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.

Age-related physiological and biological changes, such as hyperlipidemia, hypertension, and diabetic mellitus, have received strong attention as a possible background for urinary bladder disorders. [25] In the present study, blood chemical analysis revealed significantly higher serum lipid levels in the O + AL group. In addition, the insulin value significantly increased in the O + AL group compared with the Y and O + CR groups. These findings were in line with previous reports suggesting that the insulin levels increase with aging because of insulin resistance, and that CR decreased the insulin levels owing to improvement of insulin sensitivity in rats [26] and humans. [27] Furthermore, serum testosterone level significantly decreased with aging, which is similar to observations in humans. [28] Interestingly, long-term CR maintained high serum testosterone level in the present study, which is consistent with a previous report that long-term CR can prevent reductions in steroidogenesis. [29]

There are some limitations in the present study. To evaluate bladder function *in vivo* more in detail cystometric investigations would be desirable, and to explore the background of aging-induced changes, gene- and protein-molecular examinations will be required. Such further studies may reveal a possible mechanism of age-related urinary bladder dysfunction. Furthermore, under the current experimental conditions, the influence of major co-morbidities could be avoided, implying that

changes occurring under "real life" conditions would not be detected, and that the alterations demonstrated in the study may have been quantitatively underestimated. It may be that potential effects of CR could be more conspicuous if tested in animal models of disease. **Previously preventive effects of CR on chronic disease, such as type 2 diabetes and cardiovascular disease, were revealed in human and animal trials.[13, 14] It is, however, difficult to translate these results of rodent studies to human health problems. Recently, Lorenzini suggested that there are two possible interpretations of CR, one is that excess fat is deleterious for health, and the second that leanness from a normal body weight might contribute to health. [30] In the present study, we were not able to determine whether the effects of CR could be explained by any of these interpretations.**

Conclusions

The present study demonstrates for the first time that CR has a preventive effect against age-related functional and morphological changes of the rat urinary bladder. Thus, age-related impairment of detrusor contractility seems to be related to decreased expression of M_3 receptors and fibrosis of the bladder wall. These findings may contribute to an increased understanding of the mechanisms of age-related detrusor underactivity.

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Figure legends

Figure 1. Contractile responses to high K^+ (A), carbachol (CCh, B), and adenosine triphosphate (ATP, C) and relaxant responses to isoproterenol (D) in young (Y), old fed with normal food (O + AL), and old with calorie restriction (O + CR) groups of rats. Values are expressed as means ± SEM. $^*P<0.05$ compared with the Y group according to the Kruskal-Wallis H test with a post hoc test.

Figure 2. Contractile responses to electrical field stimulation (EFS, A), and their cholinergic- (B) and purinergic- (C) components in young (Y), old fed with normal food (O + AL), and old with calorie restriction (O + CR) groups of rats. $^*P<0.05$ compared with the Y group according to Kruskal-Wallis H test with a post hoc test. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared between two groups according to the Kruskal-Wallis H test with a post hoc test.

Figure 3. cDNA expressions of muscarinic 1,2 and 3 (M_1, M_2 and M_3) receptors and $P2X_1$ receptor in young (Y), old fed with normal food (O + AL), and old with calorie restriction (O + CR) groups of rats.

$^{\#}P<0.05$, $^{\#\#}P<0.01$ compared between two groups according to the Kruskal-Wallis H

test with a post hoc test.

Figure 4. Representative mictroscopic images with Masson-trichrome staining of the bladder (A: low power field (upper) and high power field (lower)) and the collagen-deposition rate in the detrusor layer (B) and whole bladder layer (C) in young (Y), old fed with normal food (O + AL), and old with calorie restriction (O + CR) groups of rats.

$^{\#}P$ <0.05, $^{\#\#}P$ <0.01 compared between two groups according to the Kruskal-Wallis H test with a post hoc test.

Table 1. Primer list for real-time RT-PCR analysis.

