Osteoarthritis and Cartilage

OARSI Osteoarthritis RESEARCH SOCIETY INTERNATIONAL

β2-Adrenergic agonist-induced hypertrophy of the quadriceps skeletal muscle does not modulate disease severity in the rodent meniscectomy model of osteoarthritis

D.P. Tonge †*, S.W. Jones ‡, T. Parr †, R. Bardsley †, M. Doherty §, R.A. Maciewicz ‖

† Nutritional Sciences, School of Biosciences, Sutton Bonington Campus, Sutton Bonington, University of Nottingham, LE12 5RD, UK
‡ Respiratory and Inflammation Research, AstraZeneca, Alderley Park, Macclesfield, Cheshire, SK10 4TG, UK
§ Academic Rheumatology, Clinical Sciences Building, City Hospital, Nottingham, NG5 1PB, UK
‖ Respiratory and Inflammation Research, AstraZeneca, Charnwood R&D, Loughborough, Leicestershire, LE11 5RH, UK

ARTICLE INFO

SUMMARY

Objective: To examine whether β2-adrenergic agonist-induced hypertrophy of the quadriceps skeletal muscle can modulate the severity of osteoarthritis (OA) in the rodent meniscectomy (MNX) model.
Methods: Male Lewis rats were subcutaneously administered with 1.5 mg/kg/day clenbuterol hydrochloride ($n = 15$) or saline vehicle ($n = 20$) for 14 days. Following pre-treatment, five animals from each group were sacrificed to assess the immediate effects of clenbuterol. The remaining animals underwent either invasive knee surgery (clenbuterol pre-treated $n = 10$; saline pre-treated $n = 10$) or a sham control surgical procedure (saline pre-treated $n = 5$). During disease initiation and progression, weight bearing was assessed by hindlimb loading. Myosin heavy chain (MHC) protein isoforms were quantified by silver stained SDS PAGE. OA severity was graded by assessment of toluidine blue stained step coronal sections of the total knee joint.
Results: Clenbuterol treatment resulted in an increase in total bodyweight, growth rate and in quadriceps skeletal muscle mass. Meniscal surgery resulted in the development of OA-like lesions, changes to weight bearing, and changes in MHC protein expression in the quadriceps. Clenbuterol-induced skeletal muscle hypertrophy had no effect on either weight bearing or articular pathology following MNX surgery.
Conclusions: Our data reveal that clenbuterol-induced skeletal muscle hypertrophy is unable to mimic the beneficial clinical effects of increased musculature derived through targeted strength training in humans, in a rodent model of MNX-induced OA. In addition we observed fibre-type switching to "slow twitch" in the quadriceps muscle during the induction of OA that warrants further investigation as to its relationship to joint stability.

© 2010 Osteoarthritis Research Society International. Published by Elsevier Ltd.
Open access under the Elsevier OA license.

Introduction

It is known that patients with knee osteoarthritis (OA) exhibit muscle weakness[1–9], which is one of the most frequent and earliest reported symptoms associated with knee OA[10]. It primarily affects the quadriceps muscle with little or no evidence of hamstring weakness[4], resulting in a reduced quadriceps to hamstring (Q/H) ratio[11].

Historically, muscle weakness has been considered a secondary effect in knee OA, resulting from disuse of the affected joint due to the presence of pain and/or inflammation, and therefore has received little attention with regards to its involvement in the initiation or progression of OA. However, there is evidence which suggests that quadriceps weakness may precede the onset of radiographic evidence of OA and pain[2], and be directly involved in its pathogenesis[6]. Firstly, quadriceps weakness is reported in those patients with radiographic signs of knee OA in the absence of pain, suggesting that the muscle weakness is unlikely to be due to disuse of a painful joint[12]. Secondly, quadriceps weakness is noted in a number of patient groups who are susceptible to developing knee OA, for example patients who have gait abnormalities resulting in increased knee loading[13], patients with anterior cruciate ligament (ACL) insufficiencies[14] and most commonly patients who have undergone partial meniscectomy (MNX) surgery as a treatment of medial meniscal tears[15]. Initially, patients who have undergone MNX have marked muscle weakness of the ipsilateral limb in the absence of manifest OA[1,16]. However, at long-term follow-up,

* Address correspondence and reprint requests to: Daniel P. Tonge, Nutritional Sciences, School of Biosciences, Sutton Bonington Campus, Sutton Bonington, University of Nottingham, LE12 5RD, UK.
E-mail address: sbxdpt@nottingham.ac.uk (D.P. Tonge).

meniscectomised patients have an elevated incidence of OA (odds ratio = 10), compared to age and sex matched subjects with no history of meniscal injury[17]. In these patient groups, quadriceps dysfunction is noted prior to any radiographic evidence of OA, again suggesting that the quadriceps dysfunction reported in knee OA is unlikely to be solely due to disuse atrophy.

Whether a strong quadriceps muscle can also be a protective factor in the initiation of OA is perhaps more debatable. A study examining the incidence of OA at a 5-year follow-up in subjects with no radiographic evidence of OA at baseline, found that those subjects who developed OA had weaker quadriceps strength at baseline[18]. Conversely, a recent longitudinal study conducted by Segal, NA and colleagues found that although extensor strength was reduced with increasing KL grade for tibiofemoral OA at baseline, neither strength nor normal Q/H ratio was protective against the development of incident radiographic OA. However, of interest, increased extensor strength *was* protective against the development of incident symptomatic whole knee OA albeit this was restricted to female participants once corrected for knee pain at baseline[19].

There is also controversy with regards quadriceps strength and disease progression in individuals with established knee OA. Whilst the assessment of patients with established knee OA showed that a strong quadriceps muscle was protective for cartilage loss at the lateral compartment of the patellofemoral joint over a 30-month period in one study[20], other studies have found this protective effect to be limited to females only[3] and one study found a detrimental effect in malaligned and lax knees[21].

Despite this, a number of studies have shown that exercises aimed at improving quadriceps function have beneficial symptomatic effects in knee OA patients[7,8,22–28]. For example, the impact of both high and low resistance training on subjects with knee OA has been recently investigated, where it was reported that improved quadriceps function was associated with a reduction in knee pain and increased physical ability[24]. Similarly, strength training (ST) and balance exercises have also been shown to significantly reduce knee pain and improve physical ability[23]. To date, only one randomised controlled trial evaluating the effects of ST programs on the onset and progression of knee OA has been conducted[7]. Here, patients with established knee OA undergoing three sessions of ST per week had reduced joint space narrowing (JSN) at 30-month follow-up compared with those patients undertaking range of motion (ROM) exercises for the same duration. However, patients who were radiographically normal at baseline exhibited a slightly elevated incidence of JSN than those undertaking ROM exercises.

Improving skeletal muscle strength and functional performance through intensive exercise regimes is often inappropriate or contraindicated for the majority of OA patients who are elderly, overweight, co-morbid and may be frail. β2-adrenergic agonists, such as clenbuterol, have potent anabolic effects on skeletal muscle, inducing increases in lean mass and contractile speed[29] similar to those observed through targeted ST protocols. Therefore, developing a pharmacological agent that is able to mimic intensive exercise regimes by improving muscle function in knee OA patients could provide a plausible route through which to modify the course of OA during both initiation, and progression[2,7,30].

Morphological studies suggest that the muscle dysfunction in knee OA is due, in part, to atrophy of the muscle fibres. A recent study noted that all patients assessed with knee OA presented with atrophy of type II fast-twitch muscle fibres, whilst less than one third of the patients presented with atrophy of type I slow-twitch muscle fibres[31] as assessed by histochemical staining. This suggests that quadriceps dysfunction in knee OA is most commonly associated with atrophy of fast-twitch type II muscle fibres, an observation supported by previous histochemical studies[32]. Therefore, a pharmacological agent that is able to selectively promote hypertrophy of skeletal muscle fibres, thus increasing muscle mass warrants investigation in an OA context. In this context, clenbuterol is a synthetic β2-adrenergic agonist, the action of which mimics adrenaline. Clenbuterol induces muscle hypertrophy *via* the stimulation of β-adrenoceptors and the subsequent activation of downstream signalling pathways[33]. In addition to marked increases in skeletal muscle mass in a number of animal models of muscle wasting[34–37], clenbuterol has also been shown to induce slow to fast fibre transitions in certain muscle groups[36,38].

To facilitate the identification of such an agent we explored whether a known modulator of muscle mass and muscle fibre type, the β2-adrenergic agonist, clenbuterol, was able to modify the severity of joint disease, assessing joint pathology and behavioural pain, in a MNX-induced rodent model of OA.

Materials and methods

Animals and housing

A total of 35 male Lewis rats (280.4 g ± 1.7) were housed in groups of six with free access to food and standard laboratory chow. Animals were subcutaneously administered the potent β2-adrenergic agonist clenbuterol HCl at a dosage of 1.5 mg/kg/day bodyweight (n = 15), or saline vehicle (n = 20) for a total of 14 days. Following pre-treatment, five animals from each group were sacrificed to assess the immediate effects of clenbuterol. The remaining animals underwent either MNX surgery (clenbuterol pre-treated n = 10; saline pre-treated n = 10) or a sham control surgical procedure (saline pre-treated n = 5). Clenbuterol hydrochloride ($C_{12}H_{18}Cl_2N_2O \cdot HCl$ MW 277.2) was sourced from Alexis pharmaceuticals, UK as a stock powder. A 1.5 mg/ml (w/v) solution was prepared in sterile physiological saline and aliquots stored at −20°C. A fresh aliquot of compound was thawed for each day of dosing to minimise any degradation over the course of the study. All sample size calculations were based on variation in surgical technique, determined from previous studies conducted by the same surgeon (un-published observations).

Surgical induction of OA (MNX)

All *in vivo* procedures were carried out in accordance to the UK Animals (Scientific Procedures) Act 1986. OA was induced *via* MNX surgery as previously described[39]. All surgery was performed under 3.5% Isofluorane inhalational anaesthetics. Rats received a dose of Cefalexin antibiotic (0.03 ml/100 g Ceporex oral drops) 1 h prior to and 12, 24, and 36 h post surgery. Animals also received subcutaneous analgesia (0.01 ml/100 g Rimadyl, Pfizer) on the induction of anaesthesia and 24 h post surgery. A small incision was made longitudinally down the medial side of the knee and a cauteriser was used to work through both the connective tissue and muscle layers until the medial collateral ligament, anchoring the medial meniscus to the tibial (TIB) plateau, was identified. The ligament was grasped at the TIB end and cut until fully transected. The ligament was then transected again at the femoral (FEM) end to remove the portion overlying the meniscus. The meniscus was freed from the fine connective tissue, allowing a full thickness, medial meniscal transection. Sham animals underwent the same surgical procedure with the omission of medial meniscal transection.

Weight bearing assessment

Weight bearing, as a surrogate of behavioural joint pain, was measured using an incapacitance meter (Linton Instrumentation, UK) as described previously[40]. The operator was blinded to both the pre-treatment regime and the previous measurements. The
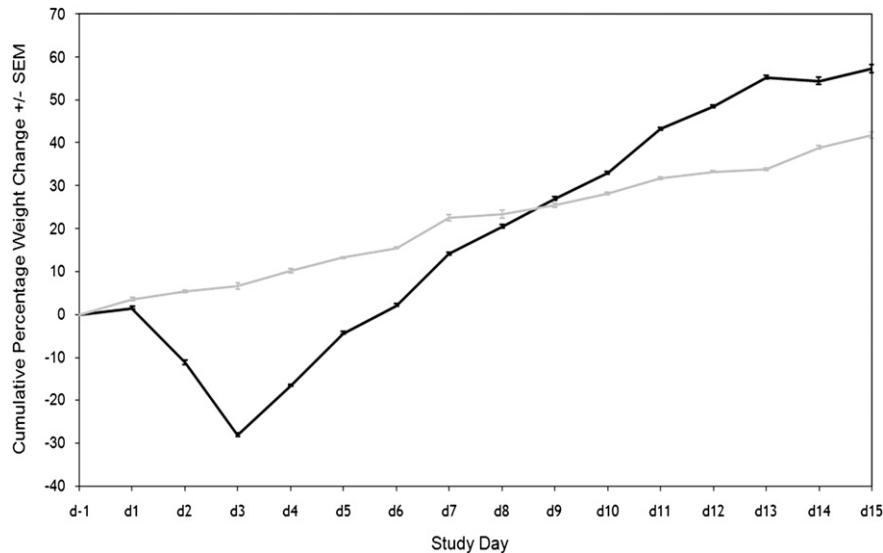
Fig. 1. The effect of subcutaneous administration of clenbuterol (1.5 mg/kg/day) on bodyweight. Male Lewis rats were randomly assigned to one of two experimental groups receiving daily subcutaneous doses of clenbuterol 1.5 mg/kg/day ($n = 5$) or saline control ($n = 5$). Cumulative weight changes were recorded daily for both clenbuterol (black lines) and saline treated (grey lines) subjects during a 14-day pre-treatment phase. Data are mean cumulative percentage ± s.e.m.

difference in weight borne by the two hind limbs was measured by placing the rat in a Perspex tube so that each hind paw was resting on a separate transducer pad. The distribution of bodyweight on each paw over 3 s was recorded and the average of three separate measurements taken. Weight readings for the left and right limbs were taken and the difference between these calculated and plotted. Measurements were taken 2 days prior to the start of the study and then on days 7, 14, 21, 28, and 35. Prior to inclusion in the study, rats were trained for 1 week to acclimatise them to the new apparatus.

Termination and histopathology

Animals were terminated 21 days post surgery with a rising concentration of CO_2 and death confirmed by cervical dislocation. Knee joints were obtained for histopathological analysis by making a full thickness cut 2 cm above and below the patella. The joints were formalin fixed and decalcified on 10% formic acid prior to processing by routine vacuum assisted wax infiltration. Toluidine blue stained step-sections were evaluated for proteoglycan loss, cartilage erosion and subchondral cartilage deposits. Sections were subjectively graded for the most severe changes in cartilage morphology assessing proteoglycan loss, erosion, proliferation and fibrillation on any single section and given a score of between 0 and 4 as follows: 0 – pathology not present, 1 – minimal change, small foci, 2 – mild change, up to 20% affected, 3 – moderate change, up to 50% affected, 4 – severe change, greater than 50% affected. The maximum score for the TIB and FEM condyles was 12 respectively, giving a total obtainable knee score of 24. Proteoglycan loss was generally associated with a loss of chondrocytes and some chondrocyte clustering. Proliferation at the joint margin only referred to proliferation at the edge of the articular cartilage plateau and not proliferation down the lateral border resulting from the operative procedure. Erosion was regarded as the frank loss of cartilage matrix not just 'shrinkage' or 'thinning'. All sections were evaluated by an experienced assessor who was blinded to the animal group in all cases.

Muscle harvest

Whole bilateral quadriceps muscle samples, inclusive of the rectus femoris, were dissected, weighed and immediately snap frozen in isopentane cooled with liquid nitrogen. Care was taken to avoid inclusion of any adipose tissue or additional muscle, most importantly the tensor fasciae latae and sartorius that are located within the dissected area.

Quadriceps muscle fibre typing

Quadriceps muscle fibres were classified based on the expression of myosin heavy chain (MHC) protein. In brief, quadriceps muscle samples (100 mg) were crushed to a powder under liquid nitrogen and homogenised in 5 ml of extraction buffer comprising 0.5 M NaCl, 20 mM pyrophosphate, 50 mM Tris, 1 mM ethyl-enediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol (DTT) in distilled water, pH 8.0. Following centrifugation at 1000g for 10 min, 500 µl aliquots of the resulting supernatant were added to an equal volume of 87% glycerol (Fluka). Preparations were vortexed to ensure thorough mixing and stored at −20°C until required. Following total protein determination[41] 50 ng protein was mixed with loading buffer (4% v/v 87% glycerol, 25 mM 1 M Tris–HCl (pH 6.8), 8% SDS and 20 mg pyronin Y, with 10% v/v beta-mercaptoethanol added prior to use). The myosin preparations were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis as described by Mizunoya[42]. Separating gels

Table I

The effect of subcutaneous administration of clenbuterol (1.5 mg/kg/day) on quadriceps muscle parameters and MHC protein expression

Parameter	Saline	Clenbuterol	P value
Bodyweight gain (%)	8.37 ± 0.62	11.46 ± 1.94	<0.001
Growth rate (g/day)	1.47 ± 0.10	4.04 ± 0.48	<0.001
Quadriceps mass (g)	4.64 ± 0.21	6.52 ± 0.21	<0.001
Quadriceps mass (% bodyweight)	1.60 ± 0.08	2.01 ± 0.12	0.020
% MHC I	4.84 ± 0.93	4.68 ± 0.92	0.988
% MHC IIa	7.25 ± 2.83	6.29 ± 2.59	0.963
% MHC IIx	22.34 ± 6.50	27.44 ± 2.88	0.631
% MHC IIb	66.55 ± 7.44	62.77 ± 4.21	0.586
Ratio [I + IIA]/[IIX + IIB]	0.13 ± 0.05	0.147 ± 0.05	0.393

Following 14 days of pre-treatment with either clenbuterol (1.5 mg/kg/day) or saline, protein extracts were prepared from quadriceps specimens and applied to an SDS PAGE gel. The different MHC protein isoforms were detected by silver staining. The % myosin data are mean densitometric units expressed as a percentage of the total MHC signal ± s.e.m.
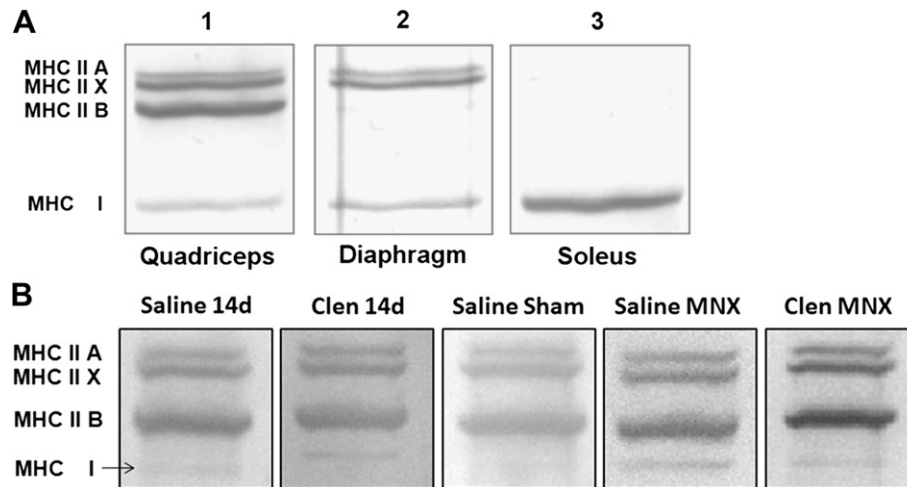
Fig. 2. Electrophoretic separation of the various myosin heavy chain protein isoforms in rat skeletal muscle. (A) Silver stained electrophoretic separation demonstrating the presence of four well-defined bands in the rat quadriceps (Lane 1). Lanes 2 and 3 contain control material from rat diaphragm and soleus respectively with known MHC protein expression profiles. (B) Silver stained electrophoretic separation demonstrating the presence of four well-defined bands in the rat quadriceps in response to various interventions.

consisted of 35% v/v glycerol, 8% w/v acrylamide (bis) 49:1 (Sigma), 0.2 M Tris–HCl (pH 8.8), 0.1 M glycine, 0.4% w/v SDS, 0.1% w/v ammonium persulphate, and 0.05% v/v N,N,N′,N′-tetramethylethylenediamine (TEMED). The stacking gel consisted of 30% v/v glycerol, 4% acrylamide (bis) 49:1, 70 mM Tris–HCl (pH 6.7), 4 mM EDTA, 0.4% w/v SDS, 0.1% w/v ammonium persulphate, and 0.05% v/v TEMED. Gels were prepared the day prior to running and stored at 4°C. The lower running buffer consisted of 0.05 M Tris–HCl, 75 mM glycine, and 0.05% w/v SDS. The upper running buffer was 6× the concentration of the lower running buffer and beta-mercaptoethanol was added before use at a final concentration of 0.12%v/v. Electrophoretic separation was performed in two stages. Sample entry into the stacking gel was run at 10 mA for 40 min, the remainder of the electrophoresis was carried out at 140 V (constant voltage) for 21.3 h. Throughout the electrophoresis, the apparatus was kept in a cold room at 4°C. Following electrophoresis, the different MHC isoforms were detected by silver staining[43] and analysed by semi-quantitative densitometry (Bio-Rad, FluorS) according to the manufacturer's instructions.

Statistical analysis

All data are reported as means ± standard error of the mean (s.e.m.). Comparisons were performed using the independent samples t-test between two groups, or analysis of variance (ANOVA) between multiple groups. Appropriate post-hoc tests were used to test for significance, with significance accepted as $P < 0.05$.

Results

Clenbuterol administration increases rodent bodyweight, growth rate and quadriceps muscle mass

Subcutaneous administration of clenbuterol at a dosage of 1.5 mg/kg/day resulted in a 35% elevation in weight gain following 14 days of treatment ($P = <0.001$) (Fig. 1), compared to saline control treated animals. Analysis of growth rate (derived from daily weight data) revealed an initial reduction in weight during the first three doses of clenbuterol followed by an increase in growth rate (g/day gained) from dose 4 onwards ($P = <0.001$) compared with saline treated controls (Table I). On evisceration, treated animals had visibly reduced adipose deposits and larger, more defined skeletal

musculature. In addition to total bodyweight, quadriceps mass was elevated (+40%) following clenbuterol treatment (6.52 g ± 0.21) compared with saline treated controls (4.64 g ± 0.21), ($P = <0.001$). Statistical significance was maintained when quadriceps mass was normalised to total bodyweight ($P = 0.020$).

MHC isoform protein expression was used as an index of muscle function. Our analysis showed that the rat quadriceps muscle is predominantly composed of "fast twitch" MHC, with a composition of 4.85% type I, 7.25% type IIA, 22.34% type IIX and 66.55% type IIB (Fig. 2). Clenbuterol administration exhibited an apparent 16% increase in total MHC protein expression relative to total protein (data not presented), although there was no change in the composition of the different MHC isoforms (Table I).

MNX surgery results in OA-like lesions, weight bearing perturbations and alters the MHC composition of the quadriceps muscle

MNX surgery was associated with a reduction in weight gain (16.92 ± 1.12%) compared with those animals undergoing the sham

Table II
The effects of MNX surgery and pre-treatment with clenbuterol (1.5 mg/kg/day) on quadriceps muscle parameters and MHC protein expression

Parameter	Sham	Saline MNX	Clenbuterol MNX	P value
Bodyweight gain (%)	24.03 ± 0.98[*]	16.92 ± 1.12	20.16 ± 1.11	0.021
Quadriceps mass (g)	5.91 ± 1.19	5.32 ± 1.72	5.60 ± 1.57	0.099
Quadriceps mass (% bodyweight)	1.69 ± 0.02	1.60 ± 0.05	1.70 ± 0.02	0.154
MHC I	3.55 ± 0.99	7.61 ± 2.15	2.68 ± 0.99	0.081
MHC IIa	7.27 ± 4.24	11.18 ± 1.86	11.69 ± 2.20	0.545
MHC IIx	24.02 ± 2.52	23.54 ± 2.21	22.14 ± 3.16	0.892
MHC IIb	68.10 ± 5.37	60.31 ± 4.07	67.53 ± 3.83	0.359
Ratio [I + IIA]/[IIX + IIB]	0.09 ± 0.04	0.18 ± 0.04	0.15 ± 0.03	0.359

Following 14 days pre-treatment with either the β2-agonist clenbuterol or saline control, animals were sub-divided into those that underwent MNX surgery (saline pre-treated $n = 10$; clenbuterol pre-treated $n = 10$) and those that underwent a sham control surgical procedure (saline pre-treated $n = 5$). Electrophoretic examination of the quadriceps muscles at 21 days post MNX surgery was performed to examine changes in the MHC protein expression profile. Protein extracts were prepared from quadriceps specimens and applied to an SDS PAGE gel and the different MHC isoforms detected by silver staining. Data are mean densitometric units expressed as a percentage of the total MHC signal ± s.e.m.
  * Saline MNX significantly different from sham procedure $P = <0.05$.

Table III
The effects of MNX and treatment regime on joint histopathology

Parameter	Saline MNX	Clenbuterol MNX	P value
Proteoglycan/chondrocyte loss (TIB)	1.90 ± 0.18	2.10 ± 0.23	0.491
Erosion/ulceration (TIB)	1.10 ± 0.35	1.30 ± 0.34	0.663
Subchondral degeneration (TIB)	0.20 ± 0.13	0.50 ± 0.27	0.485
Proteoglycan/chondrocyte loss (FEM)	0.40 ± 0.22	0.80 ± 0.25	0.202
Erosion/ulceration (FEM)	0 ± 0	0 ± 0	–
Subchondral degeneration (FEM)	0 ± 0	0 ± 0	–
Total pathology	3.20 ± 0.59	3.90 ± 0.73	0.398

Of those animals subjected to MNX surgery (Saline MNX ($n = 10$), Clen MNX ($n = 10$)), histopathological examination was performed by microscopically evaluating toluidine stained step coronal joint sections for evidence of OA on both the TIB and FEM condyles. Data are mean pathology score ± s.e.m. P values refer to differences in OA severity between saline and clenbuterol pre-treated subjects as determined by Mann–Whitney analysis.

control procedure ($24.03 \pm 0.98\%$) $P = 0.021$ at the end of the study period (Table II). Of those animals subjected to MNX surgery, histopathological examination of the ipsilateral limbs revealed evidence of OA-like lesions, predominantly localised at the TIB condyle. Microscopically, meniscectomised tibias presented with proteoglycan and chondrocyte loss, erosion and ulceration of the articular surface, but limited evidence of subchondral degeneration. FEM condyles showed evidence of proteoglycan loss; however this was less severe than that noted on the respective TIB condyles. No FEM erosion, ulceration or subchondral degeneration was noted within the experimental timeframe (Table III). sham operated control animals were free from OA 21 days post surgery.

Surgery was also associated with a reduced quadriceps mass relative to bodyweight (1.60 ± 0.05) compared with those animals that underwent a sham procedure (1.69 ± 0.02) at the end of the study although this did not reach statistical significance ($P = 0.154$). Electrophoretic examination of the quadriceps muscles at 21 days post surgery was performed to examine changes in the MHC expression profile (Fig. 2). Compared to the sham control animals, MNX operated (saline pre-treated) animals exhibited an apparent 115% increase in the protein expression of the slow twitch MHC I ($P = 0.08$), numerically in lieu of fast twitch MHC IIB (Table II).

Incapacitance assessment (as a marker of weight bearing/behavioural pain) demonstrated a 50:50 load distribution between the hind limbs prior to surgery (Fig. 3). Following MNX surgery, significantly less weight was placed on the ipsilateral limb (day 14 vs day 21, day 28, day 35; $P = < 0.001$). Weight distribution between the hind limbs of the MNX treated animals resolved over a period of 14 days post surgery, although equal distribution was not achieved within the experimental timeframe.

Clenbuterol-induced muscle hypertrophy is unable to modulate the severity of disease

Animals pre-treated with clenbuterol prior to MNX were unable to maintain the previously noted increase in quadriceps mass relative to bodyweight (1.70 ± 0.02) compared to saline pre-treated meniscectomised subjects (1.60 ± 0.05) at the end of the study ($P = 0.154$). The characteristic reduction in bodyweight gain following MNX was suppressed in response to clenbuterol pre-treatment, leading to similar weight gain to those animals undergoing the sham control procedure (clenbuterol MNX: $20.16 \pm 1.11\%$, sham control $24.03 \pm 0.98\%$). Electrophoretic analysis of MHC isoforms demonstrated that clenbuterol pre-treatment prior to MNX suppressed the increase in MHC I protein, which was previously associated with MNX surgery, and numerically, maintained the MHC IIB complement (Table II). However, 14 days pre-treatment with the β_2-agonist clenbuterol had no significant effect on TIB or FEM pathology 21 days post MNX compared to those animals pre-treated for 14 days with saline. Microscopically, the severity of proteoglycan and chondrocyte loss, ulceration and subchondral degeneration observed post surgery remained unaffected from the pre-treatment with clenbuterol (Table III).

Discussion

This is the first study to report the role of the quadriceps muscle, and the effects of induced skeletal muscle hypertrophy on the severity of joint pathology in an animal model of OA. Here, we observed a trend towards increased slow, MHC I protein in the quadriceps during the induction of OA-like cartilage lesions in the rat MNX model of OA, as determined by MHC protein expression. We also observed that the gross increase in the quadriceps muscle induced by the β_2 adrenergic agonist, clenbuterol, could not
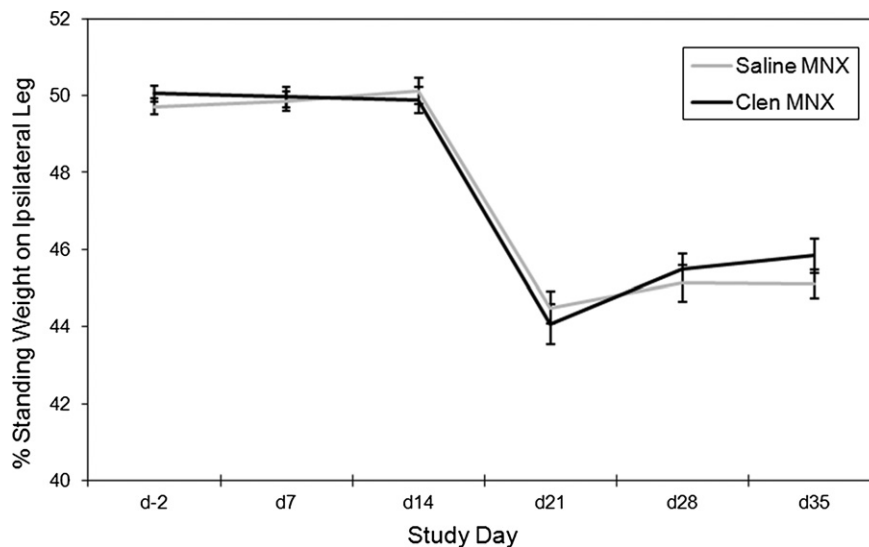

Fig. 3. The effects of MNX surgery and treatment regime on weight bearing. The effect of MNX surgery on weight bearing was assessed in both saline (grey line; $n = 10$) and clenbuterol (black line; $n = 10$) pre-treated meniscectomised animals. Two days prior to inclusion in the study, animals were acclimatised to the incapacitance equipment (d-2). Animals were pre-treated from day 1 through day 14 and surgery performed on day 15. Prior to surgery and during the 21-day development phase immediately post surgery, weight distribution was assessed using an incapacitance meter (Linton, UK) and data expressed as the percentage of total weight distributed to the operated limb ± s.e.m.

modulate the severity of joint damage or subsequent behavioural pain in this model.

The use of clenbuterol to induce skeletal muscle hypertrophy and promote lipolysis is well characterised across multiple species[44]. Here, following 2 weeks of clenbuterol treatment we attained a significant increase in the quadriceps muscle mass (+40%). However, at the study endpoint, the hypertrophic effect of clenbuterol was no longer apparent in the pre-treated animals, which presented with comparable quadriceps mass to bodyweight ratios. To determine muscle fibre composition of the quadriceps, we monitored MHC protein expression. Of interest we found that although no changes to the MHC protein composition were noted in response to clenbuterol prior to surgery, subtle effects of clenbuterol on MHC protein expression were observed in the period post surgery where clenbuterol suppressed the trend towards an MNX surgery-induced increase in MHC I. Although we only noted a trend association between pre-treatment with clenbuterol and suppression of the increase in MHC I protein observed post surgery ($P = 0.081$), this is supported by several studies that report the slow to fast fibre inducing effects of clenbuterol in rodents[44]. The fact that we did not observe these changes in the pre-treatment period may be due to the length of drug exposure and/or the muscle studied. The latter may be related to the natural distribution of MHC protein isoforms within the quadriceps muscle which comprises over 85% fast MHC IIX/MHC IIB, and less than 15% slower MHC I/MHC IIA. Such a high baseline complement of fast MHC is likely to reduce the impact of a drug such as clenbuterol with regards to its effect in inducing slow to fast fibre type transitions. Previous studies noting such changes were often conducted over a longer timeframe and tended to study the slow twitch soleus muscle, which is particularly susceptible to fibre type shifts.

As previously noted, MNX surgery was associated with a trend towards increased MHC I, indicative of a switch towards a slower more oxidative and fatigue resistant muscle type, more commonly associated with postural, anti-gravity muscles[45]. Such changes in muscle fibre type following MNX may have important implications for rehabilitation, in particular the type of muscle strengthening program recommended. One possible reason for this change in MHC composition in response to MNX surgery might be that it is a physiological attempt to increase stability of the operated joint by increasing the complement of fatigue resistant, slow muscle fibres that surround it. A further possibility is that surgery-induced changes in weight bearing led to disuse atrophy of fast MHC IIB fibres, resulting in an increase in the relative proportion of slower MHC I fibres as noted previously[46]. We must stress however, that only mild changes to weight bearing were noted ($\sim$4%) and it is unclear whether such modest perturbations are capable of inducing changes to the relative distribution of MHC.

A number of previous studies suggest that modulating muscle function through exercise may have an impact on the development and severity of OA in humans; therefore we must consider why no effect was noted in this experimental model. The experimental approach chosen in the present study was to dose with clenbuterol for up to 2 weeks prior to surgical induction of OA, but to stop administration immediately after surgery. This approach avoided any potential confounding factors of direct clenbuterol action at the joint or direct cardiovascular effects and in our opinion allowed us to better mimic the physiological impact of a hypertrophic quadriceps muscle on OA severity. However, although clenbuterol resulted in marked increases in quadriceps mass during the pre-treatment period, this effect was not maintained throughout the time course of the study and may explain the lack of effect on OA severity reported in the study. Another possibility might be the agent of choice we used in this study to induce muscle hypertrophy. Although clenbuterol is effective in this regard, it also leads

to an overall increase in animal bodyweight due to the induction of lean mass, a concurrent decline in fat mass and the relatively high mass of skeletal muscle compared to that of adipose tissue[47,48]. It is well known that weight is a risk factor for knee OA[49]. Moreover, bodyweight has been associated with the severity of OA in the Dunkin Hartley guinea pig where a 28% reduction in bodyweight led to a marked 40% decrease in OA severity[50]. By inference, it is therefore possible that this increased bodyweight in our clenbuterol treated animals compared to the control animals, at the time of induction, may have negated any potential beneficial effect of a strengthened quadriceps, and thus explains the lack of positive modulation noted during this study. A further possibility is that muscle mass is not the sole or the major determinant with regards to the role of muscles in OA. Instead, it is likely that it is the coordinate modulation of several muscle parameters such as muscle fibre type composition and size of motor units recruited during exercise that together with mass, are key in eliciting an impact on OA pathology.

In addition it is also important to note that clenbuterol has greater hypertrophic effects on slow twitch muscles compared with faster twitch muscles[35,36]. In the intact knee, agonist force is generated by the quadriceps muscle in tandem with antagonist force generation by the hamstrings producing joint stability[51]. If one considers the quadriceps/hamstrings balance (Q/H ratio) and that anabolic steroid-induced hypertrophy might elicit a greater strength increases in leg flexion over leg extension[52], it is possible that clenbuterol administration induced skeletal muscle hypertrophy disproportionately across the various muscles surrounding the knee joint, disrupting the fine balance of agonist and antagonist forces, leading to decreased joint stability. This could have a detrimental effect on the formation of joint pathology in a similar way to that observed in man with joint instability due to joint mal-alignments[53].

In summary, these findings suggest that clenbuterol-induced gross increases in skeletal musculature do not modulate the severity of OA in the rodent MNX model. We propose that animal models of OA that better mimic the human condition in terms of initiation and progression rates, and the use of pharmacological agents that more selectively target the Q/H ratio without additional weight gain, may provide a more suitable platform on which to observe subtle changes in disease progression brought about through modulating skeletal muscle function.

Conflict of interest

The authors report no conflicts of interest.

Acknowledgments

References

1. Becker R, Berth A, Nehring M, Awiszus F. Neuromuscular quadriceps dysfunction prior to osteoarthritis of the knee. J Orthop Res 2004;22:768–73.
2. Hurley M. The role of muscle weakness in the pathogenesis of osteoarthritis. Rheum Dis Clin North Am 1999;25:283.
3. Slemenda C, Heilman D, Brandt K, Katz B, Mazzuca S, Braunstein E, *et al.* Reduced quadriceps strength relative to body weight – a risk factor for knee osteoarthritis in women? Arthritis Rheum 1998;41:1951–9.

4. Brandt K, Heilman D, Slemenda C, Katz B, Mazzuca S, