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Predictive value of self-reported patient information for the identification of lumbar spinal stenosis

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Background. To our knowledge, no objective criterion has been identified for the diagnosis of lumbar spinal stenosis (LSS) and no study has evaluated the predictive value of self-reported patient information for the identification of LSS.

Objective. To develop and validate a prediction rule for the identification of LSS based on self-reported patient information alone.

Methods. Prospective derivation study using a coefficient-based multivariable logistic regression scoring method with internal validation with primary care clinics and orthopaedic departments of medical centres, as well as university and other hospitals. Participants were consecutive patients with primary symptoms of pain or numbness in the lower extremities. Physician-diagnosed LSS was the main outcome measure.

Results. Of 468 patients included in the analysis, 47.3% were diagnosed with LSS and divided into derivation and validation sets. The following items were retained at the conclusion of the derivation process: age (<60, 60–70 and >70), duration of symptoms over 6 months, symptom improvement when bending forward, symptom improvement when bending backward, symptom exacerbation while standing up, intermittent claudication and urinary incontinence. To derive a risk score for each patient, integer-based scores were assigned and summed. In the validation data sets, prevalence of LSS in patients from the first to fourth risk score quartile were 13.3%, 47.6%, 55.2% and 65.5%, respectively. Further, the likelihood ratio in the low-risk category was 0.154.

Conclusions. We developed a prediction rule for the identification of LSS based on self-reported patient information alone. Further, the likelihood ratio in the low-risk category was sufficiently low. This rule may be used for screening of LSS.

Keywords. Lumbar spinal stenosis, prediction rule, self-reported patient information.

Introduction

Lumbar spinal stenosis (LSS) is a condition caused by compression of the cauda equina or spinal nerve roots.[1] LSS may lead to substantial functional disability, intermittent claudication or vesico-rectal disturbance and an associated decrease in quality of life and increase in social burden.[2,3] Although the effectiveness of surgical treatment remains unclear due to limited scientific evidence, early diagnosis and identification of patients needing treatment is reported to improve the outcome of LSS.[4–6] However, the most common initial symptoms of LSS, namely leg symptoms, are frequently misdiagnosed.[3,7] Data on the population-based epidemiology of LSS is relatively limited possibly due in part to the difficulty of diagnosing LSS. In particular, because computed tomography and magnetic resonance imaging (MRI) are often non-specific[8–10] and therefore not sufficiently reliable for diagnosis, no objective criteria for diagnosis are available. Although

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self-reported patient information, including medical history, symptoms and signs, is a necessary component of the clinical diagnosis of LSS, its predictive value has not been extensively evaluated.

Given these conditions, the clinical prediction rule (CPR), which consists in calculating integer-based scores from information on patient symptoms and physical examination results, is a useful tool for predicting the probability of a disease.[11] Evaluation of the diagnostic performance of CPR enables the estimation of the predictive value of patient information as a group of items. Further, the predictive value of self-reported patient information in the diagnosis of LSS may potentially facilitate the conduct of community-based screening, as well as the estimation of LSS prevalence or incidence.

Here, the predictive value of self-reported patient information was evaluated for the identification of LSS in patients with leg symptoms by developing and validating the test performance of a CPR composed of self-reported patient information alone.

Methods

Subjects

The target population was identified as patients visiting primary care clinics, which were recruited for the study. Selection was limited to consecutive patients showing primary symptoms of pain or numbness in the lower extremities, including the buttocks, thighs and lower legs, aged more than 20 years, and able to visit the clinic alone without assistance. Patients having visited other hospitals or clinics for the above symptoms within the year before study participation, as well as those showing severe psychiatric disorders, namely dementia, were excluded. If necessary, the study was approved by the institutional review board of each study institution, and written informed consent was obtained from all patients.

Data collection

Data collection from patients was planned in two steps. First, eligible patients completed a self-administered questionnaire at the primary care clinics before consulting the attending physician to avoid incorporation bias. Second, patients were referred to orthopaedic departments in hospitals and medical history, physical examination, lumbar X-ray and MRI results were recorded by the orthopaedic staff physicians.

Questionnaire

The questionnaire queried patients on the association between their symptoms and posture (lying in bed, sitting on a chair, standing upright, bending forward or backward), as well as activities (walking and riding a bicycle). An additional question enquired about intermittent claudication: 'If your symptom occurs while walking, does it improve by resting?'. Other items included age, gender, time of symptom occurrence, symptom history, treatment history, sensory or motor disturbances in the legs, cauda equina syndrome (urinary or faecal incontinence, dysuria, urinary retention, nocturia and penile erection while walking), smoking habit, alcohol drinking and comorbidities. Items from the Japanese version of the modified Roland–Morris Disability Questionnaire[12] were also included by altering the expression 'low back pain' to 'pain or numbness in the lower extremities'.

LSS diagnosis process

The association between questionnaire responses and LSS diagnosis was prospectively evaluated. For all patients, orthopaedic staff physicians in each institution recorded medical history and performed a physical examination, as well as lumbar X-ray and MRI in accordance with a standardized protocol. The medical history included the type and distribution of symptoms (e.g. leg and lumbar pain), postures attenuating or exacerbating symptoms, as well as comorbidities, including diabetes and peripheral artery disease. Physical examination included the ankle pressure index and various tests designed to elicit dysfunction in the lumbopelvic region.[13] To avoid verification bias, all patients then underwent lumbar X-ray and MRI. Clinical and diagnostic test information was recorded by the attending physician in a standardized form, which was then sent for diagnosis and information verification to the study coordinator, who is an experienced orthopaedic surgeon. In the absence of a universally accepted reference standard for LSS, studies show that the opinion of expert clinicians provides a reasonable method for establishing clinical diagnosis.[14] In addition, this approach has been used in the development of classification criteria for rheumatic diseases, which similarly to LSS cannot be defined by single laboratory measurements.[15,16]

The flow of final patient diagnosis is detailed in Figure 1. First, the attending orthopaedic physician reached a clinical diagnosis based on history taking, examination and radiographic findings. The study coordinator then verified diagnosis using copies of the clinical information and radiographic images. Interobserver agreement between the attending orthopaedic physician and study coordinator was assessed by calculating agreement ratios and kappa values.[17]

Substantial discrepancy between diagnoses [interobserver agreement rate on LSS diagnosis: 60.8%, kappa value: 0.261 (95% confidence interval, CI, 0.185–0.336)] were resolved in a second diagnostic step using a consensus panel, composed of 10 orthopaedic physicians with specialist clinical experience in LSS, in which all members were either professors or associate professors at university hospitals or the head of the orthopaedic department at teaching hospitals in Japan.

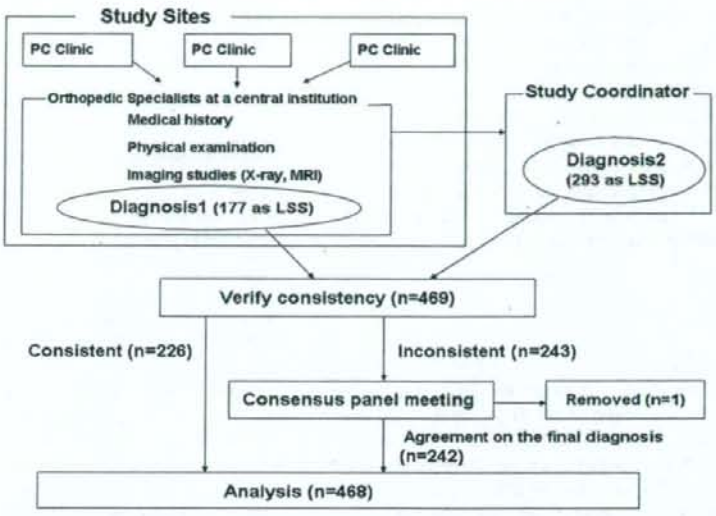

FIGURE 1 *Schema for the handling of LSS diagnoses*

For each discrepant case, panel members arrived at a four-scale score on the probability of LSS (lowest = 1 and highest = 4) based on clinical information and imaging studies. Scoring was performed without consultation with other panel members. Mean scores for each patient were then calculated, with a mean score of 3 or more confirming LSS, whereas a mean score of 2 or less was not considered as LSS. For cases with mean scores between 2 and 3, the consensus panel members carefully discussed the discrepancy and achieved final diagnosis. Cases not having reached consensus were removed from analysis.

Data analysis

Derivation. Before beginning data analysis, all observations were randomly assigned to derivation and validation sets at a 4 to 1 ratio. The univariate relationships between LSS and each item in the questionnaire from the derivation set were evaluated by univariate logistic regression analysis, and an odds ratio was generated. All items with a *P*-value of less than 0.05 in the univariate analysis were entered into a multiple logistic regression model using stepwise model selection. In addition, two items with a *P*-value of more than 0.05 but considered clinically important were also entered: 'diabetes as comorbidity', due to diabetic neuropathy being an important differential diagnosis of LSS, and 'symptom improves when bending backward', considered critical in LSS diagnosis. Only variables with a *P*-value of less than 0.05 were kept in the final model. Model calibration was evaluated using the Hosmer–Lemeshow chi-square statistic.[18]

Development of CPR. A score-based prediction rule for the final diagnosis of LSS was developed for each step based on results from multivariable logistic regression equations using a regression coefficient-based scoring method. To generate a simple integer-based point score for each predictor variable, scores were calculated by dividing the β-coefficient by the half-sum of the two smallest coefficients in the model, followed by rounding up to the nearest integer. To achieve a value of 1 for the variable with smallest coefficient score, the denominator was selected. The overall risk score for each patient was calculated by summing up the scores for each variable.[19] After the total risk score was calculated, patients were further categorized into total risk score quartiles, and their observed probabilities of LSS compared. Discriminatory performance of the rule was assessed by calculating the area under the receiver operating characteristic (ROC) curve.[20] Sensitivity and specificity were calculated at the cut-off score point of 5, which divides the second and third quartiles.