Muscarinic receptors	
M_1	5'- GTCAACACCGAGCTCAAGA- CAG-3'
	5'- CGTGGTATAGAGGTTCATGGAGAAG-3'
M_2	5'-TCCCGGGCAAGCAAGAGTAG-3'
	5'-CCATCACCACCAGGCATATTGTTA-3'
M_3	5'- AGGACTCGAGTGGGACAGCTAC-3'
	5'- ATATGGTTCAGT- CAATCCACAGTTC-3'
Purinoceptor	
$P2X_1$	5'- TCCGTCTGATCC- AGTTGGTG-3'
	5'- GATGAGGTCACTTGAGGTCTGG-3'
Gapdh	5'- ATCAACGGGAAACCCATCAC-3'
	5'-GACATACTCAGCACCAGCATCAC-3'

Table 2. Parameters of frequency volume measurements for 24 h in young (Y), old fed with normal food (O + AL), and old with calorie restriction (O + CR) groups of rats

	Y (N = 8)	O + AL (N = 8)	O + CR (N = 8)
Voiding frequency (times)	14.88 ± 1.11	14.38 ± 2.08	18.13 ± 1.53
P value	0.427 (vs O + AL)	0.113 (vs O + CR)	0.100 (vs Y)
Voided volume in a day (ml)	10.01 ± 1.26	9.69 ± 2.87	9.19± 0.77
P value	0.208 (vs O + AL)	0.294 (vs O + CR)	0.674 (vs Y)
Voided volume per micturition (ml/time)	0.70 ± 0.10	0.62 ± 0.08	0.51 ± 0.02
P value	0.753 (vs O + AL)	0.208 (vs O + CR)	0.208 (vs Y)
Mean uroflow rate (ml/s)	0.21 ± 0.02	0.18 ± 0.02	0.17 ± 0.003
P value	0.298 (vs O + AL)	0.401 (vs O + CR)	0.121 (vs Y)
Water intake (ml)	15.12 ± 4.02	12.88 ± 5.56 $^{\#}$	25.68 ± 1.35
P value	0.397 (vs O + AL)	0.012 (vs O + CR)	0.058 (vs Y)
Food intake (g)	14.20 ± 1.30	12.66 ± 2.24 $^{\#}$	19.52 ± 1.06 **
P value	0.529 (vs O + AL)	0.046 (vs O + CR)	0.006 (vs Y)

The values are expressed as mean ± SEM.

N = number of animals

$^{**}P<0.01$: significant difference from Y (Kruskal-Wallis H test with a post hoc test)

$^{\#}P<0.05$: significant difference from O + CR (Kruskal-Wallis H test with a post hoc test)

Table 3. The body and bladder weights (A), cardiac parameters (B) and blood chemical analysis (C) in young (Y), old fed with normal food (O + AL), and old with calorie restriction (O + CR) groups of rats

A: body and bladder weights

	Y (N = 16)	O + AL (N = 15)	O + CR (N = 16)
Body weight (g)	369.19 ± 7.75	407.8 ± 11.39 **, ###	255.94 ± 3.13 ***
Bladder weight (mg)	93.38 ± 2.59	108.75±3.98 **, ###	82.37 ± 1.58 **
Bladder / body weight (mg/g)	0.25 ± 0.01	0.27 ± 0.02 ##	0.32 ± 0.01 ***

B: cardiac parameters

	Y (N = 8)	O + AL (N = 8)	O + CR (N = 8)
Heart rate (bpm)	403.96 ± 17.41	352.13 ± 24.13	368.63 ± 10.39
Systolic blood pressure (mmHg)	119.54 ± 3.90	122.08 ± 7.22	119.79 ± 3.01
Diastolic blood pressure (mmHg)	96.88 ± 2.81	94.92 ± 6.16	97.96 ± 3.87
Mean blood pressure (mmHg)	85.67 ± 2.82	80.08 ± 6.83	87.25 ± 4.55

C: blood chemical analysis

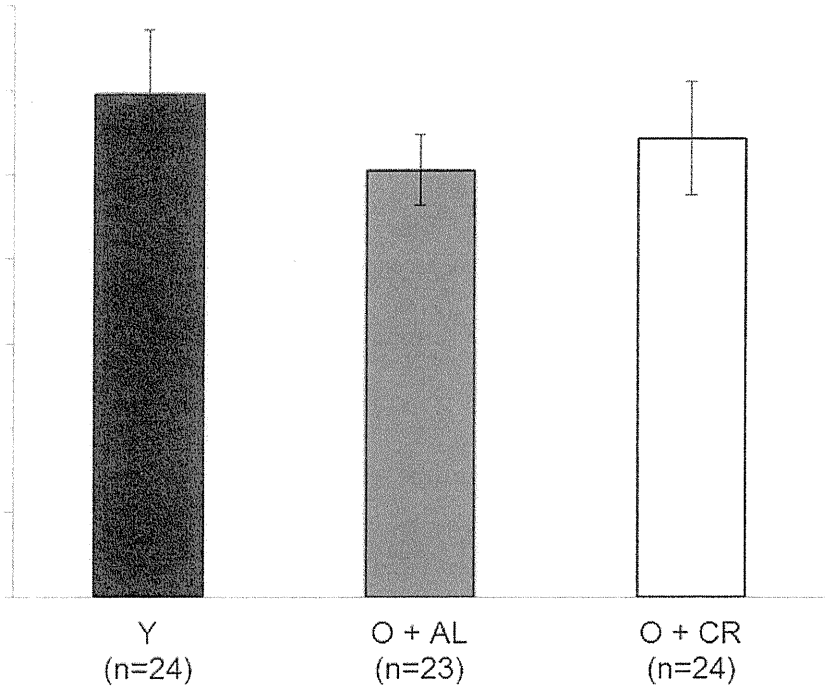
	Y (N = 8)	O + AL (N = 8)	O + CR (N = 8)
Albumin (g/dl)	3.73 ± 0.02	3.6 ± 0.11	3.61 ± 0.09
BUN (mg/dl)	22.1 ± 0.34	17.33 ± 0.57 ***	16.7 ± 0.85 ***
Creatinine (mg/dl)	0.29 ± 0.01	0.33 ± 0.01 ###	0.22 ± 0.01 **
HDL-Chol (mg/dl)	23.75 ± 0.84	30.75 ± 2.15 *	27.88 ± 2.70
LDL-Chol (mg/dl)	8.0 ± 0.24	19.75 ± 1.47 ***, ###	5.63 ± 0.32 **
Total-Chol (mg/dl)	69.63 ± 1.31	152.25 ± 13.55 ***, ###	62.38 ± 3.18
Triglyceride (mg/dl)	86.25 ± 8.86	114.75 ± 18.32 ###	32.13 ± 3.22 **
free fat acid (μEQ/l)	344.75 ± 68.28	311.13 ± 30.39 ##	153.75 ± 47.56 **
Glucose (mg/dl)	158.5 ± 11.58	150.43 ± 7.34	147.38 ± 10.91
HbA1c (%)	5.64 ± 0.13	4.67 ± 0.10 *** (n=7)	4.93 ± 0.06 ***
insulin (ng/ml)	1.31 ± 0.27	2.46 ± 0.29 *, #	1.41 ± 0.23
testosterone (ng/ml)	3.42 ± 1.02	0.28 ± 0.11 **, ##	2.05 ± 0.57
E2 (pg/ml)	25.25 ± 0.97	31.13 ± 2.12 *, ##	38.5 ± 3.96 ***
ADH (pg/ml)	110.95 ± 27.65	162.24 ± 41.78 (n=7)	126.71 ± 19.08

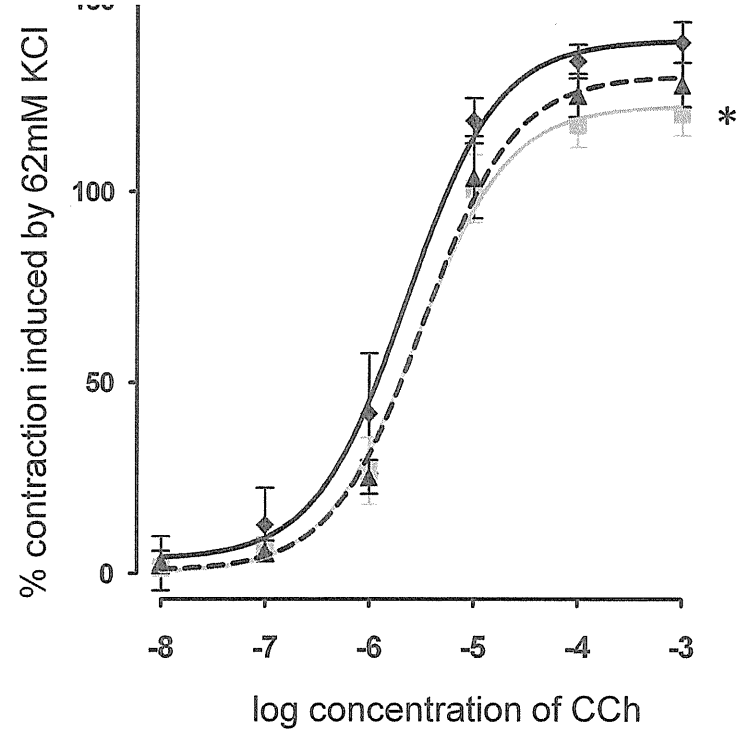
The values are expressed as mean ± SEM. N = number of animals

*P<0.05, **P<0.01, ***P<0.001: significant difference from Y (Kruskal-Wallis H test with a post hoc test)

#P<0.05, ##P<0.01, ###P<0.001: significant difference from O + CR (Kruskal-Wallis H test with a post hoc test)

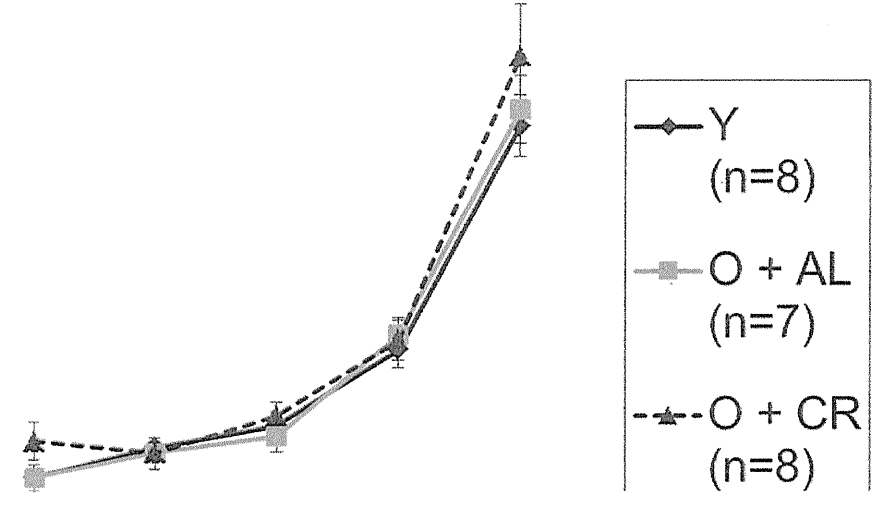
blood urea nitrogen: BUN, high density lipoprotein cholesterol: HDL- Chol, low density lipoprotein cholesterol: LDL- Chol, estradiol: E2 and antidiuretic hormone: ADH



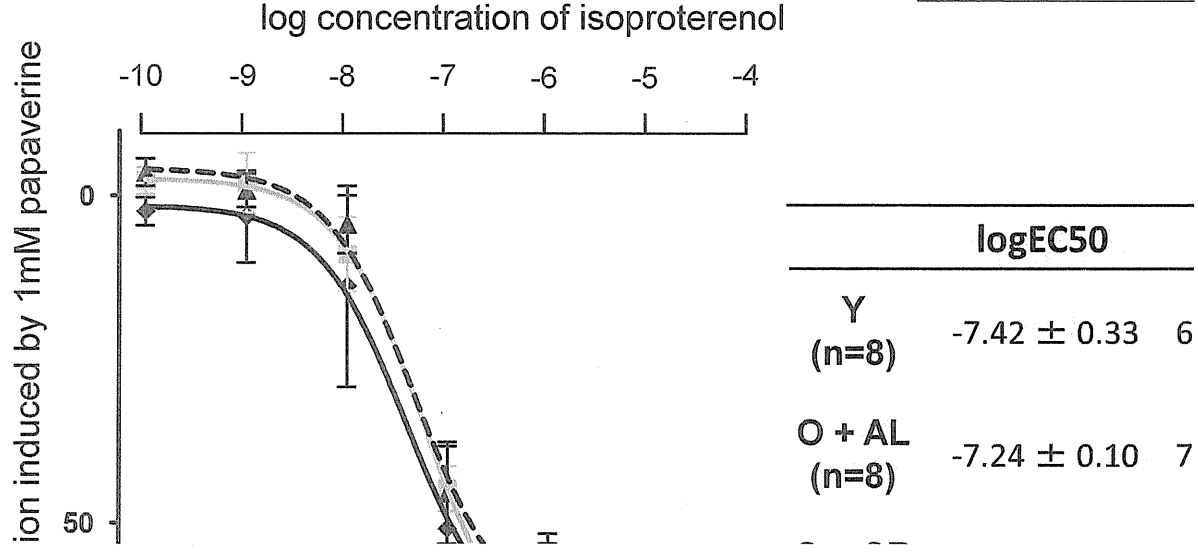


	logEC50	
Y (n=8)	-5.64 ± 0.14	13
O + AL (n=8)	-5.54 ± 0.11	122
O + CR (n=8)	-5.48 ± 0.10	13

