し、LASCの安全性に関して検討を行った。LASCはHASCに対して肺塞栓によるマウスの死亡率を低下させ、細胞凝集能も低いことが示された。また、これらの性質に関与していると考えられる因子についても網羅的に解析を行い、候補となる遺伝子を抽出することができた。実際の臨床応用を想定した検討を行うことで、最適な細胞懸濁液の作成方法を示すことができた。

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H. 知的財産権の出願・登録状況

1. 特許取得

「脂肪組織由来多分化能幹細胞を含有する細胞製剤」

特願 PTC/JP2007/065431

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金
(難病・がん等の疾患分野の医療の実用化研究事業)
分担研究報告書

多施設共同臨床試験実施に向けての基盤整備

分担研究者
水野正明　名古屋大学医学部附属病院先端医療・臨床研究支援センター　教授
安藤昌彦　名古屋大学医学部附属病院先端医療・臨床研究支援センター　准教授
加藤勝義　名古屋大学医学部附属病院先端医療・臨床研究支援センター　講師
平川晃弘　名古屋大学医学部附属病院先端医療・臨床研究支援センター　講師
清水忍　　名古屋大学医学部附属病院先端医療・臨床研究支援センター　講師

研究要旨
名古屋大学医学部附属病院における新たな治療方法の提供を推進するにあたり、脂肪組織由来幹細胞を用いた腹圧性尿失禁と強皮症を対象に、ICH-GCPに基づいた臨床試験の支援体制を提供するための整備を進めた。
まずは、腹圧性尿失禁を対象とした治験を実施するため、作成した試験計画の骨子を基に、厚生労働省医政局と医薬品医療機器総合機構（PMDA）と協議しながら、開発方針を決定した。今後、引き続きPMDAと協議しながら、本研究課題について質の高い臨床試験成績が得られるよう、臨床試験計画の作成を継続して検討し、適切な臨床試験が実施できる体制を構築することとする。

A. 研究目的

人を対象にした臨床試験においては、安全性が十分担保された環境で実施する必要がある。その規範となるのが1964年に世界医師会総会で採択されたヘルシンキ宣言であり、この宣言はその後、数回の改訂が加わり、現在に至っている。ここでは、1）科学的・倫理的に適正な配慮を記載した臨床試験実施計画書を作成すること、2）倫理審査委員会で臨床試験計画の科学的・倫理的な適正さが承認されること、3）被験者に、事前に説明文書を用いて臨床試験計画について十分に説明し、臨床試験への参加について自由意思による同意を文書で得ることが基本原則に掲げられている。この宣言に則り、我が国おいては1997年に「医薬品の臨床試験の実施の基準に関する省令（平成9年3月27日厚生省令第28号）（ICH-GCP省令）が発行され、国際基準に則った臨床試験の基盤が整備された。

一方、名古屋大学医学部附属病院は2012年度から文部科学省「橋渡し研究加速ネットワークプログラム」及び厚生労働省「臨床研究中核病院整備事業」にそれぞれ採択され、我が国における第一線の臨床研究実施施設を目指し、革新的な医薬品・医療機器の開発を行う事を目標としている。

本研究ではこの流れに沿って、脂肪組織由来幹細胞を用いた腹圧性尿失禁と強皮症における新たな治療方法の提供を推進するにあたり、信頼性の高い臨床試験成績を創出するため、ICH-GCPに基づいた臨床試験の支援体制を提供するための整備を進めた。平成24年度に研究責任者及び研究分担者に加え、臨床試験を実施する上で、必要な臨床試験支援スタッフとして、プロジェクトマネージャー、企画立案担当、生物統計家、データーマネージャー、薬事担当からなるDesign Build-up Team (DBT) を構成した。

B. 研究方法

ヘルシンキ宣言、薬事法及びICH-GCPに準拠するために制定された各種関連省令・指針を参照し、支援体制の構築を進めた。

また、保険で使用できる医療の提供を目指す開発方針の検討を行うため、研究代表者及び細胞分離装置の販売元であるサイトリ・セラピューティクス株式会社を交え、先進医療B又は医師主導治験のいずれで臨床試験を実施するべきか、厚生労働省医政局及び医薬品医療機器総合機構(Pharmaceuticals and Medical Devices Agency : PMDA) と相談を行うこととした。

(倫理面への配慮)

ICH-GCP に基づいた臨床試験の支援体制を提供するための整備を進める。また、臨床試験計画の作成に際しては、ICH-GCPをはじめ、臨床研究に関する倫理指針(平成20年7月31日全部改正)、「ヒト幹細胞を用いる臨床研究に関する指針の改正等について」(平成 22 年 11月1日 医政発1101第6号)、厚生労働大臣の定める先進医療及び施設基準の制定等に伴う実施上の留意事項及び先進医療に係る届出等の取扱いについて(平成24年7月31日)等、必要な省令、指針等に対応するよう配慮している。

C. 研究結果

本研究課題のうち、腹圧性尿失禁に対する治療については、現在、非盲検非対照試験(目標症例数30例)を、ヒト幹細胞を用いる臨床研究に関する指針(「ヒト幹細胞を用いる臨床研究に関する指針の改正等について」(平成 22 年 11月1日 医政発1101第6号))に則り、実施している(平成23年3月15日承認:厚生労働省発医政0315第3号)。この臨床試験(中間解析)に加え、それ以前に実施した探索的な臨床試験の成績[1)]を踏まえ(当該臨床試験成績(中間解析)は、科学技術部会 ヒト幹細胞臨床研究に関する審査委員会に平成25年1月21日に報告)、平成24年度に臨床試験計画書の骨子を作成した(主な検討項目としては、試験デザイン、選択基準・除外基準、用法・用量、観察期間、評価項目、統計解析方法、目標症例数)。また、細胞分離装置の販売元であるサイトリ・セラピューティクス株式会社を交え、今度の開発方針の検討も行い、治験を実施すべきか、先進医療Bとして臨床試験を実施すべきかの検討も行ったところ、先進医療Bとして、臨床試験を実施し、保険承認を目指す開発方針とすることとした。

その開発方針について、平成25年4月 9 日に厚生労働省医政局研究開発振興課の担当官と、さらに、平成25年6月 7 日には厚生労働省医政局研究開発振興課及び医薬食品局審査管理課の担

当官と、腹圧性尿失禁における先進医療Bとしての臨床試験の実施について相談したところ、本細胞分離装置（Celution®800/CRS：サイトリ・セラピューティクス株式会社）について、製造販売承認を取得するためには、先進医療Bで臨床試験を実施することは差し支えないものの、最終的には、治験を実施することが必要であるとの指導を受けた。

そのため、先進医療Bの臨床試験を経ずに、医師主導治験を行うこととし、医師主導治験を開始するためには、事前に臨床試験計画について、PMDAと相談した上で、実施する必要があると考えた。一方で、サイトリ・セラピューティクス株式会社が、当時、新たに制定される予定の「再生医療等の安全性の確保等に関する法律」の制定状況を踏まえ、法令遵守の観点から、米国本社の意向を再確認する必要があると判断されたため、サイトリ・セラピューティクス株式会社と議論を行い、方針の再確認を行った。

サイトリ・セラピューティクス株式会社の了解も得られたことから、平成25年11月12日にPMDAと薬事戦略相談（事前面談）を実施したが、PMDAから、「本品目については、機器で分離した細胞を投与するものであるが、その品目に係る非臨床試験の内容が把握できていない。臨床試験の議論に入る前に、非臨床試験のデータを精査する必要があると考えているため、まずは、非臨床試験に係る対面助言を実施し、その後、臨床試験に係る対面助言に移行して行くことが望ましいと考えている。」との助言を受けた。そこで、名古屋大学及びサイトリ・セラピューティクス株式会社が保有しているデータを改めて精査し、平成26年3月25日にPMDAと薬事戦略相談（事前面談）を再度実施した。PMDAからは、サイトリ・セラピューティクス株式会社に確認する事項があるものの、事戦略相談（対面助言）を実施することについて、了解が得られたため、これまでの情報を整理し、根拠を提示した上で、相談項目を整理することとした。

また、並行して、医師主導治験に必要な標準業務手順書の雛形の整備も行い、以下の標準業務手順書の素案を作成した。さらに、名古屋大学が保有している腹圧性尿失禁に対する有効性及び安全性に係る非臨床試験及び臨床試験成績の概要をまとめて治験薬（機器）概要書の素案を作成した。

1）試験調整委員会への業務委嘱に関する手順書、2）試験調整委員会の業務に関する手順書、3）臨床試験実施計画書及び症例報告書の見本の作成に関する手順書、4）試験薬概要書作成に関する手順書、5）説明文書及び同意文書の作成に関する手順書、6）被験者の健康被害補償に関する手順書、7）安全性情報の取扱いに関する手順書、8）記録の保存に関する手順書、9）試験薬の管理に関する手順書、10）効果安全性評価委員会に関する手順書、11）モニタリングの実施に関する手順書、12）監査の実施に関する手順書、13）総括報告書の作成に関する手順書、14）統計解析に関する標準業務手順書、15）開発業務受託機関への業務委託に関する標準業務手順書

D.　考察

新たな治療方法の提供を推進するにあたり、信頼性の高い臨床試験成績を創出するため、ICH-GCPに基づいた臨床試

験の支援体制を提供するための整備を進めることは、文部科学省・厚生労働省の「臨床研究・治験活性化 5 か年計画 2012」（平成 24 年 3 月 30 日）においても求められている。名古屋大学医学部附属病院は、2012 年度から文部科学省「橋渡し研究加速ネットワークプログラム」及び厚生労働省「臨床研究中核病院整備事業」にそれぞれ採択され、質の高い臨床研究を実施できる体制の整備を、病院長を中心に、先端医療・臨床研究支援センターにおいて、進めている。本研究課題の臨床試験を支援するために、DBT を組織し、臨床試験計画の骨子を作成した。また、細胞分離装置の販売元であるサイトリ・セラピューティクス株式会社とも連携を図ることにより、今後得られる研究成果を国民に広め、恒常的に提供できる体制も構築することができた。

一方、平成 25 年度には、先進医療 B での臨床試験又は医師主導治験を実施するため、厚生労働省医政局との事前相談、並びに PMDA と薬事戦略相談で議論しながら、最終的に開発方針として、医師主導治験を行うことを決定することができた。平成 26 年 3 月 25 日に実施した薬事戦略相談（事前面談）において、PMDA がサイトリ・セラピューティクス株式会社に確認する事項があるとしていたが、平成 26 年 4 月 16 日にサイトリ・セラピューティクス株式会社と PMDA が実施する薬事戦略相談（事前面談）を実施する予定としており、その結果を踏まえ、薬事戦略相談（対面助言を）を申し込むこととする。平成 26 年度には PMDA と薬事戦略相談（対面助言）において、治験を実施することについて、並びに当該治験デザインについて、議論を行う予定としている。

また、今後、多施設共同研究を実施するために、これまでに得られた臨床試験成績 [2] を踏まえて、臨床試験計画の作成を行う。さらに、説明文書・同意文書（見本）の作成、治験薬（機器）概要書の作成、電子的臨床試験情報収集（Electronic Data Capture：EDC）システムの構築、各種標準業務手順書の作成等を行い、他の施設の選定方法、モニタリングや監査の方法を含め、継続して検討し、適切な臨床試験が実施できる体制を構築することを目指す。

E. 結論

今回、新たな治療方法の提供を推進するにあたり、信頼性の高い臨床試験成績を創出するため、ICH-GCP に基づいた臨床試験の支援体制を提供するための整備を進め、DBT を組織した。作成した試験計画の骨子を基に、厚生労働省及び PMDA と協議しながら、開発方針を決定し、体制の構築を順次進めた。今後、本研究課題について質の高い臨床試験成績が得られるよう、臨床試験計画の作成を継続して検討し、適切な臨床試験が実施できる体制を構築する。また、試験計画については、継続して、PMDA と相談しながら進めていくこととする。

F. 研究発表

1. 論文発表

なし

2. 学会発表

1）構造並びに機能再生を目指す脂肪組織由来幹細胞治療の開発－腹圧性尿失禁に対する構造再生医療の開発－. 文部科学省 橋渡し研究加速ネットワークプログラム　厚生労働省 早期・探索的臨床試験拠点整備事業/臨床研究中核病院

G.　知的財産権の出願・登録状況（予定を含む）

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

H.　References

1)　Int J Urol 19: 652-659, 2012

2)　Int J Urol 21: 294-300, 2014

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表雑誌	巻号	ページ	出版年
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IV. 研究成果の刊行物・別刷り

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Original Article: Clinical Investigation

Regenerative treatment of male stress urinary incontinence by periurethral injection of autologous adipose-derived regenerative cells: 1-year outcomes in 11 patients

Momokazu Gotoh,[1] Tokunori Yamamoto,[1] Masashi Kato,[1] Tsuyoshi Majima,[1] Kazuhiro Toriyama,[2] Yuzuru Kamei,[2] Yoshihisa Matsukawa,[1] Akihiro Hirakawa[3] and Yasuhito Funahashi[1]

Departments of [1]Urology and [2]Plastic and Reconstructive Surgery, and [3]Biostatistics Section, Center for Advanced Medicine and Clinical Research, Nagoya University Graduate School of Medicine, Nagoya, Japan

Abbreviations & Acronyms
ADRC = adipose-derived regenerative cells
ASC = adipose-derived stem cells
FPL = functional profile length
GFP = green fluorescent protein
HoLEP = holmium laser enucleation of the prostate
ICIQ-SF = International Consultation on Incontinence Questionnaire-Short Form
MRI = magnetic resonance imaging
MSC = mesenchymal stem cells
MUCP = maximum urethral closing pressure
QOL = quality of life
RP = radical prostatectomy
SD = standard deviation
SUI = stress urinary incontinence

Correspondence: Momokazu Gotoh M.D., Ph.D., Department of Urology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-855, Japan. Email: gotoh@med.nagoya-u.ac.jp

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Objectives: To assess the efficacy and safety of a novel cell therapy for male stress urinary incontinence consisting of periurethral injection of autologous adipose-derived regenerative cells, and to determine the 1-year outcomes.

Methods: A total of 11 male patients with persistent stress urinary incontinence after prostate surgery were included in the study. The Celution system was used to isolate adipose-derived regenerative cells from abdominal adipose tissue obtained by liposuction. Subsequently, these regenerative cells, and a mixture of regenerative cells and adipose tissue were transurethrally injected into the rhabdosphincter and submucosal space of the urethra, respectively. The 1-year outcomes were assessed using a 24-h pad test, a validated patient questionnaire, urethral pressure profile, transrectal ultrasonography and magnetic resonance imaging.

Results: Stress urinary incontinence improved progressively in eight patients during the 1-year follow up, as determined by a 59.8% decrease in the leakage volume in the 24-h pad test, decreased frequency and amount of incontinence, and improved quality of life. One patient achieved total continence. The mean maximum urethral closing pressure and functional profile length increased from 35.5 to 44.7 cmH_2O, and from 20.4 to 26.0 mm, respectively. Magnetic resonance imaging showed the sustained presence of the injected adipose tissue, and enhanced ultrasonography showed a progressive increase in blood flow to the injected area in all patients. No significant adverse events were observed peri- or postoperatively.

Conclusion: Periurethral injection of autologous adipose-derived regenerative cells might represent a safe and feasible treatment modality for male stress urinary incontinence.

Key words: adipose-derived regenerative cells, adipose-derived stem cells, cell therapy, prostatectomy, stress urinary incontinence.

Introduction

Cell therapy can be used for the regeneration of damaged tissues in several diseases, and it has been experimentally investigated in many fields. MSC are multipotent adult stem cells that can proliferate into a variety of cell types in culture.[1–4] Experimental studies have reported the use of MSC obtained from the bone marrow, adipose tissue or skeletal muscle for the treatment of SUI, with the objective of regenerating the sphincter.[5–11]

Multipotent stem cells are 100-fold more abundant in adipose tissue than in the bone marrow. The human body is rich in adipose tissue, and adipose tissue can be easily and safely harvested in large quantities with minimal morbidity. Therefore, adipose tissue has attracted attention as a source for cell therapy. ASC showed the ability to differentiate into bone, cartilage, adipose, nerve, blood vessel and muscle cells.[1,3,4,12] We therefore developed a treatment method for SUI caused by sphincter deficiency, based on the regeneration of the sphincter using ASC. The rationale and efficacy of periurethral injection of cultured ASC for the treatment of SUI were showed in animal experiments.[13] However, the clinical application of cell therapy requires the use of autologous cells, and the procurement of enough cells without the need for cell culture. We used ADRC to develop a novel treatment strategy for the regeneration of the urethral sphincter, which meets these criteria. We previously reported the favorable short-term outcomes of three cases of the first in-human study using this treatment strategy.[14] Here, we report the 1-year outcomes in 11 male patients enrolled in the preliminary clinical study.

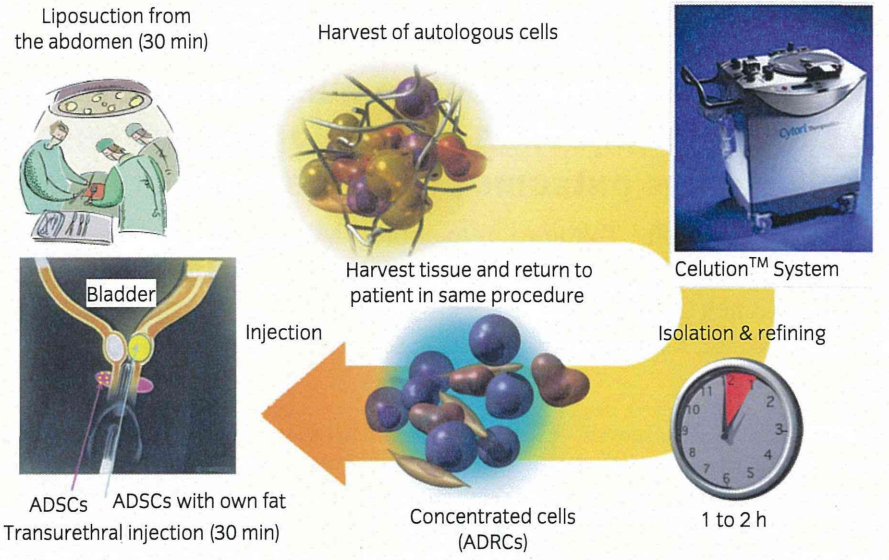

Fig. 1 A brief outline of the treatment strategy. The autologous tissue source was harvested from the abdomen by liposuction. ADRC were isolated from the tissue without culture using the Celution system. Finally, ADRC were transurethrally injected into the urethral sphincter. This treatment can be completed as a single surgical procedure within 3 h.

Table 1 Patients' characteristics, and outcomes on isolation and injection of ADRC

Patients	Age (years)	Duration of SUI (months)	Type of prostate surgery	Time for ADRC isolation (min)	Time for ARDC injection (min)	No. isolated cells	Viable cells (%)
1	68	20	HoLEP	98	25	7.5×10^6	93.8
2	76	54	RP	110	31	2.2×10^7	91.2
3	85	104	RP	118	54	2.2×10^7	90.1
4	72	74	RP	103	17	7.3×10^6	91.4
5	78	78	RP	110	27	2.4×10^7	89.7
6	78	52	RP	80	33	3.3×10^7	90.2
7	71	29	RP	79	40	7.5×10^6	93.8
8	74	66	HoLEP	74	30	2.5×10^6	94.2
9	75	40	RP	117	48	1.8×10^7	90.0
10	79	61	RP	73	39	2.3×10^7	88.6
11	77	74	RP	119	30	3.3×10^7	90.9
Mean (SD)	76 (5)	59 (24)		98 (18)	34 (11)	1.8×10^7 (1.1)	91.3 (1.9)

Methods

The present study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine, and by the committee of the Japanese Ministry of Health, Labor and Welfare according to the Guidelines on Clinical Research using Human Stem Cells. Written informed consent was obtained from the patients.

A brief outline of the treatment strategy in the present study is shown in Figure 1. The autologous tissue source was harvested from the abdomen by liposuction. ADRC were isolated from the tissue without culture using the Celution (Cytori Therapeutics, San Diego, CA, USA) system. Finally, ADRC were transurethrally injected into the urethral sphincter. This treatment can be completed as a single surgical procedure within 3 h.

Patients

A total of 11 patients were treated and followed up for more than 1 year (Table 1). The causes of SUI were sphincter deficiency after radical prostatectomy in nine patients and holmium laser enucleation of the prostate in two patients. The inclusion criteria for the patients with prostate cancer were persistence of SUI for more than 2 years after surgery, localized prostate cancer of good risk with a preoperative prostate-specific antigen level of <10 ng/mL and Gleason score of 6 points or less, negative surgical margin on pathological examination of the resected prostate specimen, and no evidence of recurrence or metastasis with undetectable levels of prostate-specific antigen.

Harvesting adipose tissue (liposuction)

Under spinal anesthesia, 250 mL of adipose tissue was harvested from the anterior abdominal wall by making two 3-mm incisions. Ringer's lactate was first infused in the subcutaneous layer, and the adipose tissue was harvested. The suctioned adipose tissue was placed in saline, and allowed to stand for settling of the blood and cellular debris; adipose tissue floated at the top of the mixture.

Isolation of ADRC

ADRC were isolated from the harvested adipose tissue using the Celution system,[15] which is a commercially available kit designed to isolate ADRC from human adipose tissue in a short time. This instrument allows the isolation of therapeutic doses of autologous ADRC after liposuction without the need for culture. The nucleated cell composition of ADRC includes approximately 0.6–1.6% ASC, as well as both mature and progenitor endothelial and smooth muscle cells, CD45+ hematopoietic cells and resident tissue macrophage/monocytes, pericytes, and preadipocytes, as well as other less well characterized stromal fibroblastic cell populations.[15] The final concentrated cell output was measured using a NucleoCounter (Chemometec, Allerod, Denmark), which exclusively detected nucleated cells. By using the Celution system, we could finally obtain a 5-mL solution containing concentrated ADRC.

Periurethral injection of ADRC

After liposuction and isolation of ADRC, transurethral endoscopic injection of ADRC was carried out. For periurethral injection, two distinct formulations were produced: 1 mL of the isolated ADRC fraction alone was preserved for direct injection, and another 4 mL of the fraction were mixed with intact autologous adipose cells, yielding a total of 20 mL of this combined solution.

A 22-Fr rigid endoscope was used for injecting the processed ADRC solution. Under endoscopic vision, a puncture needle was passed through the endoscope into the urethra at the region of the external urethral sphincter. The 18-G needle was 35 cm in length and graduated in centimeters, and was specially ordered. The ADRC solution was injected after puncturing the urethra at the region of the external urethral sphincter under endoscopic vision. Initially, a 1-mL solution was injected to a depth of 5 mm into the rhabdosphincter at 5 and 7 o'clock positions. Subsequently, 20 mL of the formulation containing ADRC and adipose tissue was equally injected into the submucosal spaces at 4, 6, and 8 o'clock positions to facilitate complete coaptation of the urethral mucosa. After the solution was injected, a 6-Fr urethral balloon catheter was placed and removed the next day.

Primary outcome measure

The amount of incontinence was evaluated by a 24-h pad test, and the total daily leakage volume was calculated. The 24-h pad test was carried out consecutively for 4 days in each evaluation period (baseline, 2 weeks, and 1, 3, 6, 9 and 12 months). Changes in the mean daily leakage volume during the 4 days from baseline to 12 months after treatment were evaluated as a primary outcome measure.

Secondary outcome measures

Urethral sphincter function was objectively assessed by urethral pressure profile using a urodynamic system (MMS, Enschede, the Netherlands). MUCP and FPL were measured at baseline, 2 weeks, and 1, 3 and 6 months after treatment.

Subjective symptoms and QOL were evaluated using a validated disease-specific questionnaire, the ICIQ-SF.[16,17] In the ICIQ-SF, the therapeutic effects in terms of frequency of urinary incontinence (0–5 point scores), amount of leakage (0–6 point scores) and impact on everyday life (0–10 point scores) were examined, and the total score ranging from 0 to 21 points was calculated. A high score indicates unfavorable conditions. These parameters were assessed at baseline, and repeated at 2 weeks, and 1, 3, 6, 9 and 12 months after treatment.

The blood flow to the area where the ADSC were injected was assessed using contrast-enhanced transrectal ultrasonography after intravenous injection of perflubutane at each evaluation period. Power Doppler imaging was obtained 5 min after perflubutane injection.

The morphological condition of the injected area was monitored using MRI at baseline, and 3, 6 and 12 months after treatment.

Voiding function was evaluated by uroflowmetry, and measurement of post-void residual urine volume at baseline, 2 weeks, and 1, 3, 6 and 12 months.

Statistical analyses

For each patient, the change and/or percent change between baseline and 6 (or 12) months with respect to the primary and secondary outcome measures were calculated. The means (standard deviations) of them were calculated and the Wilcoxon signed-rank test was carried out. A two-sided $P < 0.05$ was considered to be statistically significant. The analyses were carried out using SAS (version 9.3; SAS Institute, Cary, NC, USA).

Results

Liposuction was carried out in the abdomen without significant morbidity, and 250 mL of adipose tissue was harvested in all cases. The isolated adipose tissue solution contained 7.3×10^6 to 3.3×10^7 ADRC (6.7×10^6 to 3.0×10^7 viable cells; Table 1). The mean time of cell extraction was 98 min, and the mean time of periurethral injection was 34 min (Table 1).

The means of change and percent change between baseline and 12 months for daily leakage volume in 11 patients were −86.8 g ($P = 0.054$) and −40.9% ($P = 0.010$), respectively. These improvements were mainly attributable to a progressive decrease of daily leakage volume in eight patients (Table 2). In contrast, no change in daily leakage volume was observed in the remaining three patients. In the eight patients showing decreased leakage volume, urinary incontinence improved immediately after injection, deteriorated approximately 1 month after injection, and progressively improved thereafter up to 6–12 months (Fig. 2).

The means of change and percent change between baseline and 12 months for MUCP in 11 patients were 9.2 cmH2O ($P = 0.017$) and 38.8% ($P = 0.019$), respectively. The mean MUCP increased both in the patients with improved and those with unimproved leakage volume on the 24-pad test (Fig. 3a). The means of change and percent change between baseline and 12 months for FPL in 11 patients were 5.6 mm ($P = 0.006$) and 40.1% ($P = 0.006$), respectively. The mean FPL increased in patients with improved leakage volume, but not in those with unimproved leakage volume (Fig. 3b). No significant increase of post-void residual urine volume was observed in any of the

Table 2 Outcomes on incontinence, urethral sphincter function and post-void residual urine

Patients	Daily leakage volume (g)				MUCP (cmH2O)				FPL (mm)				PVR (mL)			
	Baseline	12 Months	Change	Percent change (%)	Baseline	6 Months	Change	Percent change (%)	Baseline	6 Months	Change	Percent change (%)	Baseline	12 Months	Change	Percent change (%)
Improved																
1	49.5	20.0	−29.5	−59.6	50	44	−6.0	−12.0	39	40	1.0	2.6	50	30	−20.0	−40.0
2	32.0	0.0	−32.0	−100.0	29	42	13.0	44.8	14	32	18.0	128.6	40	30	−10.0	−25.0
3	37.7	14.4	−23.3	−61.8	21	36	15.0	71.4	20	27	7.0	35.0	20	10	−10.0	−50.0
4	100.3	63.3	−37.0	−36.9	49	60	11.0	22.4	26	30	4.0	15.4	40	20	−20.0	−50.0
5	171.7	86.3	−85.4	−49.7	48	51	3.0	6.3	16	20	4.0	25.0	36	40	4.0	11.1
6	122.3	55.3	−67.0	−54.8	40	53	13.0	32.5	20	30	10.0	50.0	25	23	−2.0	−8.0
10	900.0	385.0	−515.0	−57.2	35	31	−4.0	−11.4	13	20	7.0	53.8	12	0	−12.0	−100.0
11	430.0	180.0	−250.0	−58.1	17	47	30.0	176.5	7	15	8.0	114.3	20	0	−20.0	−100.0
Mean (SD)	230.4 (299.9)	100.5 (128.1)	−129.9 (172.5)	−59.8 (18.1)	36.1 (12.9)	45.5 (9.4)	9.4 (11.6)	41.3 (61.5)	19.4 (9.7)	26.8 (8.0)	7.4 (5.1)	53.1 (45.6)	30.3 (13.0)	19.1 (14.7)	−11.3 (8.9)	−45.2 (39.7)
Unimproved																
7	376.2	389.0	12.8	3.4	25	36	11.0	44.0	20	24	4.0	20.0	30	40	10.0	33.3
8	604.0	595.0	−9.0	−1.5	28	42	14.0	50.0	29	28	−1.0	−3.4	40	55	15.0	37.5
9	345.3	437.0	91.7	26.6	49	50	1.0	2.0	20	20	0.0	0.0	0	15	15.0	NE
Mean (SD)	441.8 (141.3)	473.7 (107.8)	31.8 (53.0)	9.5 (15.0)	34.0 (13.0)	42.7 (7.0)	8.7 (6.8)	32.0 (26.2)	23.0 (5.2)	24.0 (4.0)	1.0 (2.7)	5.5 (12.6)	23.3 (20.8)	36.7 (20.2)	13.3 (2.9)	35.4 (3.0)
All																
Mean (SD)	288.1 (276.9)	202.3 (210.2)	−86.8 (164.6)	−40.9 (36.3)	35.5 (12.3)	44.7 (8.5)	9.2 (10.2)	38.8 (53.0)	20.4 (8.6)	26.0 (7.1)	5.6 (5.4)	40.1 (44.5)	28.5 (14.7)	23.9 (17.3)	−4.5 (13.7)	−29.1 (48.8)
P-value			0.054	0.010			0.017	0.019			0.006	0.006			0.307	0.100

P-value was calculated by the Wilcoxon signed rank test.

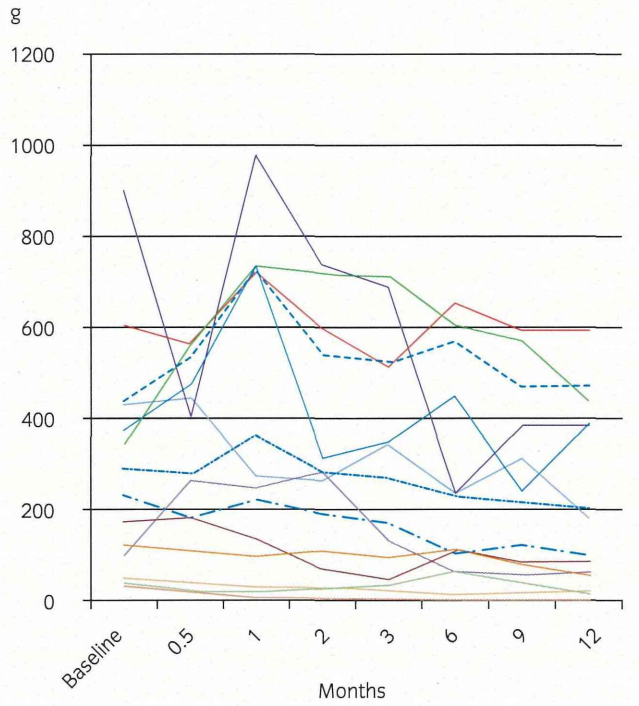

Fig. 2 Changes in daily leakage volume by 24-h pad test. In eight patients, leakage volume decreased progressively over time up to 12 months with a mean reduction rate of 59.8%; however, no change in leakage volume was achieved in three cases. In the eight patients showing decreased leakage volume, urinary incontinence improved immediately after injection, deteriorated approximately 1 month after injection and progressively improved thereafter up to 6–12 months. One patient achieved total continence. ——, 1; ——, 2; ——, 3; ——, 4; ——, 5; ——, 6; ——, 7; ——, 8; ——, 9; ——, 10; ——, 11; ——, All ($n = 11$); ——, Improved ($n = 8$); ——, Unimproved ($n = 3$).

patients, changing from 28.5 mL at baseline to 23.9 mL 3 months after treatment. Additionally, no significant change in maximum flow rate was detected.

The outcomes on subjective symptoms and QOL assessed by the ICIQ-SF are summarized in Table 3. In all patients, the frequency of incontinence was significantly improved ($P = 0.016$), whereas the remaining three parameters were not (i.e. $P = 0.311$ for total score, $P = 0.500$ for amount of leakage and $P = 0.645$ for QOL, respectively). The eight patients with improved leakage volume showed improvement in the total score and all subscores of the ICIQ-SF, such as frequency of incontinence, amount of leakage and QOL. However, the three patients who did not show changes in the leakage volume showed no improvement in the parameters of ICIQ-SF (Table 3).

In all patients, enhanced ultrasonography showed a sequential increase in the blood flow to the area where the ADSC were injected (Fig. 4), which was maintained during the entire follow-up period. MRI showed a bulking effect at the site of adipose tissue injection, which persisted even at 3 months after injection.

No significant adverse event occurred during the liposuction and ADSC injection procedures. No severe side-effects, such as pelvic pain, inflammation or de novo urgency, were observed after the operation in all cases during the follow-up period. Serum prostate-specific antigen level did not increase from the

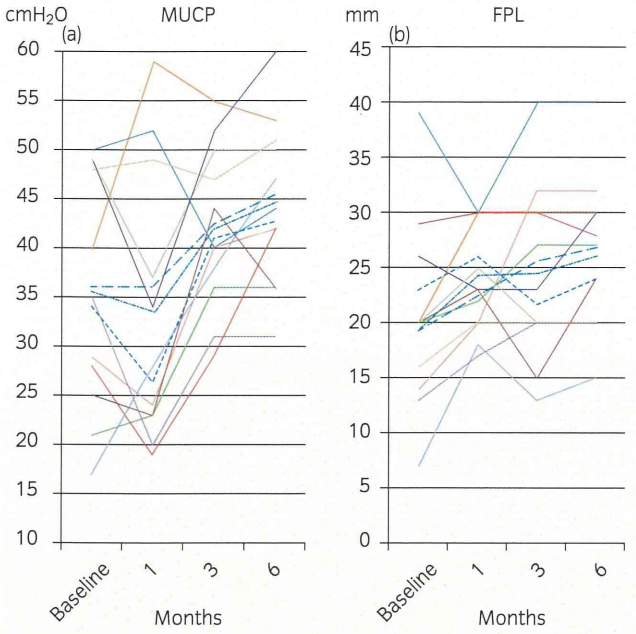

Fig. 3 Changes in (a) MUCP and (b) FPL on urethral pressure profile. (a) Mean MUCP progressively increased both in the patients with improved and those with unimproved leakage volume on the 24-pad test. (b) In contrast, mean FPL increased in patients with improved leakage volume, but not in those with unimproved leakage volume. ——, 1; ——, 2; ——, 3; ——, 4; ——, 5; ——, 6; ——, 7; ——, 8; ——, 9; ——, 10; ——, 11; ——, All (n = 11); – –, Improved (n = 8); – – –, Unimproved (n = 3).

baseline level in any of the patients. In four patients (cases 2, 3, 9, 11), mild subcutaneous hemorrhage at the abdomen was a complication of liposuction, which spontaneously disappeared within 1 month.

Discussion

The pathophysiology of male SUI after prostate surgery is urethral sphincter dysfunction caused by a reduction in the volume of skeletal and smooth muscle cells, impaired blood flow, and denervation at the sphincter. Because the treatment of sphincter dysfunction requires an increase in the muscle cell population, the promotion of revascularization and the reconstruction of innervation, regenerative treatment using stem cells could be developed into an ideal treatment modality for this condition. At the experimental level, the treatment of SUI using stem cells derived from the bone marrow, skeletal muscles and adipose tissue has been reported in several studies.[5–11] These studies suggested the potential and promising role of stem cell therapy for the treatment of SUI. In contrast, regenerative treatment of SUI in humans has been reported in a limited number of clinical studies.