Validation of CPR. The prediction rule in the validation set was validated, and patients were categorized in risk score quartiles using the same derivation process. The observed probabilities of LSS in patients were compared and likelihood ratios calculated. Discriminatory performance of the rule was assessed by calculating the area under the ROC curve. Sensitivity and specificity were calculated at the cut-off score point of 5. All statistical analyses were performed using Stata, version 9.2 (Stata Corp., College Station, TX).

Sample size. Sample size was determined to enable the detection of a significant difference in LSS prevalence between patients clinically showing an improvement in symptoms when bending forward and those showing no improvement. LSS prevalence in groups showing 'symptom improvement' and 'no symptom improvement' was 60% and 41%, respectively, which revealed that a sample size of at least 308 patients was needed to reach a statistical power of 0.90 at a 0.05 significance level (two-sided test).

Results

Patient characteristics

From December 2002 to December 2004, a total of 469 patients were evaluated by 104 orthopaedic surgeons in 22 clinics and 50 hospitals in various sites around Japan. Ages ranged from 20 to 96 years, with a mean age of 65.2 years, and 54.2% of patients were male. Although recruitment of all patients was planned to be carried out at primary care settings, protocol violation occurred in some institutions, in which patients were recruited from the orthopaedic department of the hospital. However, recruitment of at least one-third of patients at primary care clinics was clearly confirmed. Of the 469 participants, diagnosis from the two observers was consistent in 226, of whom 126 were diagnosed with LSS. Of the 243 discrepant cases, only nine cases' mean scores were between 2 and 3, and thus were discussed by the consensus panel, of which agreement was reached in 8. In total, the consensus panel discussion diagnosed 96 cases with LSS. The interobserver agreement rate between attending physicians and expert panel members was 85.7%, with a kappa value of 0.71 (95% CI, 0.621–0.799). In contrast, the agreement rate between the study coordinator and expert panel members was 67.5%, with a kappa value of 0.36 (95% CI, 0.272–0.445).

The consensus panel failed to reach agreement in one case only, which was removed from analysis, leaving 468 cases. Results showed a 47.3% prevalence of LSS in the patient sample group. Other diagnoses included lumbar disc herniation (17.7%), diabetic neuropathy (2.8%) and peripheral artery disease (8.3%). In the remaining patients (23.7%), no specific diagnosis other than 'not LSS' was determined (Table 1).

Derivation. From the total patient sample, 374 and 94 patients were selected as the derivation and validation set, respectively. First, 26 variables with a *P*-value less than 0.05 were identified (Table 2). Second, the following variables with *P*-values less than 0.05 were retained in the multivariable model as independent predictors: age more than 60 years (with a higher risk for more than 70 years), symptom present for more than 6 months, symptom improvement when bending forward, symptom improvement when bending backward, symptom exacerbation while standing up, intermittent claudication and urinary incontinence (Table 3). For the final model, results show a Hosmer–Lemeshow statistic of 2.47 ($P = 0.9632$), which indicates the predicted probabilities of LSS statistically agreed with the observed frequencies.[18,21]

TABLE 1 *Patient characteristics*

Variable	($N = 469$)	
Age (mean ± SD)	65.2 ± 13.7	
Sex (male)	54.2%	
Clinical diagnosis of patients	*n*	%
LSS	222	47.3
LDH[a]	83	17.7
Diabetic neuropathy	13	2.8
Peripheral artery disease	39	8.3
Other[b]	111	23.7
Undetermined	1	0.2

[a]Lumber disc herniation.
[b]Unknown or unspecified, but regarded as non-LSS.

Development of CPR. An integer-based score, derived in the second step from the β-coefficient, is shown in Table 3 and Appendix, from which a mean overall risk score of 5.05 (range: –2 to 10) was obtained for each patient in the derivation set. These patients were categorized into risk score quartiles: the first (Q1), second (Q2), third (Q3) and fourth (Q4) quartiles were defined by risk scores of 2 or less, 3–4, 5–6 and 7 or more, respectively. Of the 374 patients in the derivation set, Q1 showed a 17.7% (9/51) probability of LSS, whereas Q2, Q3 and Q4 showed 25.3% (25/99), 50.8% (62/122), and 77.5% (79/102), respectively. Table 4 shows the model performance indices. For the derivation set, calculations show an area under the ROC curve of 0.77 (Fig. 2). Sensitivity and specificity at the cut-off score point of 5 were 0.81 and 0.58, respectively. Further, the likelihood ratio in Q1 of the derivation set was 0.24.

Validation of CPR. In the validation set, results show a mean overall risk score for each patient of 5.21 (range: 0–10). Of the 94 patients in the validation set, Q1 showed a 13.3% (2/15) probability of LSS, whereas Q2, Q3 and Q4 showed 47.6% (10/21), 55.2% (16/29) and 65.5% (19/29), respectively (Fig. 3). In addition, calculations show an area under the ROC curve of 0.67 (Fig. 2), indicating a slight decrease in performance compared with the derivation set. However, no statistical significance was observed between the area under the ROC curves of the derivation and validation sets ($P = 0.125$). Sensitivity and specificity at the cut-off score point of 5 were 0.75 and 0.51, respectively. Further, the likelihood ratio in Q1 of the validation set was 0.15.

TABLE 2 *Univariate correlates of LSS in the derivation set*

Variable	LSS ($N = 199$) (%)	Not LSS ($N = 175$) (%)	Odds ratio (95% CI)	*P*-value
Age				
<60	14.9	41.7	Reference	–
60–70	29.1	29.2	2.81 (1.57 to 5.01)	<0.001
>70	56.0	29.2	5.39 (3.12 to 9.32)	<0.001
Onset				
Under 6 months	34.3	52.8	Reference	–
Over 6 months	59.4	42.2	2.17 (1.41 to 3.32)	<0.001
Missing data	6.3	5.0	1.93 (0.77 to 4.80)	0.16
RDQ[a]				
I change my position frequently to try and get my lower extremities comfortable (yes, %)	73.1	61.3	1.72 (1.11 to 2.67)	0.016
I walk more slowly than usual because of my lower extremities (yes, %)	86.3	73.4	2.28 (1.34 to 3.89)	0.002
Because of my lower extremities, I use a handrail to get upstairs (yes, %)	66.3	52.3	1.80 (1.18 to 2.73)	0.006
Because of my lower extremities, I have to hold on to something to get out of an easy chair (yes, %)	43.4	32.2	1.62 (1.06 to 2.47)	0.025
I only stand for short periods of time because of my lower extremities (yes, %)	71.4	57.3	1.86 (1.21 to 2.87)	0.005
I only walk short distances because of my lower extremities (yes, %)	76.0	63.3	1.83 (1.17 to 2.88)	0.008
I sit down for most of the day because of my lower extremities (yes, %)	37.7	23.1	2.01 (1.28 to 3.16)	0.002
Symptoms				
Treatment for symptoms needs to be repeated every year (yes, %)	40.0	19.6	2.71 (1.72 to 4.34)	<0.001
Symptoms improve when bending forward (yes, %)	42.9	24.6	2.30 (1.48 to 3.57)	<0.001
Symptoms improve when bending backward (yes, %)[b]	15.4	21.6	0.66 (0.40 to 1.13)	0.128
Symptoms exacerbated while standing up				
No	7.4	19.1	Reference	–
Yes	86.3	72.9	3.04 (1.56 to 5.95)	0.001
Missing data	6.3	8.0	2.01 (0.74 to 5.42)	0.168
Back pain occurs while walking				
No	16.6	25.6	Reference	–
Yes	78.9	70.4	1.73 (1.04 to 2.90)	0.036
Missing data	4.6	4.0	1.74 (0.60 to 5.18)	0.31
Numbness of lower legs occurs while walking				
No	20.0	31.7	Reference	–
Yes	74.9	63.3	1.87 (1.16 to 3.02)	0.011
Missing data	5.1	5.0	1.62 (0.60 to 4.36)	0.34
Intermittent claudication				
No	25.7	34.7	Reference	–
Yes	69.7	55.8	1.69 (1.07 to 2.66)	0.025
Missing data	4.6	9.6	0.65 (0.26 to 1.60)	0.345
Walking is easier when bending forward (yes, %)	55.4	39.2	1.93 (1.28 to 2.91)	0.002
Urinary symptoms				
Wake up to urinate at night (yes, %)	86.3	72.9	2.34(1.38 to 3.99)	0.002
Urinary incontinence (yes, %)	28.0	17.1	1.89 (1.15 to 3.10)	0.012
Alcohol intake				
No	44.0	27.1	Reference	–
Yes	49.1	60.8	0.50 (0.32 to 0.78)	0.002
Missing data	6.9	12.1	0.35 (0.16 to 0.76)	0.008
Comorbidity				
Hypertension	43.4	29.7	1.82 (1.19 to 2.80)	0.006
Cataract	33.7	20.1	2.02 (1.27 to 3.23)	0.003
Gastroenteropathy	22.3	13.1	1.91 (1.11 to 3.29)	0.02
orthopaedic disease	18.3	9.1	2.25 (1.21 to 4.17)	0.01
Diabetes[b]	16.6	16.6	1.00 (0.58 to 1.73)	0.998

[a]modified Roland–Morris Disability Questionnaire.
[b]Variables show $P > 0.05$, but are considered clinically significant.