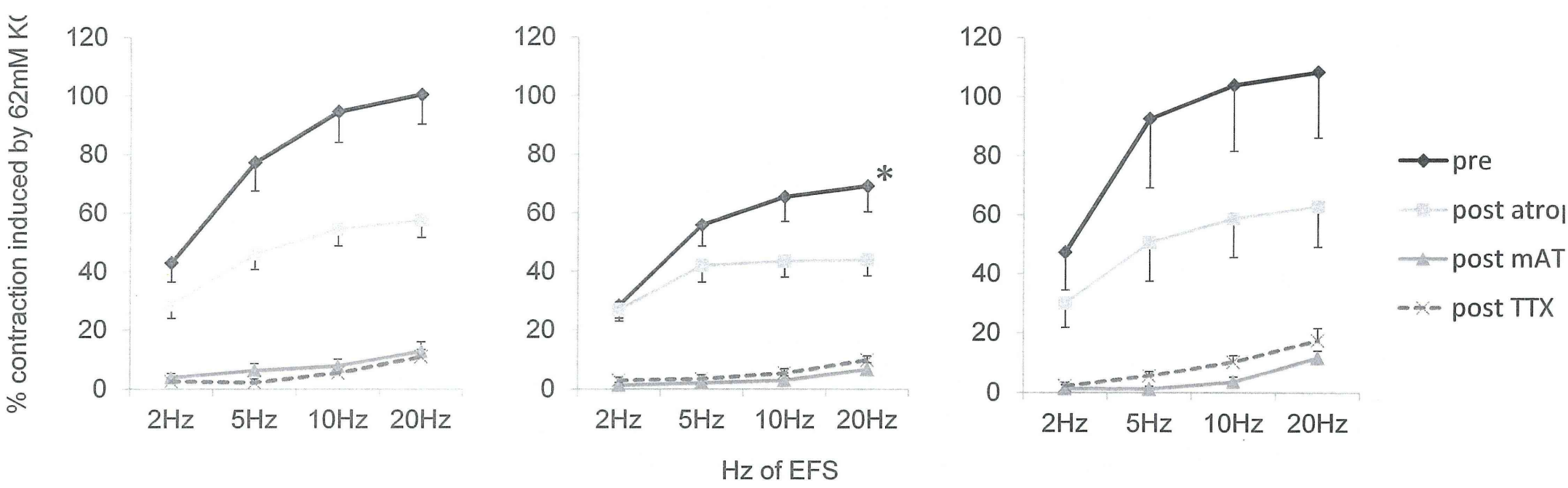
ntractile response to ATP



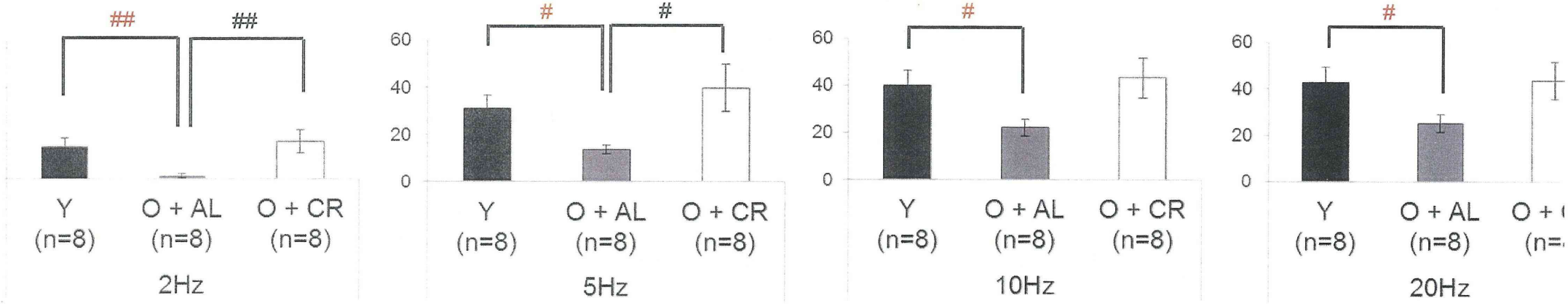
D Dose-response curve for isoproterenol



	logEC50	
Y (n=8)	-7.42 ± 0.33	6
O + AL (n=8)	-7.24 ± 0.10	7



B Atropine sensitive (cholinergic) component



C Purinoceptor desensitization sensitive (purinergic) component