A few clinical studies have described the use of autologous adult muscle-derived stem cells for the treatment of female SUI, which has shown promising efficacy and safety.[18,19] Carr and Chancellor carried out a prospective, dose-ranging study to assess the 12-month safety and potential efficacy of autologous muscle-derived cell therapy in 38 female patients with SUI.[19] A muscle biopsy sample was obtained from the quadriceps femoris and cultured to harvest autologous muscle-derived cells. Using a cystoscope-assisted periurethral approach, the cells were injected into the external urethral sphincter. Among 29 patients receiving two treatments, 88.9% of those who received a high-dose injection of muscle-derived cells and 61.5% of those who received low-dose injection showed a 50% or greater reduction in urine leakage in 1-h pad tests. Additionally, 77.8% of the high-dose group and 53.3% of the low-dose group had a 50% or greater reduction in the frequency of SUI.

Because adipose tissue contains abundant multipotent stem cells, therapeutic levels of ADRC can be isolated using the Celution system, as shown in the present study. Clinical studies assessing the application of ADRC have been carried out for breast reconstruction[20] and cardiac infarction.[21] These clinical studies support the feasibility of cell therapy using ADRC. Our group carried out the first in-human study using cell therapy with ADRC for SUI, and we described the short-term results of three cases in a previous report.[14] In the present study, we extended our previous clinical findings by including a larger number of patients and investigating the 1-year outcomes. Although this is a preliminary study, we obtained promising results as described in the present report.

The improvement of urinary incontinence observed in the present study can be explained by several mechanisms. In most cases, SUI improved initially within a week after injection, deteriorated subsequently and progressively improved thereafter. This clinical course suggests the involvement of a specific mechanism in which a bulking effect produced by the injected adipose tissue fraction mixed with ADRC plays an important role. The injected adipose tissue fraction, which was processed to isolate ADRC, contained 30% of lactated Ringer's solution. Absorption of the solution could be responsible for the temporary deterioration in the condition during the initial week. Among the ADRC, the ASC subpopulation might have contributed to the progressive improvement in sphincter function, which was reflected in the increased MUCP and FPL, and the decreased frequency and amount of SUI. The persistent bulking effect indicated the survival and growth of the injected adipose tissue, which could be attributed to the presence of ASC.

ASC can differentiate into mature adipose tissue and possibly into contractile cells. In our preclinical experimental study,[13] cultured rat ASC were injected into the proximal urethra after bilateral transection of the pelvic nerves. The leak point pressure was significantly higher in the rats undergoing ASC injection than in those undergoing injection of collagen or vehicle. Additionally, when GFP expressing cultured ASC were injected into the urethra of female nude rats, alpha smooth muscle actin-positive cells were stained in the merged distribution (70%) with the GFP expressing ASC 12 weeks after injection, suggesting the possible differentiation of ASC into smooth muscle cells. Previous studies on rats showed that cultured ASC injected into the injured urethra differentiated into contractile cells with smooth muscle cell features.[22] We also confirmed in pigs that injected ADRC isolated by the Celution system differentiated into smooth muscle cells (unpubl. data).

Environmental effects caused by the injected ASC could also be responsible for the improvement in SUI. Cultured ASC are known to secrete a large number of angiogenesis-related cytokines.[23] In our experimental study, injection of cultured human ASC into the ischemic hind limb of nude rats produced human hepatocyte growth factor and vascular endothelial growth factor, and improved blood flow (unpubl. data). In the

Table 3 Means and standard deviations on subjective symptoms and QOL assessed by the ICIQ-SF

	Total			Frequency of leakage			Leakage amount			QOL		
	Baseline	12 Months	Change	Baseline	12 Months	Change	Baseline	12 Months	Change	Baseline	12 Months	Change
Improved patients	13.8 (3.7)	11.3 (6.1)	−2.5 (5.8)	4.6 (0.5)	3.4 (1.7)	−1.3 (1.5)	4 (1.1)	3.3 (1.8)	−0.8 (1.5)	5.1 (2.9)	4.6 (3.2)	−0.5 (3.8)
Unimproved patients	19.7 (1.5)	19.7 (1.5)	0.0 (2.6)	5 (0)	4.3 (0.6)	−0.7 (0.6)	5.7 (0.6)	6 (0)	0.3 (0.58)	9 (1.7)	9.3 (1.2)	0.3 (2.5)
All patients	15.4 (4.2)	13.5 (6.5)	−1.8 (5.2)	4.7 (0.5)	3.6 (1.5)	−1.1 (1.3)	4.5 (1.2)	4 (2)	−0.5 (1.4)	6.2 (3.1)	5.9 (3.5)	−0.3 (3.4)

Total score: 0–21, frequency of leakage: 0–5, leakage amount: 0–6, QOL: 0–10.

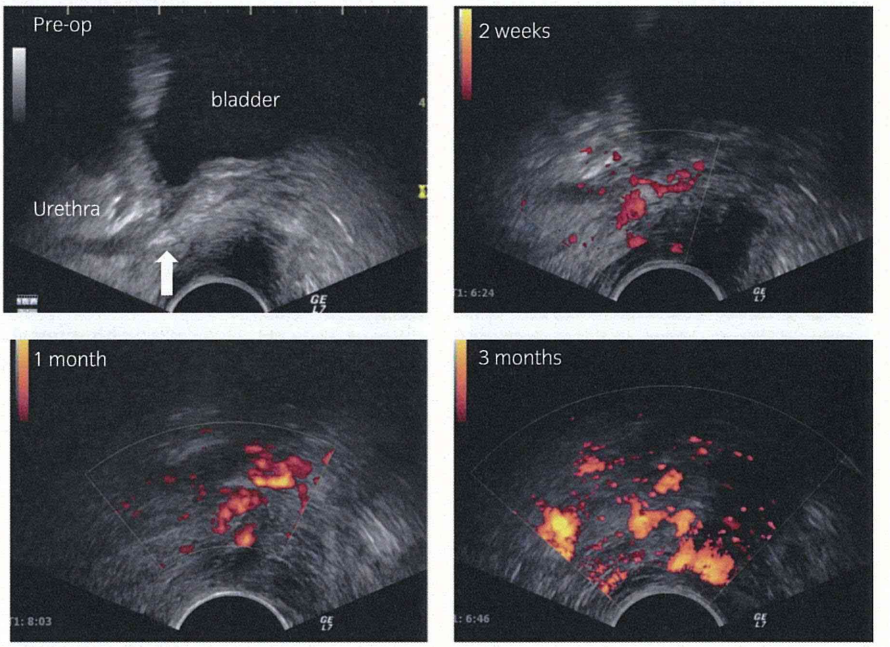

Fig. 4 Contrast-enhanced transrectal ultrasonography to assess the blood flow of the periurethral area after ADRC injection. The bladder and urethra was visualized as a sagittal section. The blood flow around the injected area visualized as orange color was progressively increased after the injection of ADRC up to 3 months in case 1.

present study, increased blood flow to the injected area, which was observed by ultrasonography, was maintained throughout the follow-up period. This increase in blood flow could be related to the promotion of angiogenesis by cytokines secreted by the injected ADRC, and could have positively affected the regeneration of the injected adipose tissue and impaired sphincter function.

