and patient selection, the risk of disease recurrence remains high; the 5-year recurrence-free survival (RFS) and overall survival (OS) after RC are reportedly 48% to 70% and 57% to 60%, respectively.[3-5] Once patients experience recurrence after RC, their median survival time (MST) is approximately 15 months,[6] and even shorter if they do not undergo salvage chemotherapy.[7]

Postoperative cisplatin-based systemic chemotherapy has been used to prevent recurrence.[8] Although 2 previous randomized controlled trials (RCTs) showed a survival benefit for presurgical chemotherapy (neoadjuvant chemotherapy [NAC]),[9,10] it has not been widely adopted in practice.[8] In contrast, postoperative systemic chemotherapy (adjuvant chemotherapy [AC]) has been used more often; David et al reported that in stage III bladder cancer, NAC and AC are applied in 1.4% and 10.4% of cases, respectively.[11] A recent meta-analysis of 9 RCTs including 945 participants revealed a statistically significant benefit of AC on OS in muscle-invasive bladder cancer (hazard ratio [HR], 0.77; 95% confidence interval, 0.59-0.99; $P = .049$).[12] However, most RCTs failed to prove the benefit of AC, and the optimal targets of AC have never been identified.[13-15]

In our institution, 3 cycles of cisplatin-based AC have been offered to patients with non–organ-confined bladder cancer (pT3-4

Table 1 Clinicopathologic Characteristics of the Patients

Variable	AC After RC	RC Alone	*P* Value
No. of Patients	39	22	
Male/Female	34/5	21/1	.404
Age, Median (IQR)	62 (58-69)	72 (63-79)	.004
BMI, Median (IQR)	22.5 (21.4-25.3)	21.4 (18.8-23.4)	.024
Form of Urinary Diversion			.023
Ileal conduit (%)	24 (61.5)	12 (54.5)	
Ileal neobladder (%)	12 (30.8)	4 (18.2)	
Continent reservoir (%)	2 (5.1)	0 (0.0)	
Ureterocutaneostomy (%)	1 (2.6)	6 (27.3)	
Estimated Blood Loss, Median (IQR) (mL)	1620 (1215-2290)	1550 (1240-2280)	.781
Allogeneic Blood Transfusion (%)	11 (28.2)	13 (59.1)	.029
Postoperative GFR <60 mL/min (%)	16 (41.0)	9 (40.9)	.993
Postoperative Complications of Clavien-Dindo Classification Grade 3	2 (5.1)	6 (27.3)	.038
Performance Status			
≤1	36	16	.089
2	3	6	
pT Stage (%)			
<pT2	3 (7.7)	0 (0.0)	.256
pT2	2 (5.1)	3 (13.6)	
pT3	23 (59.0)	16 (72.7)	
pT4	11 (28.2)	3 (13.6)	
Nuclear Grade (%)			
Grade 1/Grade 2	4 (10.3)	3 (13.6)	.746
Grade 3	33 (84.6)	19 (86.4)	
pN (%)			
pN0	18 (46.2)	12 (54.5)	.621
pN1	9 (23.1)	6 (27.3)	
pN2	10 (25.6)	4 (18.2)	
pN3	2 (5.1)	0 (.0)	
Lymph Nodes Removed, Median (IQR)	17 (10-25)	14 (9-24)	.373
Metastatic Lymph Nodes, Median (IQR)	1 (0-2)	0 (0-1)	.739
Lymphovascular Invasion (%)	36 (92.3)	15 (68.2)	.015
Positive Surgical Margins (%)	5 (12.8)	2 (9.1)	.661
Cisplatin-Based AC			
MVAC/GC (%)	28 (71.8)/11 (28.2)		
Median AC cycles (IQR)	3 (2-3)/3 (3-3)		

Abbreviations: AC = adjuvant chemotherapy; BMI = body mass index; GC = gemcitabine and cisplatin; GFR = glomerular filtration rate; IQR = interquartile range; MVAC = methotrexate, vinblastine, doxorubicin, and cisplatin; RC = radical cystectomy.

or pN1-3, or both). In this study, the outcomes were compared between patients with non–organ-confined bladder cancer receiving AC and those not receiving AC, and the predictors of survival benefit were analyzed.

Patients and Methods

This study was approved by our institutional review board (No. 3124). We reviewed medical records of all patients undergoing RC with curative intent at our institution from 1990 to 2012 and collected data for locally advanced (pT3-4) or node-positive (pN1-3) disease, or both, for the present study. Patients who received NAC or perioperative radiotherapy or those with early recurrence or mortality (within 8 weeks after RC) were excluded. It has been our policy to recommend 3 cycles of AC for patients with non–organ-confined bladder cancer, although not all patients choose to receive treatment.

Cystectomy specimens were restaged based on the 2009 Union for International Cancer Control TNM system.[16] Glomerular filtration rate was evaluated by the revised formula for Japanese participants,[17] and body surface area was evaluated by the formula of Du Bois.[18]

Until 2008, the AC protocol had been MVAC (methotrexate, vinblastine, doxorubicin, and cisplatin), and thereafter GC (gemcitabine and cisplatin) was used. Three cycles of AC were offered, but some patients failed to complete the protocol because of poor tolerance, and a few underwent 4 courses at the physician's discretion. The dose of chemotherapeutic agents was reduced by 25% to 50% in cases of impaired renal function or severe adverse events, or both, in the previous courses.

Follow-up comprised physical examination, serum biochemical profile, urine cytologic analysis, chest radiography or computed tomography, and abdominopelvic computed tomography. Bone scintigraphy was performed when clinically indicated.

Clinicopathologic characteristics were compared between the 2 groups using the χ^2 test or the Mann-Whitney *U* test. RFS and CSS from the date of surgery were calculated using the Kaplan-Meier method, and the difference was tested with a log-rank test. Multivariate analyses were carried out using a Cox proportional hazards regression model. StatView-J 5.0 (SAS Institute, Inc, Cary, NC) was used. *P* values less than .05 were considered significant.

Results

Of the 187 patients undergoing RC, 64 were identified as having non–organ-confined bladder cancer. Three patients were excluded from the analysis because of early recurrence (n = 2) and postoperative mortality (n = 1). Of the remaining 61 patients, 39 (64%) received AC after RC (AC group), and 22 (36%) did not (non-AC group) because they declined AC treatment (n = 10), had a protracted recovery after RC (n = 5), or had insufficient renal function (n = 3), comorbidities (n = 2), or advanced age (n = 2). AC was initiated between 6 and 10 weeks after RC and was completed in 28 patients (72%).

Clinicopathologic characteristics of the patients are shown in Table 1. The median age was 64 years (interquartile range [IQR], 59-75 years), and the median follow-up period was 29 months (IQR, 17-59 months). The patients receiving AC were significantly younger (*P* = .004) and had a higher body mass index (BMI) (*P* = .024) and a lower perioperative blood transfusion rate (*P* = .029). Two (5.1%) patients in the AC group (ileus and postoperative bleeding) and 6 (27.3%) in the non-AC group (ileus in 4, intestinal fistula in 1, and lymphocele in 1) had postoperative complications of grade 3 or more according to the Clavien-Dindo classification (*P* = .038).[19] There were no significant differences in pT stage, pN stage, nuclear grade, and renal function between the 2 groups (Table 1).

During follow-up at a median of 29 months, 25 of 39 (64%) patients in the AC group and 15 of 22 (68%) patients in the non-AC group ultimately experienced recurrence/metastasis. Salvage systemic chemotherapy was administered in 68% (17 of 25) and 47% (7 of 15) of the AC and non-AC groups, respectively (*P* = .205). Nineteen (49%) patients in the AC group and 15 (68%) patients in the non-AC group died of recurrent bladder cancer.

RFS was better for the AC group, but the difference was not statistically significant (MST, 23.7 vs. 11.4 months; *P* = .154)

Figure 1 **(A) Recurrence-Free Survival (RFS). The AC Group Showed Better RFS Than Did the Non-AC Group, but the Difference Was Not Statistically Significant (Median Survival Time [MST] 23.7 vs. 11.4 Months, Respectively; *P* = .154). (B) Cancer-Specific Survival (CSS) of Patients Having Adjuvant Chemotherapy (AC) After Radical Cystectomy (RC) (AC Group) and Those Having RC Alone (Non-AC Group). CSS Was Significantly Better for the AC Group Than for the Non-AC Group (MST, 57.4 vs. 17.9 Months, Respectively; *P* = .008)**

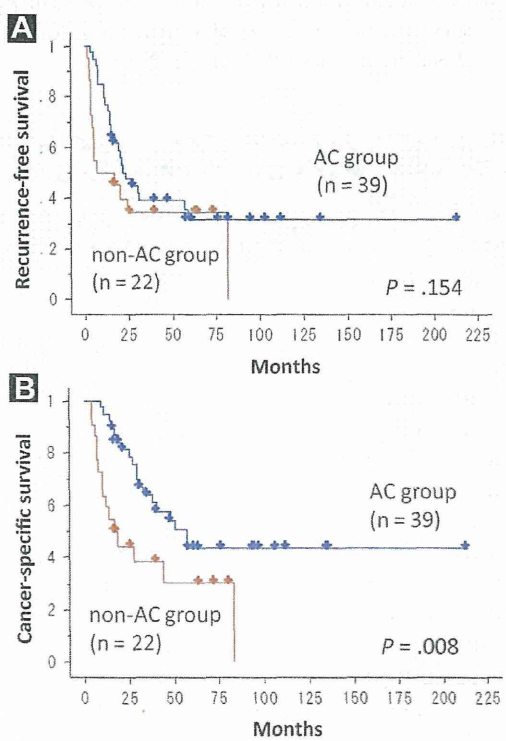


Table 2 Univariate and Multivariate Cox Regression Analysis of Recurrence-Free Survival

Variable	Referent	Univariate			Multivariate		
		HR	95% CI	*P* Value	HR	95% CI	*P* Value
Age, years		0.997	0.937-1.028	.825	0.984	0.946-1.022	.403
Female Sex	Male	0.593	0.182-1.934	.386			
Performance Status 2	≤1	1.416	0.592-3.386	.434	1.324	0.496-3.534	.576
BMI		0.945	0.842-1.060	.334	0.901	0.779-1.043	.165
GFR ≥60 mL/min	<60	0.986	0.520-1.870	.965			
Transfusion Yes	No	1.567	0.836-2.932	.161	1.691	0.851-3.360	.134
Complication Grade 3	No	0.914	0.324-2.577	.865			
≥pT3	≤pT2	1.134	0.443-2.902	.793			
Grade 3	Grade 1/2	1.560	0.551-4.405	.403			
LVI Positive	Negative	2.198	0.781-6.172	.136	7.366	2.037-26.64	.002
≥pN1	pN0	1.582	0.846-2.959	.151			
Metastatic LNs		1.084	1.010-1.164	.026			
Removed LNs		0.991	0.968-1.015	.461			
Positive Surgical Margin	Negative	2.725	1.180-6.289	.019	3.204	1.314-7.816	.011
AC	Non-AC	0.630	0.331-1.198	.159	0.325	0.147-0.717	.005

Abbreviations: AC = adjuvant chemotherapy; BMI = body mass index; CI = confidence interval; GFR = glomerular filtration rate; HR = hazard ratio; LVI = lymphovascular invasion; LNs = lymph nodes.

(Figure 1A). By multivariate analysis, AC was an independent predictive factor for RFS (HR, 0.325; $P = .005$), along with surgical margin status and lymphovascular invasion (LVI) (Table 2).

CSS was significantly longer in the AC group (MST, 57.4 vs. 17.9 months; $P = .008$) (Figure 1B). Negative surgical margins ($P = .041$) and administration of AC ($P = .011$) were factors associated with CSS by univariate analyses. By multivariate analysis, AC was significantly associated with better CSS (HR, 0.186; $P < .001$), along with negative surgical margins, negative LVI, and increased body mass index (BMI) (Table 3).

Node-positive and node-negative cases were also evaluated separately. As shown in Figure 2A, of 31 patients with pTanypN+ disease, 21 (68%) who received AC had significantly better CSS compared with 10 (32%) who were not given AC ($P = .029$). In contrast, in patients with pT3-4pN0 disease (n = 30), AC was not associated with any survival benefit ($P = .103$) (Figure 2B).

