-Wallis H test with a post hoc test for multiple comparisons between groups. Statistical analysis was performed using Statistical Package for Social Sciences, version 22 (SPSS, Chicago, IL).** *P* values <0.05 was considered statistically significant.

Results

FV measurements (Table 2)

The water intake was significantly higher in the O+CR group than the O+AL group, and tended to be higher than the Y group (p=0.058). The O+CR group also showed a significant higher food intake than the Y and O+AL groups. There were no significant differences in any of the urodynamic parameters investigated among the three groups (N = 8 in each group).

In Vitro Functional Studies on Detrusor Strips

Contractile responses

There were no significant differences in contractile responses to high K^+ (n = 23 – 24, N = 7 - 8 in each group) or ATP (n = 7 - 8, N = 7 - 8 in each group) among the three groups (Figures 1A and 1C, respectively).

The Emax value for CCh of the O + AL group was significantly lower than that of the Y group, whereas the Emax values were not significantly different between O + CR and Y groups. There were no significant differences in the CCh pEC50 values among the groups (n = 8, N = 8 in each group, Figure 1B).

The amplitudes of contractions induced by EFS were lower in the O + AL

group (n = 8, N = 8) than in the Y group (n = 8, N = 8), and the difference was significant at 20 Hz, whereas there were no significant differences between the Y and O + CR groups (n = 8, N = 8 in each, Figure 2A). The cholinergic component, **which was sensitive to atropine administration,** was significantly less in the O + AL than in the Y group at all frequencies. The response in the O + AL group was significantly lower than those of the O + CR group at 2 and 5 Hz (Figure 2B), whereas the purinergic component, **which was desensitized by repeated administrations of mATP,** showed no significant differences among the three groups (n = 8, N = 8 in each, Figure 2C).

Relaxant responses

There were no significant differences in the relaxant responses to isoproterenol among the three groups (n = 8, N = 8 in each, Figure 1D).

Real-time RT-PCR analysis (Figure 3, n = 8, N = 8 in each group))

The expression of the M_2 receptor was significantly lower in the O + AL and O + CR groups than the Y group, and no significant differences were found between O + AL and O + CR groups. The expression of the M_3 receptor was significantly lower in the O + AL group than the Y group, but the M_3 receptor expression in the O + CR group was not significantly different from that in the Y group. There were no significant

differences in the expressions of M_1 or $P2X_1$ receptors among the three groups.

Histological examinations

The collagen-deposition was significantly higher in both detrusor and whole bladder wall of the O + AL group compared with the Y and O + CR groups. The values were not significantly different between the Y and O + CR groups (Figures 4A-C, n = 7 - 8, N = 7 - 8 in each group).

Body and bladder weights, heart rate and blood pressure

Body and bladder weights of the O + AL group were significantly higher than those of the Y and O + CR groups, and additionally those of the O + CR group were significantly lower than those of the Y group (Table 3A, N = 15 - 16 in each group).

There were no significant differences in heart rate and blood pressure among the three groups (Table 3B, N = 8 in each group).

Blood chemical analysis (Table 3C, N = 15 - 16 in each group)

Compared with the Y group, the O + AL group had significantly higher values of LDL- and total-Chol, and insulin, and lower values of testosterone. The O + CR

group showed significantly lower values of LDL-Chol, triglyceride and free fatty acid than the Y group. The O + AL group showed significantly higher values of LDL- and total Chol, triglyceride, free fatty acid and insulin, and lower values of testosterone and E2 compared with the O + CR group.

Discussion

In the present study, the contractile responses to high K^+ of the detrusor strips were similar in all groups, indicating no impairment of the intrinsic contractile properties of the detrusor smooth muscle with aging. This is supported by findings in isolated strips of human bladder, showing no decline in the detrusor contractility with age. [19] In contrast, the contractile responses to muscarinic stimulation and EFS were impaired in the O + AL group compared with the Y group, which is consistent with a previous report in rats. [7] The contractile responses to EFS after atropine and mATP pre-treatment suggested that this was mainly due to an impairment of the cholinergic component. This finding is consistent with a previous study reporting a decreased cholinergic component and a compensatory increased purinergic component of contraction in the aged human bladder, [5] **although the compensatory increase of purinergic component was not observed in the aged rats.**

To disclose a mechanism behind the decreased cholinergic contractility of the O + AL group, the expressions of muscarinic (M_1-M_3) and purinergic ($P2X_1$) receptors were evaluated by real time RT-PCR. These experiments revealed that the expression of muscarinic M_3 receptors, but not $P2X_1$ receptors, significantly decreased in the O + AL group compared with the Y group, suggesting that the impairment of the detrusor

contractility with aging is, at least partly, due to a reduced expression of muscarinic M_3 receptors.

A previous report indicated that the collagen deposition in the bladder wall increased with aging in Wistar male rats. [7] In the human bladder, widespread degeneration of muscle cells and axons were proposed to correlate with the impaired detrusor contractility of the aged detrusor. [20] Other previous histological examinations indicated an age-related increase of collagen deposition, [3] which may lead to contractile impairments. [4] In agreement with these previous findings, the present histological analysis indicated that fibrosis is progressing with age in the rat bladder. Thus, age-related impairment of detrusor contractility may be linked with fibrosis directly or indirectly.

In contrast to the changes in detrusor contractility and collagen deposition with aging demonstrated in the present study, FV measurement failed to show significant influence of aging on voiding behaviors **except for feeding behaviors.** The reasons why the functional and morphological changes *in vitro* were not reflected in *in vivo* voiding behavior are not known. It may be speculated that *in vivo* there are compensatory mechanisms keeping voiding behavior within normal limits. **Another possible reason is that the insufficient number of FV measurement might mask the**

age-related changes of in vivo voiding behaviors. In a previous study, aged rats showed lower voiding frequency with higher voided volume per micturition, [21] and another previous study using cystometry demonstrated that aged rats showed higher bladder capacity and post-void residual volume compared with young rats. [7] These discrepancies may be due to experimental differences including the strains and ages of the rats investigated.

The relaxant responses to isoproterenol were not different between the groups. This is in contrast to previous investigations showing that β-adrenoceptor mediated relaxation is decreased with aging. [22, 23] Again, the discrepancies may be due to differences in experimental techniques, species and ages of animals used.

Interestingly, the present *in vitro* functional investigation clearly demonstrated that long-term CR had a preventive effect against the age-related detrusor contractile impairments. In addition, long-term CR restored the reduced M_3 receptor expression and decreased collagen deposition to the levels of the Y group. A possible prophylactic effect of CR on age-related fibrosis has been demonstrated in the rat aorta and heart. [24] Our findings are the first demonstration of the preventive effects of CR against the age-related impairments of the detrusor contractility that may be linked to decreased M3 receptor expression and progressive fibrosis.