Discussion

Summary of main findings

To our knowledge, this study is the first to evaluate the predictive value of self-reported patient information for the identification of LSS in patients with leg symptoms. Results show that the likelihood ratio in the low-risk category was sufficiently low to allow patients with a low-risk score to be excluded from diagnosis (Table 4). Although based on self-reported information, items in the present study are closely consistent with those previously reported as associated with LSS diagnosis.[1,22]

TABLE 3 *Multivariable predictors of diagnosis of LSS and associated risk scoring system*[a]

Characteristic	Regression β-coefficient (95% CI)	Assigned risk score[b]
History		
Age		
60–70	1.21 (0.58 to 1.85)	2
>70	1.79 (1.19 to 2.39)	3
Onset over 6 months	0.49 (0.001 to 0.97)	1
Symptoms		
Symptoms improve when bending forward	0.97 (0.46 to 1.49)	2
Symptoms improve when bending backward	−0.99 (−1.64 to −0.34)	−2
Symptoms exacerbated while standing up	1.22 (0.49 to 1.96)	2
Intermittent claudication (+)	0.57 (0.04 to 1.09)	1
Urinary incontinence (+)	0.63 (0.05 to 1.20)	1

[a]Hosmer–Lemeshow statistics, 2.47 ($P = 0.9632$).
[b]Scores were assigned by dividing the β-coefficient by 0.53 and rounding to the nearest integer.

TABLE 4 *Performance indices of the CPR*

Index	Derivation indices		Validation indices	
Area under ROC curve	0.77 (0.72 to 0.81)		0.67 (0.57 to 0.78)	
Sensitivity/specificity[a]	0.81/0.58		0.75/0.51	
Risk category (score)	LSS/total	LR[b]	LSS/total	LR[b]
Q1 (2 or less)	9/51	0.24	2/15	0.15
Q2 (3 or 4)	25/99	0.38	10/21	0.91
Q3 (5 or 6)	62/122	1.18	16/29	1.23
Q4 (7 or more)	79/102	3.91	19/29	1.90

[a]For a threshold with a score of 5: in the derivation set, the positive and negative predictive values were 63% and 77%, respectively; in the validation set, positive and negative values were 60% and 67%, respectively.
[b]Likelihood ratio.

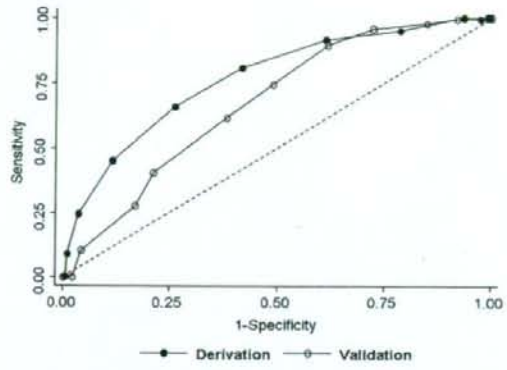

FIGURE 2 *ROC curves for LSS. The areas under ROC curves for the derivation and validation set were 0.77 (range: 0.72–0.81) and 0.67 (range: 0.57–0.78), respectively*

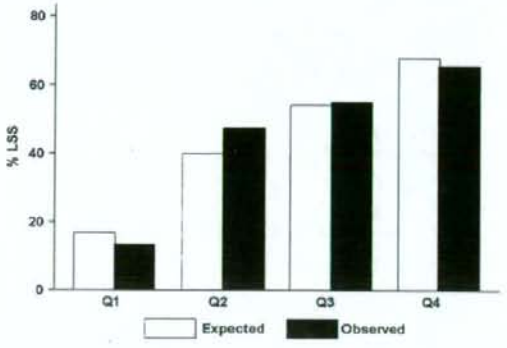

FIGURE 3 *Expected and observed LSS risk quartiles in the validation set patients. Risk categories: Q1 = score of 2 or less; Q2 = score of 3–4; Q3 = score of 5 – 6 and Q4 = score of 7 or more*

Evaluation of predictive value of patient histories

CPRs usually consist of items related to medical history, physical information and diagnostic tests. To diagnose physical conditions, rules which use only self-reported information from patients are rare. Although evaluated as a group of items in the present study, medical history as a diagnostic tool is often assessed as an independent item. For example, the Journal of the American Medical Association publishes a periodical feature entitled the 'Rational Clinical Examination Series',[23] in which the predictive value of clinical information is evaluated, such as that of symptoms for the diagnosis of heart failure or influenza.[24,25] A limitation of the individual evaluation of every piece of clinical information is the overpowering sensitivity or specificity for single items due to the dependence of final diagnosis on information from a number of diverse sources.[24] In the present study, the value of self-reported information from patients was evaluated as a group of items, which may potentially improve the value of clinical information in use.

Cauda equina syndrome

An additional item, urinary incontinence, is also contained in the present prediction rule. A great number of patients presenting with an advanced form of LSS manifest varying degrees of bladder dysfunction, including urinary incontinence,[26] with more severe clinical signs of cauda equina syndrome in patients with neuropathic bladder than in those without.[27] Early diagnosis and decompression was reported to improve the outcome in patients with cauda equina syndrome, which includes bladder dysfunction[28]. Given this finding, the careful history and physical examination of patients with suspected cauda equina syndrome is recommended.

Application for primary care practice

A CPR composed only of self-reported patient information may be useful for a variety of purposes. First, with the recent ageing population, the lack of epidemiological data on LSS may hamper appreciation of its importance in policymakers. Given the ease of use and distribution of self-reported information in the community, our tool may be used to estimate the incidence or prevalence of LSS in the general population. Second, this method may be used by patients with leg symptoms for self-screening of LSS. An ecology of medical care study revealed that only a small portion of patients reporting symptoms visit a hospital.[29] Given this observation, our tool may facilitate the decision to seek medical care for patients. Third, because of the simplicity of our tool, assessing the need to refer patients to an orthopaedic specialist may be facilitated for non-orthopaedic specialists. Wider application of our tool is predicated on its validation in a variety of settings.

Limitations

Although our method shows potential in its ease of use, several limitations should be noted. First, no consensus has been reached on an objective standard for LSS diagnosis, which essentially remains clinical. In the absence of explicit criteria, however, expert opinion is a reasonable strategy for the diagnosis of clinical syndromes[14] and has been previously used in a number of disorders.[15,16] In the present study, inconsistent diagnosis from two observers were resolved by a consensus panel composed of 10 orthopaedic specialists, who may in turn qualify the reference standard used to diagnose LSS.

Second, because a number of patients were recruited from hospitals, LSS prevalence in the present study appears higher than that using patients recruited from primary care clinics. Unlike in the UK, no official gatekeeping system by GPs is available in Japan, with patients free to choose hospitals or clinics when seeking medical care.[30] A study showed that among 1000 Japanese residents, 232 visited a primary care physician and 88 a hospital-based outpatient clinic as a first visit in a month.[31] These findings show that in some situations, hospitals play a role as primary care providers. Despite this situation, further investigation is necessary to validate our rule in other primary care populations.

Third, the likelihood ratio in the high-risk category was low, which suggests that this rule is possibly weak for a definite diagnosis of LSS. However, especially in primary care settings, a screening tool is crucial to eliminate the possibility of specific diseases in diagnosis. Further, the likelihood ratio in the low-risk category was sufficiently low, which implies that this rule useful in safely excluding LSS patients.

Conclusion

The present study is the first to report a prediction rule for LSS identification based only on self-reported patient information. Results show that the likelihood ratio in the low-risk category was sufficiently low. Our rule may be used for LSS screening and is suggested to improve the quality of LSS diagnostic practice in primary care.

Declaration

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Ethical approval: This study was approved by the institutional review board of each study institution.
Conflicts of interest: None.

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Appendix. Questionnaire used to identify patients with possible LSS among those with pain or numbness in the lower extremities (including thigh, buttock and lower legs).

Q1. How old are you?	
under 60 years old	0
60 years to 70 years old	+2
over 70 years old	+3
Q2. How long have you had your symptom?	
less than six months	0
Six months or more	+1
Q3. Does your symptom improve when you bend forward?	
no	0
yes	+2
Q4. Does your symptom improve when you bend backward?	
no	0
yes	-2
Q5. Does your symptom occur while you stand up?	
no	0
yes	+2
Q6. If your symptom occurs while walking, does it improve by resting?	
no	0
yes	+1
Q7. Do you have urinary incontinence?	
no	0
yes	+1

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Analgesic effect of percutaneously absorbed non-steroidal anti-inflammatory drugs: an experimental study in a rat acute inflammation model

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Abstract

Background: External medication that is absorbed percutaneously may be used to reduce inflammation and relieve pain from acute injuries such as ankle sprains and bruises. The plaster method of percutaneous absorption for non-steroidal anti-inflammatory drugs (NSAIDs) was established in Japan in 1988. However, due to the possibility of a placebo effect, the efficacy of this method remains unclear. This experimental study was conducted to control for the placebo effect and to study the efficacy of the plaster method in relieving pain by using a rat model of inflammation.

Methods: Male Wistar-Imamichi rats were used. A yeast suspension was injected into the right hind paw to induce inflammation. A sheet (2.0 × 1.75 cm) containing the drug was adhered to the inflamed paw. Five treatment groups were used, and each sheet contained a single drug: loxoprofen sodium (loxoprofen-Na) (2.5 mg); felbinac (1.75 mg); indomethacin (1.75 mg); ketoprofen (0.75 mg); or base only (control, 0 mg). Mechanical pain threshold, expression of c-Fos in the dorsal horn, and amount of prostaglandin (PG) E_2 in the inflamed paw were evaluated.

Results: Pain threshold increased after treatment, and was significantly increased in the loxoprofen-Na group compared with the control group ($p < 0.05$). Amounts of PGE_2 were significantly decreased in the loxoprofen-Na and indomethacin groups compared with the control group ($p < 0.05$). Expression of c-Fos was significantly decreased in the loxoprofen-Na group compared with the control group ($p < 0.05$).

Conclusion: Percutaneously absorbed NSAIDs have an analgesic effect, inhibit expression of c-Fos in the dorsal horn, and reduce PGE_2 in inflamed tissue, indicating the efficacy of this method of administration for acute inflammation and localized pain.