Discussion

The role of adjunct chemotherapy with RC in the treatment of muscle-invasive bladder cancer remains controversial.[8] At our

Table 3 Univariate and Multivariate Cox Regression Analysis of CSS

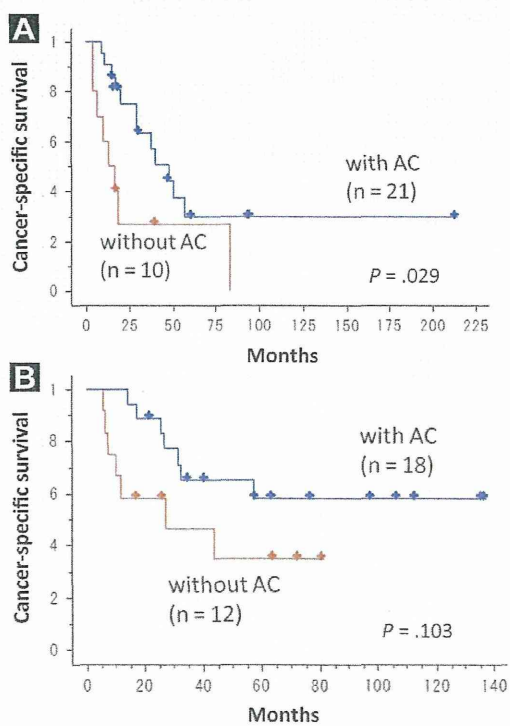
Variable	Referent	Univariate			Multivariate		
		HR	95% CI	*P* Value	HR	95% CI	*P* Value
Age, years		1.012	0.978-1.046	.494	1.003	0.961-1.047	.884
Female Sex	Male	0.521	0.124-2.185	.373			
Performance Status 2	≤1	1.072	0.377-3.053	.896	0.604	0.184-1.985	.406
BMI		0.892	0.783-1.018	.089	0.822	0.690-0.980	.029
GFR ≥60 mL/min	<60	0.870	0.438-1.730	.692			
Transfusion Yes	No	1.782	0.902-3.521	.096	1.972	0.908-4.282	.086
Compllicatioin Grade 3	No	0.919	0.278-3.033	.889			
≥pT3	≤pT2	0.882	0.341-2.284	.796			
Grade 3	Grade1/2	2.976	0.707-12.50	.137			
LVI Positive	Negative	2.146	0.654-7.042	.208	18.31	4.089-81.99	<.001
≥pN1	pN0	1.692	0.852-3.356	.133			
Metastatic LNs		1.055	0.983-1.133	.139			
Removed LNs		0.991	0.966-1.016	.472			
Positive Surgical Margin	Negative	2.538	1.037-6.211	.041	2.733	1.065-7.015	.037
AC	Non-AC	0.411	0.207-0.814	.011	0.186	0.081-0.430	<.001

Abbreviations: AC = adjuvant chemotherapy; BMI = body mass index; CI = confidence interval; GFR = glomerular filtration rate; HR = hazard ratio; LNs = lymph nodes; LVI = lymphovascular invasion.

institution, all patients with locally advanced or node-positive bladder cancer (pT3-4 or N+, or both) are offered 3 courses of cisplatin-based AC, although approximately one third decline treatment. In this study, comparing the prognoses of those receiving AC (AC group) and those not receiving AC (non-AC group), we found a significant survival advantage for the AC group. The advantage, represented as 57.4 versus 17.9 months in the MST of CSS ($P = .008$), remained significant after adjusting for possible confounding factors, and was even more evident for node-positive bladder cancer ($P = .029$).

The prognosis of patients undergoing RC clearly depends on the pathologic stage. Those with organ-confined disease (pT2 or less) have an acceptable 5-year OS of 75% to 83%, whereas the 5-year survival rate is reportedly 47% to 65% for patients with locally advanced disease (pT3-4) and 22% to 40% for patients with node-positive disease (pN1-3).[3-5] AC has been used to improve such an ominous prognosis for locally advanced or node-positive bladder cancer. Leow et al found favorable outcomes in patients with node-positive disease in their meta-analysis study of RCTs, suggesting that nodal involvement could be an indication for AC.[12] Svatek et al retrospectively analyzed their large cohort and found that bladder cancers of advanced T stage (≥ T3) with nodal involvement were most likely to benefit from AC.[20] Our results are consistent with their conclusions.

Figure 2 **(A) Cancer-Specific Survival (CSS) of Patients With Positive Nodes. Subgroup Analysis of 31 Node-Positive Cases: The AC Group (n = 21) had Significantly Better CSS Compared With the Non-AC Group (n = 10) (Median Survival Time [MST], 47.8 vs. 15.2 Months, Respectively; $P = .029$). (B) CSS of Patients With pT3-4 pN0. Analysis of Node-Negative Cases (n = 30) Yielded No Significant Benefit for AC**



One of the issues affecting AC is delayed commencement of chemotherapy because of protracted recovery after RC. In fact, patients who experienced grade 3 postoperative complications were less likely to undergo AC in our study. A large cohort study at Memorial Sloan-Kettering Cancer Center found that 298 of 1142 (26%) patients required readmission after RC and 347 (30%) may have skipped AC because of postoperative complications.[21] Furthermore, toxicity can make it difficult to complete multiple cycles of AC.[12] Of 102 candidates, 78%, 74%, and 62% of patients achieved 2, 3, and 4 cycles, respectively, mostly as a result of treatment-related toxic effects in the largest RCT of AC that had an intended 4 cycles per protocol.[15] In our study, 28 patients (72%) finished 3 cycles of AC.

Another important issue is the relative effectiveness of AC versus NAC, with the latter now strongly recommended, based on RCTs,[9,10] by the European Association of Urology guidelines[22] and the National Comprehensive Cancer Network guidelines.[23] Nevertheless, NAC has been less commonly adopted in practice than has AC[7,10,22]; 17% and 35% of patients received cisplatin-based NAC and AC, respectively, even in a high-volume tertiary referral center committed to multimodality therapy.[24] Underuse of NAC may result from concerns with overtreatment in overstaged patients or disease progression in unresponsive patients. Wosnitzer et al. observed no statistically significant difference in survival between patients receiving NAC and those receiving AC, stating that chemotherapy sequence relative to surgery appeared less important than the actual execution of perioperative chemotherapy.[25] Development of efficient predictors of therapeutic efficacy including molecular markers[26,27] would facilitate more rational use of chemotherapy.

Our study has several limitations. First, this is a retrospective study with a relatively small sample size. Although AC remained a statistically significant factor predictive of CSS even by multivariate analysis, different characteristics of the 2 groups, including age, postoperative complications, and salvage chemotherapy, might confound the study end points. Furthermore, other unexamined factors might affect the comparison. Second, the AC regimen was changed during the study period. MVAC (methotrexate, vinblastine, doxorubicin, and cisplatin) was used through 2008, and GC (gemcitabine and cisplatin) replaced it in 2009. Different profiles of adverse events might have affected treatment decisions, although equivalent efficacies were shown in the locally advanced or metastatic setting for these 2 protocols.[6] Third, there was a transition of the surgical procedure during the study period. For example, the standard upper boundary of the lymph node dissection was the iliac bifurcation until 2004. Since 2005, more than 80% of the patients underwent lymph node dissection up to the aortic bifurcation. Despite such modification, our policy of AC for the management of non–organ-confined bladder cancer had not changed.

Conclusion

In summary, our results suggest that postoperative cisplatin-based AC gives patients a survival advantage in locally advanced or node-positive bladder cancer, especially in node-positive cases. Further clinical trials targeting such patients are warranted.

Clinical Practice Points

- A survival benefit of presurgical chemotherapy (NAC) in the treatment strategy for muscle-invasive bladder cancer has been established by 2 previous RCTs. In contrast, the role of post-surgical chemotherapy (AC) remains to be determined.
- Although a recent meta-analysis of 9 RCTs revealed a statistically significant benefit of AC on overall survival in muscle-invasive bladder cancer, individual RCTs failed to prove the benefit of AC, and the optimal targets of AC have never been identified.
- In our retrospective study, postoperative cisplatin-based AC improved survival in locally advanced or node-positive bladder cancer, especially in node-positive cases. The survival benefit of AC was retained even after adjustment with other confounding factors on multivariate analysis.

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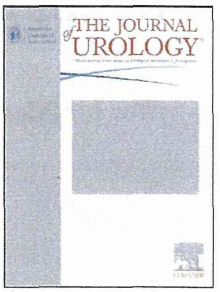

Long-term caloric restriction in rats may prevent age-related impairment of *in vitro* bladder function

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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