The present study had some limitations. This was a preliminary, single-arm, non-comparable study. The sample size was small. The favorable outcomes of the present preliminary study suggest that a phase III trial with a randomized, comparable protocol and an appropriate sample size is required. The lack of improvement in three cases in the present study could not be explained.

In conclusion, periurethral injection of autologous ADRC is a safe and feasible treatment modality for male SUI, and probably for female SUI. Our method has the advantages that the cells used are autologous and do not require culture, and the treatment is carried out in the context of a single surgical procedure.

Acknowledgments

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Conflict of interest

None declared.

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Intravesical Application of Rebamipide Suppresses Bladder Inflammation in a Rat Cystitis Model

Yasuhito Funahashi,* Masaki Yoshida, Tokunori Yamamoto, Tsuyoshi Majima, Shun Takai and Momokazu Gotoh

From the Department of Urology, Nagoya University Graduate School of Medicine, Nagoya and National Center for Geriatrics and Gerontology (MY), Ohbu, Japan

Purpose: We examined the effects of intravesical application of rebamipide (Otsuka Pharmaceutical, Tokyo, Japan) on bladder inflammation and overactivity in a chemically induced cystitis model.

Materials and Methods: Female Sprague Dawley® rats under isoflurane anesthesia were injected with 150 mg/kg cyclophosphamide in the peritoneum, and 1 mM or 10 mM rebamipide or vehicle was administered in the bladder and remained for 1 hour. Control rats were injected with saline in the peritoneum and vehicle was administered in the bladder. The bladder was harvested at 48 hours. Hematoxylin and eosin staining was performed and the inflammation grade was assessed. The amount of myeloperoxidase was measured using enzyme-linked immunosorbent assay. Proinflammatory cytokines were quantified using reverse transcriptase-polymerase chain reaction. Cystometrogram was done in awake rats 48 hours after cyclophosphamide treatment to measure voiding reflex parameters.

Results: Histological evaluation revealed that bladder inflammation in cyclophosphamide treated rats was suppressed by rebamipide in a dose dependent manner. Up-regulated myeloperoxidase, IL-1β, IL-6 and TNF-α expression in cyclophosphamide treated rats was also suppressed in rebamipide treated rats. Cystometrogram demonstrated that the intercontraction interval decreased in cyclophosphamide treated rats but was prolonged by rebamipide.

Conclusions: Intravesical application of rebamipide suppressed bladder inflammation and overactivity in a dose dependent manner. This may provide a new treatment strategy for chemotherapy associated cystitis.

Key Words: urinary bladder, overactive; inflammation; cystitis; rebamipide; cyclophosphamide

Abbreviations and Acronyms

CYP = cyclophosphamide
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
ICI = intercontraction interval
IL = interleukin
MPO = myeloperoxidase
TNF = tumor necrosis factor

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* Correspondence: Department of Urology, Nagoya University Graduate School of Medicine, 65 Tsuruma-cho, Showa-ku, Nagoya 466-8550, Japan (telephone +81-52-744-2985; FAX: +81-52-744-2319; e-mail: yfunahashi@med.nagoya-u.ac.jp).

REBAMIPIDE has been widely used for acute and chronic gastritis and ulcer disease. Its mechanism includes suppression of inflammation, proinflammatory cytokines and chemokines, inhibition of inflammatory cell activation and migration, inhibition of free radicals and reactive oxygen species, acceleration of wound healing and mucosal barrier repair, and stimulation of prostaglandin and mucus glycoprotein synthesis.[1–3]

Because these bioregulation effects of rebamipide, including cytoprotective, wound healing and anti-inflammatory properties, may be common in various organs, rebamipide may have protective and healing actions in various tissues. Most orally administered rebamipide is not

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absorbed from the intestine[4] and rebamipide acts on damaged organs by direct contact. Thus, it must be administered locally to exert its effects. Several animal studies demonstrated its anti-inflammatory effects in the stomach as well as the colon,[5] intestines,[6] lungs[7] and conjunctiva[8] in animal models when administered directly to these target organs. Rebamipide was also recently approved for dry eyes in the form of eye drops and groups reported the potency of a rebamipide enema against inflammatory bowel disease.[9–11]

Based on this background we hypothesized that rebamipide may effectively suppress bladder inflammation when given intravesically. Specifically, we applied rebamipide directly in the bladder in a chemically induced cystitis rat model and investigated its therapeutic effects on bladder inflammation and overactivity.

MATERIALS AND METHODS

All animal experiments were performed in accordance with institutional guidelines and approved by the Nagoya University Institutional Animal Care and Use Committee.

Cystitis Model

Female Sprague Dawley rats weighing 230 to 260 gm were injected with CYP (150 mg/kg) in the peritoneum while under isoflurane anesthesia. The bladder was compressed and emptied. Rebamipide (300 μl, 1 mM or 10 mM) or vehicle composed of 1.000 gm polyvinyl alcohol, 0.146 gm sodium citrate, 0.715 gm sodium chloride and 0.180 gm potassium chloride in distilled water (adjusted to pH 6, total amount 100 ml) was administered in the bladder. Rats were kept supine for 1 hour under anesthesia. Control rats were injected with saline in the peritoneum and vehicle was administered in the bladder. The rats were divided into 4 groups, including control, CYP, CYP plus 1 mM rebamipide and CYP plus 10 mM rebamipide.

Animal Perfusion and Tissue Preparation

At 48 hours after intraperitoneal CYP administration 24 rats (6 per group) were anesthetized with isoflurane, followed by intracardiac perfusion with cold heparinized saline. The bladder was excised immediately.

Histological Analysis

The excised bladder was immediately frozen in frozen section compound (Leica Microsystems, Wetzlar, Germany), cut into 8 μm sections and mounted on slides. Tissue sections were fixed in 4% paraformaldehyde for 10 minutes, stained with hematoxylin and eosin, dehydrated through a graded ethanol series, cleared in xylene and coverslipped with mounting medium (Merck, Darmstadt, Germany). Bladder histological changes were graded as 0—no evidence of inflammatory infiltration or interstitial edema, 1—mild (few inflammatory cells and little or no interstitial edema), 2—moderate (infiltration of a moderate number of inflammatory cells and moderate interstitial edema) or 3—severe (many diffuse infiltrating inflammatory cells and severe interstitial edema). A single blinded research technician at our laboratory graded bladder histology.

MPO Assay

After histological evaluation the bladder was kept at −80C until mRNA or protein analysis was done. Half of the bladder tissues were homogenized in RIPA lysis buffer. The homogenate was centrifuged at 10,000 × gravity for 10 minutes and supernatants were stored at −80C until assayed. MPO concentration was measured using an enzyme-linked immunosorbent assay kit (Hycult® Biotech). Protein concentration was determined using a Bio-Rad® kit with bovine serum albumin as the standard. MPO concentrations were standardized to tissue protein levels and expressed as ng/mg total protein.

Cytokine mRNA Quantification

Total RNA was extracted from the other half of the bladder tissue using TRIzol® reagent according to the manufacturer protocol. RNA concentration was estimated by measuring absorbance at 260 nm with a NanoDrop™ 2000c. The 260/280 nm absorbance ratio was used to check for purity. RNA (1 μg) was reverse transcribed into cDNA using SuperScript™ II. Primer and probe sets designed for TaqMan® Gene Expression Assays were used for IL-1β, IL-6, TNF-α and GAPDH. mRNA levels were quantified with a Mx3000P™ Real-Time PCR System in a 20 μl volume using PCR Master Mix (Applied Biosystems®). We amplified cDNA for 40 cycles at 95C for 15 seconds and 60C for 60 seconds. Reaction specificity was confirmed by melting curve analysis. Samples were quantified using the threshold cycle ratio in regard to GAPDH.

Cystometry

At 48 hours after intraperitoneal CYP administration 6 rats per group were anesthetized with isoflurane. Via a lower midline abdominal incision we exposed the bladder and inserted PE-50 tubing (Clay Adams, Parsippany, New Jersey) in the bladder through the dome. Anesthesia was ended and unrestrained conscious rats were placed in a recording cage. After 2-hour acclimation saline was infused transvesically at 0.04 ml per minute and the rats voided spontaneously through the urethra. At least 4 reproducible micturition cycles were recorded after an initial 60-minute stabilization period. Chart™ 7 was used for data collection and analysis. We evaluated baseline pressure, voiding pressure threshold, peak voiding pressure and ICI.

Statistics

All values are expressed as the mean ± SE. The nonparametric Mann-Whitney U test was used to assess differences between groups. All tests were 2-sided with $p < 0.05$ considered statistically significant. Statistical analysis was done with SPSS®.

RESULTS

Histopathology

Inflammatory changes, including severe submucosal edema and inflammatory cell infiltration accompanied by urothelial injury, were observed in the

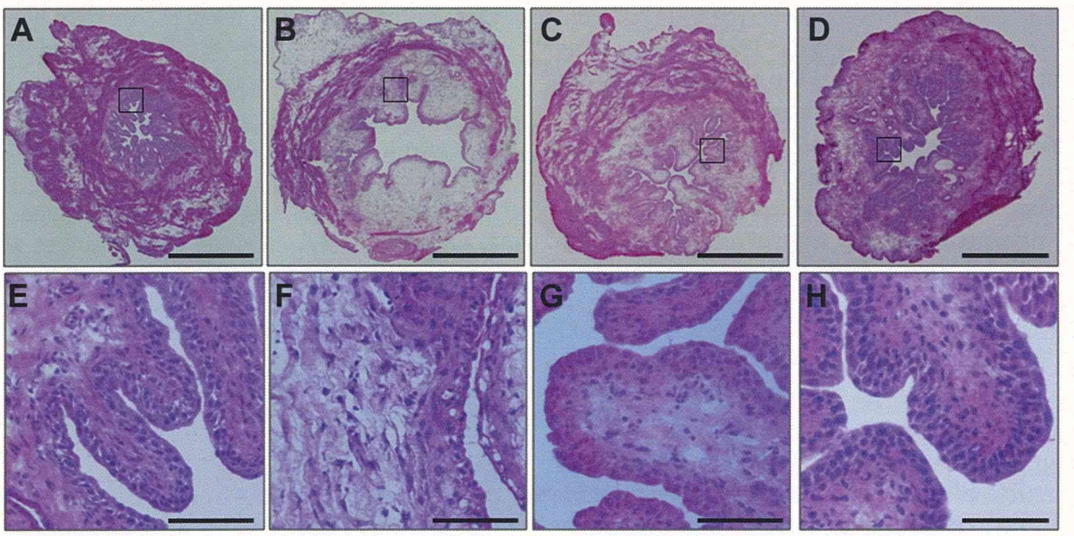

Figure 1. Bladder histological findings. Compared with controls (*A* and *E*) severe submucosal edema and inflammatory cell infiltrates were observed in CYP treated rats (*B* and *F*). Inflammation was ameliorated by intravesical administration of 1 (*C* and *G*) and 10 (*D* and *H*) mM rebamipide. H&E, reduced from ×40 (*A* to *D*) and ×400 (*E* to *H*). Scale bar indicates 2 mm (*A* to *D*) and 100 μm (*E* to *H*).

bladder of CYP injected rats. Bladder submucosal edema was reduced in rats treated with 1 mM rebamipide and apparently suppressed in those treated with 10 mM rebamipide. Rebamipide application decreased inflammation grade in a dose dependent manner (figs. 1 and 2, *A*).

MPO Assay

The amount of MPO in the bladder was significantly higher in the CYP group than in the control group ($p < 0.01$). Rebamipide dose dependently decreased MPO, demonstrating anti-inflammatory effects consistent with histological findings (fig. 2, *B*).

Proinflammatory Cytokine mRNA Expression

We quantified IL-1β, IL-6 and TNF-α mRNA levels in the bladder. Similar to the amount of MPO, these proinflammatory cytokines were also up-regulated in the CYP group. This was ameliorated by intravesical rebamipide (fig. 3).

Cystometry

CYP injected rats showed bladder overactivity with significantly shortened ICI ($p < 0.001$). Rebamipide

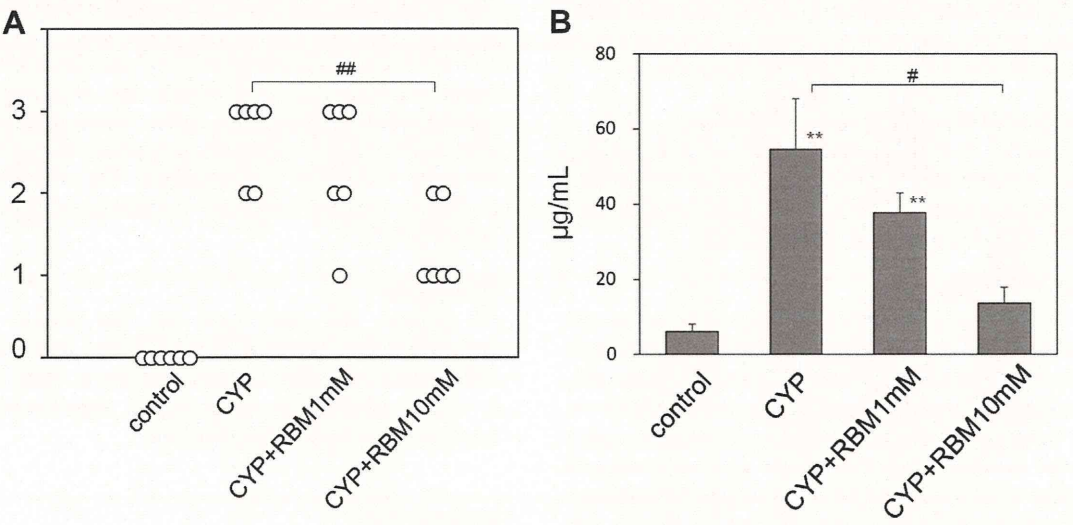

Figure 2. *A*, inflammation findings were scored from 0 to 3. Compared to controls inflammation scores were significantly higher in other groups. Intravesical administration of 10 mM rebamipide (*RBM*) decreased inflammation score. Pound signs indicate $p < 0.01$ between groups. *B*, MPO assay revealed that MPO was increased in CYP treated rats and suppressed by 10 mM rebamipide. Asterisks indicate $p < 0.01$ vs control. Pound sign indicates $p < 0.05$ between groups.