Background

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in clinical situations worldwide to reduce inflammation and pain. These agents are administered

orally, intravenously, intrarectally and percutaneously. Topical applications for percutaneous absorption comprise several forms, such as ointments, lotions, aerosols, liniments, cataplasms and plasters, and pressure-sensitive adhesives. A plaster method of percutaneous absorption for NSAIDs was developed in Japan in 1988. This application is used to reduce inflammation and to relieve pain from acute injuries such as sprains and bruises. This differs from patch-type drugs for systemic administration. However, in clinical situations, external medicines are not typically used as a main conservative treatment because the efficacy of this method in humans remains unclear due to the possibility of a placebo effect. Since the effects of plaster treatment can be separated from the placebo effect, an experimental study was needed to evaluate the efficacy of this method. Few preclinical experiments have investigated the analgesic effects of percutaneous absorption with these types of drugs.

NSAIDs inhibit the biosynthesis of prostaglandins (PGs), and PGE_2 is one of the inflammatory mediators associated with inducing peripheral hyperalgesia [1]. PGE_2 levels can be used to determine the effect of the medication as an index of inflammation. In addition, the *c-Fos* gene is expressed following noxious input, and level of expression offers a marker of signals to sensory cells in the spinal cord [2-4]. The purpose of this study was to investigate the effects of plaster treatment on mechanical hyperalgesia, expression of c-Fos in the spinal cord, and the amount of PGE_2 in a rat model of inflammation.

Methods

A total of 90 male Wistar-Imamichi rats (4–5 weeks old; Imamichi Institute of Animal Reproduction, Japan) were used in this study. Animals were housed in plastic cages at room temperature with a 12:12 light:dark cycle and *ad libitum* access to food and water. All experiments were approved by the Animal Studies Committee at Fukushima Medical University.

Acute inflammatory model

Animals were anesthetized using 99% diethyl ether (Wako Pure Chemical Industries, Osaka, Japan). A suspension of 10% or 20% brewer's yeast (Sigma-Aldrich, MO, USA) at 0.1 ml/hind paw was injected intradermally into the right hind paw. According to Randall and Selitto [5], 20% brewer's yeast induces inflammation. Pain threshold is reduced at 1 h and is further decreased at 2 and 4 h. A return to baseline is seen by 48 h after induction of inflammation. However, efficacy of treatment with use of 20% yeast was only found according to PGE_2 level, and not pain threshold or c-Fos expression (data not shown). As the degree of inflammation using 20% yeast thus appeared too strong to evaluate pain threshold and c-Fos expression by the percutaneous drug delivery system, 10% yeast was used. To prevent ingestion of the drug sheet, an Elizabethan collar comprising a plastic sheet was applied around the neck of each rat (Fig. 1).

Treatment groups

Animals were divided into 5 groups and treated with different percutaneously absorbed drugs. All drugs were clinically applied in the form of a patch sheet (10.0 × 14.0 cm) to the affected area. Drug dose per sheet was 100 mg loxoprofen sodium (loxoprofen-Na), 70 mg felbinac, 70 mg indomethacin or 30 mg ketoprofen. Since the density of each drug imbedded in the sheet differed, the applied dose was controlled by standardizing sheet size in this study at 2.0 cm × 1.75 cm, based on the relative difference between human and rat body sizes. The sheet for rat treatment contained 2.5 mg loxoprofen-Na, 1.75 mg felbinac, 1.75 mg indomethacin or 0.75 mg ketoprofen. Control rats received an application of a base sheet, representing a sheet without NSAIDs (control; 0 mg). This base sheet contained several substances to improve drug absorption, control drug release and minimize skin irritation. To exclude the influence of such substances, treatment groups were compared to the control group treated with the base sheet alone. A sheet of each drug was applied to the paw for treatment and covered by a net to prevent the sheet from coming off (Fig. 2).

Measurement of pain threshold

Pain threshold was measured using 30 rats (n = 6 per group). Treatments were started 4.5 h after 10% yeast injection. The pain threshold began to decrease at 1 h and decreased further by 2 and 4 h, returning to baseline by 48 h after induction of inflammation [5]. In addition, swelling of the paw is reportedly maximized at 2–5 h after inflammation is induced and is maintained for more than 5 h [6]. As a result, to investigate the efficacy of NSAID sheets, treatments were started on the basis of active inflammation according to the clinical situation, and pain

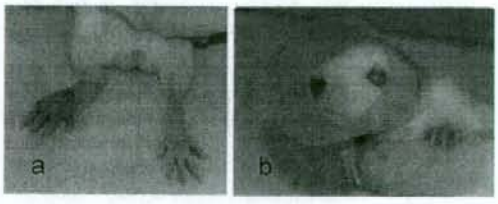

Figure 1
Acute inflammatory model and plastic Elizabethan collar for the rat neck. a) A suspension of yeast was injected intradermally into the right paw (*). b) A plastic Elizabethan collar was fixed around the neck of each rat to prevent ingestion of the drug sheet.

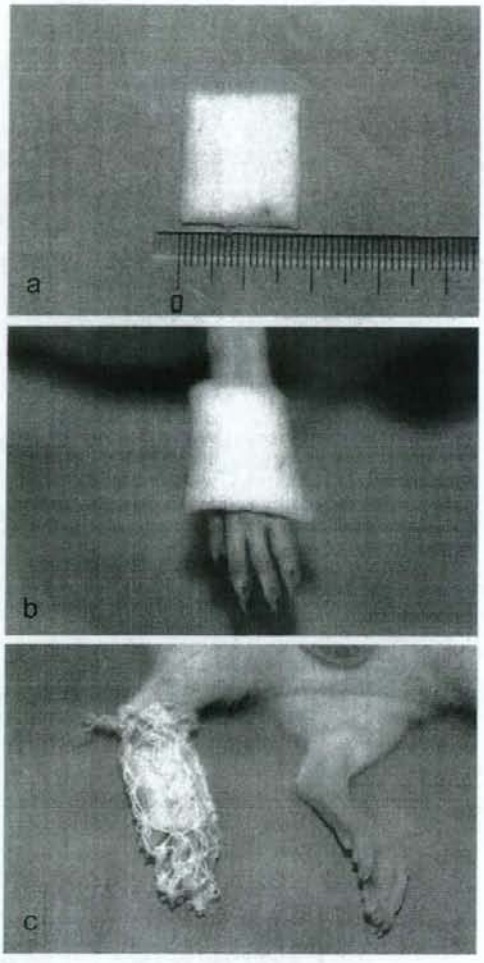

Figure 2
Sheet application. a) Sheet size was 2.0 cm × 1.75 cm. b) The sheet was applied to the paw. c) The foot was covered by a net to prevent the sheet peeling off.

thresholds were measured. Experiments were performed according to the method of Randall and Selitto [5], with the same modifications used by Winter and Flataker [6]. Just before treatment, pain thresholds were measured on bilateral paws. Pain thresholds were measured using the Analgesy-Meter (Ugo Basile, Comerio, Italy), which is able to gradually apply pressure to the paw, and the degree of pressure is shown on a graduated scale. Struggling or vocalization was considered as a positive pain response. Two sheets were adhered to the dorsal and plantar sides of paw. Pain thresholds were measured 3 h after treatment to compare groups. Measurement of pain thresholds was performed once on each rat at each time point. Data are shown as mean ± standard deviation. Statistical analysis was performed using the Bonferroni/Dunn test. Values of $p < 0.05$ were considered statistically significant.

Amount of PGE_2 in inflamed tissue

Levels of PGE_2 were assayed using 35 rats (n = 6 in each treatment group; n = 5 for treatment-naïve rats). Induced inflammation was not administered to naïve rats, and they were not given any treatment. Paws were treated with treated or control sheets immediately after injection of 20% yeast suspension. One sheet was adhered to the dorsal side of the paw.

The amount of PGE_2 was measured 0.5 h after treatment. Since PGE_2 induces peripheral hyperalgesia [1], production of PGE_2 might start shortly after injection. To determine whether drugs inhibit PGE_2 production, drugs sheets were adhered immediately after injection. In addition, if drugs inhibit production of PGE_2, expression of c-Fos would be related to this response, so we chose the same period for these measurements. Rats were euthanized using 99% diethyl ether, then inflamed paws were isolated 0.5 h after treatment, immediately frozen with liquid nitrogen and stored at -80°C. Whole paws were crushed in a Cryo-press (Microtec, Tokyo, Japan), then homogenized with a Polytron PT-3100 homogenizer (Kinematica, NJ, USA) in ice-cold phosphate-buffered saline (PBS) supplemented with 10 mM EDTA and 100 μM indomethacin (Sigma-Aldrich). The homogenate was centrifuged, and the supernatant fraction was stored at -20°C until the PGE_2 assay. PGE_2 content was assayed using a PGE_2 EIA kit (Cayman Chemical Company, MI, USA). All raw data are shown in the graph. Statistical analyses were performed using the Bonferroni/Dunn test. Values of $p < 0.05$ were considered statistically significant.

Expression of c-Fos in the dorsal horn

Histological findings were analyzed using 25 rats (n = 5 per group). Paws were treated with treated or control sheets immediately after 10% yeast suspension injection. Two sheets were adhered on the dorsal and plantar sides of the paw. Perfusion was conducted 0.5 h after treatment. Expression of c-Fos is known to peak at 30 min in the superficial laminae of the dorsal horn after nociceptive stimulation [7]. We therefore investigated expression of c-Fos 30 min after stimulation and treatment. After perfusion with 200 ml of 4% paraformaldehyde-0.1 M PBS, the spinal cord at the L5 level was removed. This section of spinal cord was then immersed in 4% paraformaldehyde-0.1 M PBS solution for 1 h, in a 10% sucrose-PBS

solution for 24 h, and in a 20% sucrose-PBS solution for 24 h. A 40-μm frozen section was made from the spinal cord using a microtome. Sections were collected as floating sections in 0.1 M PBS, and these were immersed in 0.1 M PBS including 0.2% Triton X for 3 days. Sections were immersed in 1% blocking serum for 30–60 min, then reacted with anti-c-Fos antibody (1:3000; Santa Cruz Biotechnology, CA, USA) at a temperature of 4°C for 48 h. Sections were then reacted using the avidin-biotin complex method (Vector Laboratories, CA, USA) for 30 min, followed by washing in biotinylated IgG antibody (Vector Laboratories) for 30 min. Afterwards, sections were stained using a 3,3'-diaminobenzidine, 0.0045% hydrogen peroxide solution and placed on a glass slide to keep dry.

A microscope connected to a computer was used at 400× magnification. The c-Fos-immunoreactive neurons were observed and counted on the computer monitor with a KS 100 imaging system (Carl Zeiss, Hallbergmoos, Germany). After confirmation of layers in the spinal dorsal horn in accordance with reports by Molander et al. [8], images at 400× magnification were imported into the computer to check for c-Fos-immunoreactive neurons. From 30 sections per sample test rat, 5 sections were chosen with the maximum number of c-Fos-immunoreactive neurons for layers I–VI. The spinal dorsal horn was divided into 3 groups: layers I–II; III–IV; and V–VI. The mean number of c-Fos-immunoreactive neurons for layers I–II was set as a measured value for a test rat. Statistical analysis was performed using a Bonferroni/Dunn test. Values of $p < 0.05$ were considered statistically significant.

Results

Measurement of pain threshold

Pain threshold of normal paws (contralateral side) was 255.0 ± 17.0 g, compared to 136.0 ± 4.0 g for inflamed paws 4.5 h after injection (0 h after treatment). Pain threshold in the control group decreased to 90 ± 17 g, while that in the other 4 groups was increased 3 h after treatment (Fig. 3). In the loxoprofen-Na group, pain threshold was significantly increased compared with the control group ($p < 0.05$).

Amount of PGE_2

Amount of PGE_2 was 1.01 ± 0.25 ng in treatment-naïve rats, and 8.97 ± 3.1 ng in the control group. Amounts of PGE_2 were 3.34 ± 1.53 ng in the loxoprofen-Na group, 10.49 ± 8.78 ng in the felbinac group, 1.16 ± 0.13 ng in the indomethacin group and 4.72 ± 2.42 ng in the ketoprofen group. Significant differences in amounts of PGE_2 were seen between control group and the loxoprofen-Na and indomethacin groups ($p < 0.05$) (Fig. 4).

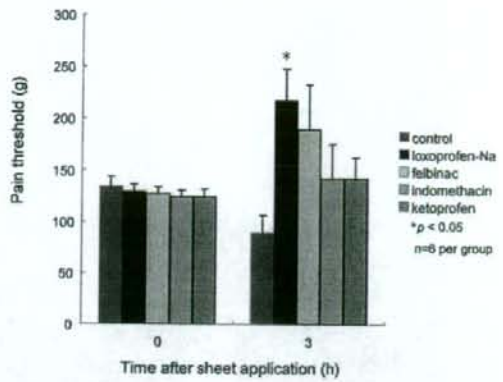

Figure 3
Pain threshold in inflamed paws (n = 6 per group). No difference was seen among groups at 4.5 h after injection of yeast suspension (0 h). Pain threshold increased 3 h after treatment in treated groups. In the loxoprofen-Na group, pain threshold significantly increased compared with the control group (*$p < 0.05$).

Expression of c-Fos

Immunoreactivity to c-Fos was observed in superficial laminae of the dorsal horn in all groups (Fig. 5). Numbers of c-Fos-immunoreactive cells in the 4 treatment groups were decreased compared with the control group (Table 1). In particular, c-Fos-immunoreactivity was significantly

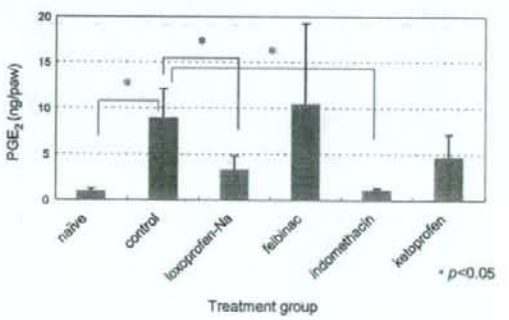

Figure 4
PGE_2 in inflamed paws. In the control group, the amount of PGE_2 increased 0.5 h after inducing inflammation. A significant difference in the amount of PGE_2 was identified between the control group and treatment-naïve animals (*$p < 0.05$). The amount of PGE_2 in the groups treated with loxoprofen-Na and indomethacin was decreased compared with the control group (*$p < 0.05$) (n = 5 for naïve group; n = 6 for treatment groups).

Table 1: Number of c-Fos-immunoreactive cells in superficial laminae of the ipsilateral dorsal horn (n = 5 per group)

	Control	Loxoprofen-Na	Felbinac	Indomethacin	Ketoprofen
Mean ± SEM	134.8 ± 4.5	113.7 ± 4.7	125.3 ± 5.4	127.5 ± 4	127.2 ± 3.5
p value vs. control		0.0008*	0.1268	0.2164	0.2227

The number of c-Fos-immunoreactive cells was decreased in the loxoprofen-Na group compared with the control group (* $p < 0.05$).

less in the loxoprofen-Na group than in the control group ($p < 0.05$) (Table 1).

Discussion

NSAIDs are the most widely used drugs for reduction of inflammation and pain in clinical situations [9]. However, side effects upon oral administration of NSAIDs include gastrointestinal disturbance and hepatic dysfunction. Reducing these side effects while maintaining the drug's therapeutic effects is important, as is exposing the target region to the drugs for a suitable length of time. To reduce side effects and enhance therapeutic effects, changing the route or method of administration is effective even without changing the chemical structure. Drugs can be given orally, intravenously, intrarectally or percutaneously. Blood concentrations of NSAIDs rise after oral administration, and systemic side effects may result. In contrast, percutaneous absorption only acts locally and is expected to increase drug concentrations and produce higher effects at the site of inflammation, thus reducing side effects throughout the whole body. Percutaneously delivered NSAIDs were thus developed to reduce inflammation and pain. Percutaneous absorption allows drugs to permeate the skin and affect the local area [10]. However, this method is not used as main clinical treatment due to the placebo effect. Depending on the substances facilitating permeability of NSAIDs through the skin, patients feel coldness or warmth on the skin and can also notice various smells. These factors can act to create placebo effects, influencing assessment of the effect of plasters that contain NSAIDs. The present experimental study was performed to exclude such possible placebo effects.

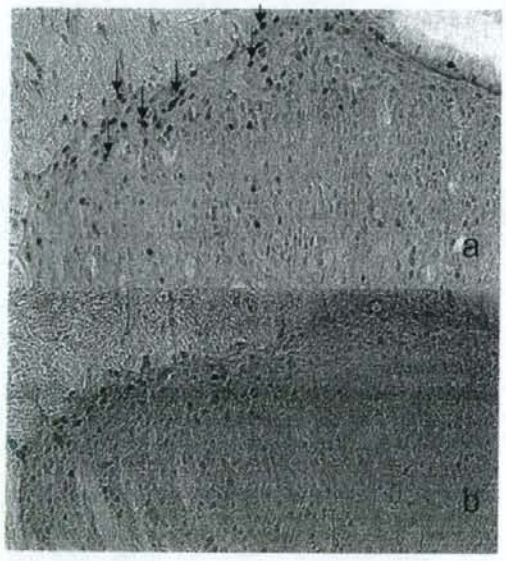

Figure 5
Immunohistochemical findings of c-Fos-immunoreactive cells in the dorsal horn ipsilateral to the inflamed hind paw (n = 5 per group). Neurons displaying c-Fos immunoreactivity (arrows; brown staining) in the superficial layer (I–II) for control (a) and loxoprofen-Na-treated (b) groups.

Changes in the diameter of the paw in this acute inflammatory model have been reported in experimental studies after oral administration of NSAIDs, but not following percutaneous absorption [11]. However, no reports have described analgesic effects after topical administration of NSAIDs in an experimental study. In this study, percutaneous absorption of NSAIDs displayed analgesic effects compared with the control group. Loxoprofen sodium was particularly effective. Although inflammation had already developed before treatment, percutaneously absorbed NSAIDs were effective in increasing pain thresholds locally. This result suggests that the local concentration of NSAIDs in inflamed tissue increases following permeation through the skin and is sufficient to achieve analgesic effects.

Analgesic effects of NSAIDs are associated with inhibition of PG production. In particular, PGE_2 and PGI_2 increase the effect of bradykinin, a pain-inducing substance. PGE_2, one of the principal inflammatory mediators, reportedly contributes to the induction of peripheral hyperalgesia and allodynia [1]. In the present study, yeast-induced inflammation increased PGE_2 content in the paw. This increase of PGE_2 may contribute to local inflammation and decreased pain thresholds. The amount of PGE_2 decreased after treatment, so percutaneous absorption of NSAIDs may have inhibited PGE_2 production to reduce pain in the inflamed hind paw. Treatment was started just

after inducing inflammation, and the time interval between treatment and PGE_2 measurement was 30 min. Percutaneously absorbed NSAIDs thus appear to work quickly. PGE_2 levels at later post-treatment time points were not measured, which was a limitation to this study. However, inhibition of PG biosynthesis is known to indirectly inhibit bradykinin generation. Since increases in pain threshold remained present 3 h after initiation of treatment in this study, PGE_2 biosynthesis was assumed to be inhibited even after 30 min.

Expression of the c-Fos gene is an effective way to signal sensory cells in a spinal cord and trigeminal nuclei excited by noxious inputs. In particular, c-Fos-activated cells are found in the ipsilateral superficial layer (I and II) in the dorsal horn of the spinal cord 2 h after noxious input such as mustard oil, formalin and carrageenin [2-4,12,13]. A previous study identified c-fos mRNA in the spinal cord after the formalin test, and expression of c-Fos peaked at 30 min in the superficial laminae of the dorsal horn (laminae I–II) and at 1–3 h in the deep laminae (laminae V–VI) [7]. Expression of c-Fos mRNA in those regions appears to correspond to early- and late-phase responses to the formalin test [14]. Some reports have evaluated NSAID effects for oral and intravenous administration using c-Fos expression in the spinal cord [9,14-21]. However, no previous studies have investigated the expression of c-Fos after treatment by percutaneously absorbed NSAIDs. In the present study, percutaneous local absorption of NSAIDs into inflamed tissue inhibited expression of c-Fos in the spinal cord compared with controls. Loxoprofen-Na was especially effective in decreasing c-Fos expression. These results suggest that loxoprofen-Na can be effective in the early stage of inflammation induction compared with other drugs. Treatment for >25 min prior to induction of inflammation has been performed to evaluate expression of c-Fos [11,19-21]. In this study, treatment was started just after inducing inflammation, and only one treatment group could prevent c-Fos expression. However, other time points and durations of effect were not investigated and thus this may be limitations to this study. In addition, dose and drug metabolism of NSAIDs differ depending on whether treatment is oral or by percutaneous absorption, so the effect of percutaneously absorbed NSAIDs cannot be directly compared with oral NSAIDs. Nevertheless, NSAIDs administered systemically affect not only inflamed tissues, but also tissues where prostanoids play physiological roles. This means that oral administration of NSAIDs carries risks of adverse effects such as gastric ulcer and edema. In contrast, percutaneously absorbed NSAIDs affect only the local area, so the risk and severity of adverse effects might be reduced compared with oral NSAIDs.

We investigated the effect of percutaneously absorbed NSAIDs by measuring pain threshold, amount of PGE_2 and expression of c-Fos in the dorsal horn. No previous reports have used these three categories to investigate the effects of percutaneously absorbed NSAIDs. We found that these effects are not placebo effects, and percutaneous absorption of NSAIDs can be expected to be useful as a main method of conservative treatment instead of oral administration. In particular, percutaneous absorption of NSAIDs is likely to be useful for patients with localized disease or risk factors, and for elderly individuals with a higher risk of side effects.

Conclusion

Percutaneously absorbed NSAIDs have analgesic effects, inhibit expression of c-Fos in the dorsal horn, and reduce PGE_2 levels in inflamed tissues. This type of topical application may be useful for acute inflammation and localized pain.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

All authors participated in the design of the study. MSE performed the studies and drafted the manuscript. MSH and SKO performed statistical analyses. SKO and SKI participated in coordination and helped to draft the manuscript. All authors have read and approved the final version of the manuscript.

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Cross talk between Smad transcription factors and TNF-α in intervertebral disc degeneration

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Abstract

The transforming growth factor-β (TGF-β) and the tumor necrosis factor-α (TNF-α) families are known to play important roles in intervertebral disc degeneration (IVD). However, molecular interactions between the TGF-β and TNF-α signaling pathways have yet to be elucidated. The purpose of this study was to analyze the expression patterns of Smad transcription factor signaling associated with IVDs with aging and to examine the modulation of Smad signaling by TNF-α in IVD cells using SD rats. According to these experimental results, BMP signals in the TGF-β family were more likely to be a key factor in IVD degeneration by aging, and it was predicted that besides the involvement of catabolic factors like MMPs and ADAMS-TS, there may be a decrease in expression of anabolic factors through cross talk of signaling between TNF-α and TGF-β pathway in pathogenesis of disc degeneration.

Keywords: Intervertebral disc; Disc degeneration; Nucleus pulposus; TNF-alpha; Smad

Low back pain, a common locomotor dysfunction, occurs because of intervertebral disc (IVD) degeneration resulting from aging, kinetic load, or social factors, such as smoking [1,2].

Clinically, spinal fusion is frequently used to treat disc disorders; however, postoperative problems, such as instability of the adjacent intervertebral discs, often occur. Motion preservation, through the development of a new treatment method inhibiting IVD degeneration prior to surgery, has been investigated and various biological strategies aimed at the restoration and regeneration of IVD degeneration have been suggested [3]. These strategies currently include intradiscal injections of cytokines or growth factors, gene transfection of IVD cells, artificial IVDs using tissue engineering, and cell transplantation therapy [4–7]. These reports demonstrate that growth factors belonging to the transforming growth factor-β (TGF-β) super family activate IVD cells and are also involved with bone, cartilage and stem cell differentiation. TGF-β super family proteins increase the synthesis of the extracellular matrix and PGs essential to maintaining IVD homeostasis and are key factors in IVD degeneration and regeneration.

The intracellular effectors of TGF-β signaling are members of the Smad family of proteins and consist of five receptor-regulated Smads (R-Smads): Smad1, Smad2, Smad3, Smad5, Smad8, one cooperating Smad (Co-Smad): Smad4, and two inhibitory Smads (I-Smads): Smad6, Smad7 [8]. Among the R-Smads, Smad2 and Smad3 are phosphorylated, mainly by stimulation by TGF-β, whereas Smad1, Smad5 and Smad8 are phosphorylated mainly by stimulation by the bone morphogenetic proteins (BMPs) [9]. Moreover, among the I-Smads, Smad6 selectively inhibits BMP signals, whereas Smad7 inhibits both TGF-β and BMP signals [8,9]. In addition, Smads are activated by a receptor transfer into nuclei where they modulate transcription [8,9]. This pathway is relatively simple, but other signal-transducing pathways further modulate the activation and functions of the Smads. To elucidate the molecular biological process of IVD degeneration, it is

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necessary to analyze the differentiation of IVD cells, the transcription factors that are important for degeneration, and the cross talk between transcription factors.

It is known that an inflammatory cytokine, tumor necrosis factor-α (TNF-α), inhibits the expression of PGs and type II collagen by increasing the expression of matrix metalloproteinases (MMPs) and ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs), which are among the major factors in IVD degeneration [10]. However, there are no reports in the literature describing intracellular signaling cross talk between the TGF-β family and the TNF-α signal pathway, including in the disc degenerative process.

Therefore, the purposes of this study were to analyze the genes related to intracellular Smad signaling in the NP and AF of IVDs, to study the influences of the TGF-β super family and Smads on IVD degeneration due to aging (Study 1), to study the influence of TNF-α on Smad gene expression and to compare cross talk between a TGF-β family and TNF-α signals from the perspective of Smad gene expression (Study 2).

Materials and methods

The animal experiments were carried out according to a protocol approved by the Animal Experimentation Committee at our institution.

Study 1: disc harvest. A total of 64 female Sprague–Dawley (SD) rats were used for the aging study. The "young group" consisted of 32 female Sprague–Dawley (SD) rats that were 12 weeks old; the "old group" consisted of 32 female SD rats that were 32 weeks old. All animals were purchased from Japan Crea (Tokyo, Japan). The rats were euthanized by injection of an excess amount of pentobarbital sodium (Nembutal®, Abbott. Lab) (100 mg/kg). Following euthanasia, coccygeal intervertebral discs were harvested under sterile conditions (each rat yielded one sample that included three vertebrae). The tissues from the three vertebrae dissected from each rat were pooled to produce one sample each for the nucleus pulposus (NP) and annulus fibrosus (AF). These pooled NP and AF tissues were used for biochemical analyses (six samples each), histology (six samples each), and reverse transcription polymerase chain reaction (RT-PCR) analysis (20 samples each).

Measurement of proteoglycan content. The NP disc tissues ($n = 6$ samples each per group) harvested for the biochemical analyses were digested with papain (Sigma–Aldrich, MO, USA). The papain digests were then analyzed to determine the total sulfated PG content by the dimethylmethylene blue (DMMB) dye-binding method [11]. The ratio of the absorbance measured at 530 and 595 nm by a plate reader (Spectra MAX250, Molecular Devices) was calculated to determine the PG content of each sample. The total amount of PG per NP disc was normalized to the total mass per NP disc (wet weight). All assays were performed in duplicate.

Histological examination. The three spinal segments were harvested together with their cranial and caudal vertebral bodies. The spinal segments from each animal ($n = 6$) in each group (young and old) ($n = 18$ IVDs per group) were fixed, and then calcified. Each specimen was cut longitudinally at the center of the disc for histologic evaluation. Sample discs ($n = 18$) from each group (young and old) were processed individually for paraffin wax embedding. These were stained with hematoxylin and eosin and Safranin-O for evaluation. Two histologists who are familiar with human and animal IVD specimens performed the evaluation of these sections. The intra-observer reliability, based on readings at two time intervals one month apart, was $\kappa = 0.90$, showing excellent agreement.

Reverse transcription polymerase chain reaction (RT-PCR). RT-PCR was used to determine the level of gene-specific PCR primers for the following in Table 1. β-Actin expression was used as the internal control. Briefly, total RNA was extracted from the separately NP and AF tissues from 20 rats in each age group with an SV Total RNA Isolation System (Promega, Madison, WI). PCR amplification was carried out using a two-step protocol, comprising 10 min of preheating at 95 °C for activation of AmpliTaq Gold DNA polymerase (Applied Biosystems), followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. The PCR products were separated by electrophoresis on non-denaturing 1.2% TBE polyacrylamide gels and then stained with ethidium bromide. The gels were subsequently scanned with a UV Densitograph System (Atto Biotechnologies, Inc., Tokyo, Japan) and band intensities were quantified by densitometry and normalized for β-actin gene levels using the CS Analyzer software program (Version 2.01, Atto). The semi-quantitative data are representative of three independent experiments ($n = 3$).

Statistical analysis. The results are expressed as means ± standard deviation (SD) of the experiments. Statistical significance was determined using a two-way analysis of variance (ANOVA) with Fisher's PLSD test as a post hoc test. The Statview program was used for statistical analyses. Significance was accepted at $p < 0.05$. Error bars in the figures represent the standard deviation (SD).

Study 2: disc harvest and culture. The NP and AF were macroscopically dissected from the coccygeal discs of 12-week-old female SD rats ($n = 12$; Japan Crea) after euthanasia. NP tissues were first digested with 0.025% Trypsin–EDTA for 15 min; the isolated cells were then cultured in monolayer with Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen, Corp., Carlsbad, CA) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C, 5% CO_2. AF tissues were first digested with pronase E (0.04%) (Kakenkagaku, Tokyo, Japan) for approximately 30 min and then with 0.125% collagenase P (Boehringer Mannheim, Mannheim, Germany) for approximately 1 h. The digested tissue was filtered with a 100-μm cell strainer, and then spread onto culture plates. The cells were cultured in monolayer with DMEM supplemented with 10% FBS and antibiotics at 37 °C, 5% CO_2 [12]. The cultures were maintained for two weeks to permit the formation of tissues with properties similar to those of native NP and AF. TNF-α protein (#24783, Upstate, NY) was added to the cultures at a concentration of 50 ng/ml for either 24 or 48 h before harvesting.

Reverse transcription polymerase chain (RT-PCR) reaction. At harvesting, total RNA was isolated by first mincing the cells using a sterile scalpel. The pieces were then snap-frozen and pulverized in liquid nitrogen and the tissue powder was homogenized with Isogen reagent (Nippongene, Tokyo, Japan) and then further purified using RNeasy spin columns (Qiagen, Valencia, CA). RNA samples were then reverse transcribed to cDNA using oligo dT primer and Multiscribe Reverse Transcriptase followed by the SV Total RNA Isolation System. RT-PCR analysis was carried out as mentioned above. (The upstream and downstream primer sequences for each primer are as mentioned above) (Table. 1).

Results

Study1: proteoglycan content. The biochemical analysis showed that the PG content of the NP tissues of young rats differed significantly from that of the old rats (young: 1.48 ± 0.35 μg/mg mass ratio; old: 0.84 ± 0.20 μg/mg mass ratio ($p < 0.05$, two-way ANOVA) (Fig. 1A).

Histological examination. The histological structure and morphology of the NP and AF were well preserved in both age groups. However, an age-related decrease in sulfated PGs stained by Safranin-O was detected in the NP region of old rat discs compared to that in young rat discs (Fig. 1B). The histological structure exhibited a decreased number of notochordal cells with aging. The notochordal

Table 1
Reverse transcriptase-polymerase chain reaction (RT-PCR) primers

Smad ligands	
TGF-β1 (NM021578)	5′-atacgcctgagtggctgtct-3′ 5′-tgggactgatcccattgatt-3′
TGF-β2 (NM031131)	5′-aatcctagccagggacgttt-3′ 5′-tgcaggagcaaaaaggttct-3′
TGF-β3 (NM013174)	5′-gcaacttggaggagaactgc-3′ 5′-gtcagaggctccaggtcttg-3′
BMP-4 (NM012827)	5′-tctggtctccgtccctaatg-3′ 5′-aaacttgctggaaaggctca-3′
BMP-7 (XM342591)	5′-gaaaacagcagcagtgacca-3′ 5′-ggtggcgttcatgtaggagt-3′
GDF8 (NM019151)	5′-agagagaggcgaatgtggaa-3′ 5′-tcactgctgtcatccctctg-3′
Activin (AF140031)	5′-taacgaaggcaaccagaacc-3′ 5′-ggtatgccagccactacgtt-3′
R-Smads	
Smad1 (NM013130)	5′-cagcagctaccccaactctc-3′ 5′-ccgtcatgttcgtcatgttc-3′
Smad2 (NM019191)	5′-ggaacctgcattctggtgtt-3′ 5′-acgttggagagcaagcctaa-3′
Smad3 (NM013095)	5′-cttggtgcagagacctgtca-3′ 5′-caggtgggatcctatgtgct-3′
Smad5 (AB010955)	5′-gaatgccacgtttcctgatt-3′ 5′-aggggtatcagctgggagtt-3′
Smad8 (AF012347)	5′-gacagcagcatctttgtcca-3′ 5′-cgtgcacatcttcgtcagtt-3′
Co-Smad	
Smad4 (NM019275)	5′-tcgattcaaaccatccaaca3′ 5′-gccctgaagctatctgcaac-3′
I-Smads	
Smad6 (XM345947)	5′-attttgttgcagggatctgg-3′ 5′-ggcgaaacgatgctagagac-3′
Smad7 (AF042499)	5′-tcctgctgtgcaaagtgttc-3′ 5′-tctggacagtctgcagttgg-3′
β-actin (EF156276)	5′-agccatgtacgtagccatcc-3′ 5′-ctctcagctgtggtggtgaa-3′

cells were identified morphologically by their larger size and large number of vacuoles compared to the NP cells.

Reverse transcription polymerase chain reaction (RT-PCR). Semi-quantification of the RT-PCR results, normalized to the β-actin control, demonstrated that the mRNA expression of the Smad ligands, TGF-β2, TGF-β3, and BMP-7, was significantly decreased with aging in the NP (TGF-β2: young, 1.05 ± 0.67 vs. old, 0.68 ± 0.59; TGF-β3: young, 0.28 ± 0.27. vs. old, 0.10 ± 0.11; BMP-7: young, 0.17 ± 0.17 vs. old, 0.08 ± 0.08, $p < 0.05$. β-Actin value = 1.0) (Fig. 2A). BMP-4 and BMP-7 mRNA expression in the AF tissues from young rats showed a statistically significant decrease from the level seen in old rats (BMP-4: young, 0.48 ± 0.51 vs. old, 0.01 ± 0.03; BMP-7: young, 0.49 ± 0.47 vs. old, 0.05 ± 0.06, $p < 0.05$. β-Actin value = 1.0) (Fig. 2B). Among the R-Smads, Smad3 and Smad5 mRNA expression decreased with aging in the NP (Smad3: young, 0.47 ± 0.32 vs. old, 0.27 ± 0.33; Smad5: young, 0.52 ± 0.17 vs. old, 0.33 ± 0.23, $p < 0.05$. β-Actin value = 1.0) (Fig. 3A); Smad3 mRNA expression increased in the AF with aging (Smad3: young, 0.30 ± 0.25 vs. old, 0.66 ± 0.27, $p < 0.05$. β-Actin value = 1.0) (Fig. 3B). Interestingly, a clear difference was noted between the NP and AF for the expression of Smad5 mRNA with a 10-fold higher expression in the NP and a very low expression in the AF. The results of the Co-Smad analysis revealed that the expression of Smad4 mRNA was comparable to the increased expression of R-Smad seen with aging (Smad4: NP, young, 0.44 ± 0.12 vs. old, 0.58 ± 0.20, $p < 0.05$; AF, young, 0.39 ± 0.19 vs. old, 0.55 ± 0.44, $p < 0.05$. β-Actin value = 1.0). The mRNA expression of the I-Smads, Smad6, and Smad7, in NP and AF tissues did not differ significantly between young and old rats; however, Smad6 mRNA expression in both age groups in both tissues was significantly higher than Smad7 mRNA expression (Smad6: NP, young, 0.74 ± 0.15 vs. old, 0.79 ± 0.17, $p = 0.142$; AF, young, 0.67 ± 0.30 vs. old, 0.79 ± 0.43, $p = 0.083$. Smad7: NP, young, 0.10 ± 0.13 vs. old, 0.07 ± 0.08, $p = 0.274$; AF, young, 0.01 ± 0.03 vs. old, 0.06 ± 0.08, $p < 0.05$. β-Actin value = 1.0) (Fig. 3A and B). These results were established by three independent experiments.

Study2: reverse transcription polymerase chain reaction. RT-PCR results demonstrated that the expression of

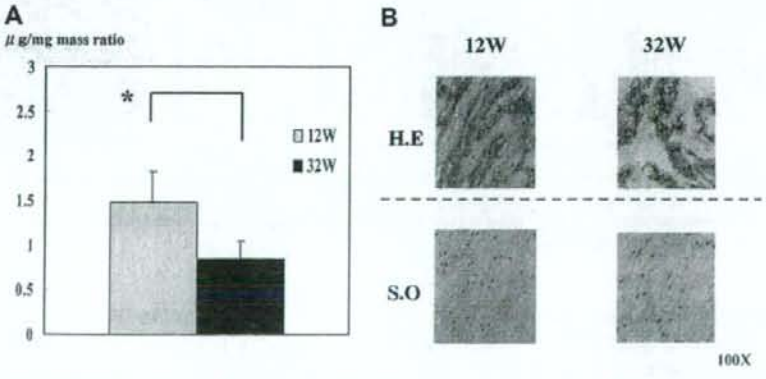

Fig. 1. The biochemistry and histology of young and old rat intervertebral discs (IVDs). (A) Proteoglycan (PG) content of young and old rat IVDs. The PG content of NP discs from young female Sprague–Dawley (SD) rats ($n = 6$; 12-week-old) and old ($n = 6$; 32-weeks-old) was measured by the dimethylmethylene blue (DMMB) dye-binding assay. Differences among the groups are indicated by an asterisk ($^*p < 0.05$). The error bars represent the standard deviation (SD). (B) Histology of young and old rat intervertebral discs. Female Sprague–Dawley (SD) rats that were 12-week-old ($n = 6$) and 32-week-old ($n = 6$) were used. The sections were stained with hematoxylin and eosin (HE) and Safranin-O (S-O) for evaluation (100×).

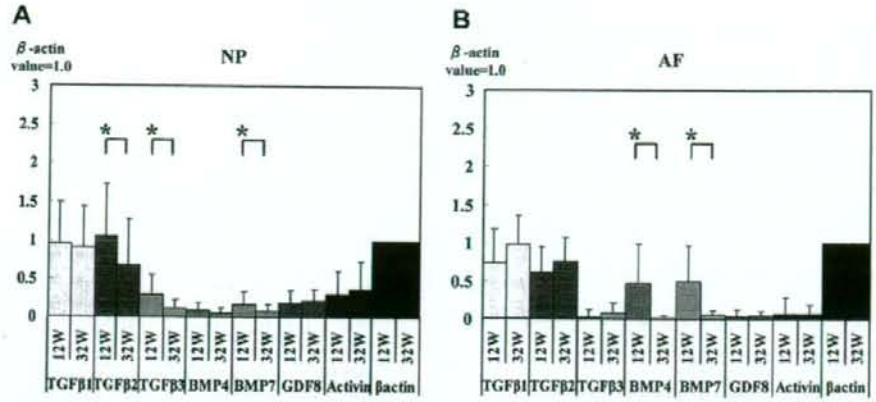

Fig. 2. The mRNA expression of the Smad ligand genes. The RT-PCR analysis shows the semi-quantification of Smad ligand mRNA expression in nucleus pulposus (NP) and annulus fibrosus (AF) tissues from each age group ($n = 20$ samples/group), normalized to β-actin, which served as an internal control (β-actin value = 1.0). Differences among the groups are indicated by an asterisk ($^*p < 0.05$). The error bars represent the standard deviation (SD). (A: NP. B: AF.)

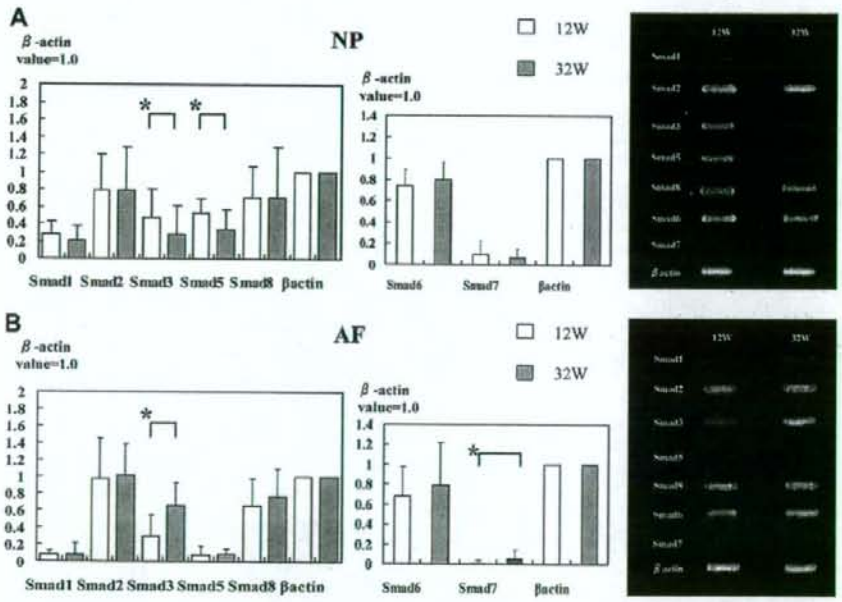

Fig. 3. The mRNA expression of the receptor-regulated Smad (R-Smad) and inhibitory Smad (I-Smad) genes in young and old rat tissues. The RT-PCR analysis shows the semi-quantification of R-Smad and I-Smad mRNA expression in the young and old groups ($n = 20$ samples/group), normalized to β-actin, which served as an internal control (β-actin value = 1.0). Differences among the groups are indicated by an asterisk ($^*p < 0.05$). The error bars represent the standard deviation (SD). (A: NP. B: AF.)

R-Smad mRNA in TNF-α-treated NP cells (NP + TNF) decreased when compared with NP cells that were not treated with TNF-α (NP); these decreases were significant for Smad3, Smad5, and Smad8 (Smad3: NP, 0.45 ± 0.34 vs. NP + TNF-α, 0.14 ± 0.07; Smad5: NP, 0.50 ± 0.34. NP + TNF-α, 0.14 ± 0.10; Smad8: NP, 0.59 ± 0.35 vs. NP + TNF-α, 0.30 ± 0.10, $p < 0.05$. β-Actin value = 1.0) (Fig. 4A). On the other hand, in TNF-α-treated AF cells (AF + TNF-α), the expression of Smad1, Smad3, and Smad5 mRNA was significantly increased when compared to AF cells that were not treated with TNF-α (AF); Smad8 mRNA expression was significantly decreased in TNF-α-treated cells when compared to TNF-α untreated cells (Smad1: AF, 0.08 ± 0.02 vs. AF + TNF-α, 0.52 ± 0.13;

Smad3: AF, 0.59 ± 0.07 vs. AF + TNF-α, 0.93 ± 0.18; Smad5: AF, 0.25 ± 0.04 vs. AF + TNF-α, 0.69 ± 0.35; Smad8: AF, 0.83 ± 0.06 vs. AF + TNF-α, 0.28 ± 0.11, $p < 0.05$. β-Actin value = 1.0) (Fig. 4B). Confirming what was found in Study 1 for the I-Smads, Smad6 mRNA expression was markedly higher in both NP and AF tissues, compared with Smad7 mRNA expression (Smad6: NP, 0.46 ± 0.08 vs. NP + TNF-α, 0.36 ± 0.06, $p < 0.05$; Smad7: NP, 0.04 ± 0.02 vs. NP + TNF-α, 0.04 ± 0.05, $p = 0.914$; Smad6: AF, 0.20 ± 0.08, AF + TNF-α, 0.36 ± 0.07, $p < 0.05$; Smad7: AF, 0.01 ± 0.02 vs AF + TNF-α, 0.01 ± 0.01, $p = 0.932$. β-Actin value = 1.0) (Fig. 4A and B). Furthermore, Smad6 mRNA expression was significantly decreased in TNF-α-treated NP cells when compared to TNF-α untreated NP cells.

Discussion

There have been several reports on the expression of endogenous anabolic cytokines in IVDs or articular cartilage. Matsunaga et al. reported that TGF-β exists in the IVDs of young mice and that its expression decreases with aging [13]. Chambers et al. compared mice from a strain that develops osteoarthritis of the knee joint with age- and sex-matched mice that do not develop OA and reported that the gene expression of TGF-β increased in the degenerative group [14]. Recently, Murakami et al. compared the differences in gene expression of anabolic cytokines [BMP-2, BMP-7, TGF-β, insulin-like growth factor (IGF-1)] in IVDs using 6-month-old (young model) and 3-year-old (old model) rabbits, and reported that the gene expression of BMP-2, BMP-7, and TGF-β increased with aging [15]. When comparing the expression of anabolic cytokines, it is important to note that there may be species differences (i.e., between mice and rabbits) as well as differences in tissues (i.e., between cartilage and IVD). Intervertebral disc of rabbits and rats differ as well, because notochordal cells remain throughout life in rabbits, but not in rats (or in humans) [16]. In this study, we analyzed, for the first time, the genes related to intracellular Smad signaling in IVDs to determine the influences of the TGF-β super family and Smads on IVD degeneration due to aging. Our results on the gene expression of anabolic cytokines in rats differ from the results of the IVDs from the rabbits used by Murakami et al. This may have occurred because of species differences between rats and rabbits. In Murakami's study, in addition to the influence of the small number of animals used ($n = 6$), the interstitial cell bioactivity among individuals and sites, as well as times were concerns, therefore we set the number of samples at $n = 32$ for gene expression in order to obtain an adequate statistical sample. Whether 32-week-old rats (eight months after birth) are an appropriate aging model may be questioned. Our selection was based on many studies that used a "mature" SD rat model in which the SD rats were 16-week-old. In addition, in preliminary experiments we

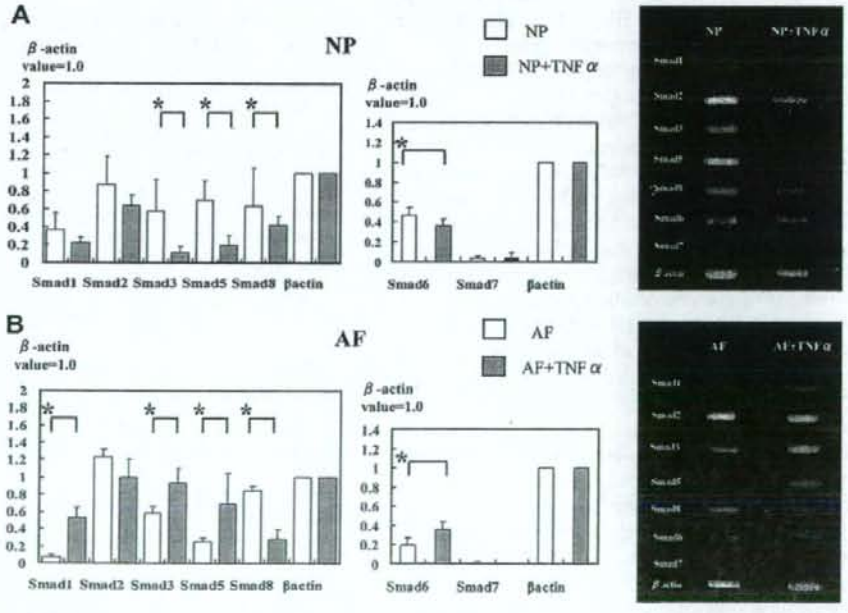

Fig. 4. The influence of TNF-α protein on the expression of intervertebral disc (IVD) R-Smad and I-Smad mRNA. The RT-PCR analysis shows the semi-quantification of R-Smad and I-Smad mRNA expression in TNF-α untreated cells and treated cells. ($n = 12$), normalized to β-actin, which served as an internal control (β-actin value = 1.0). Differences among the groups are indicated by an asterisk ($^*p < 0.05$). The error bars represent the standard deviation (SD). (A: NP. B: AF.)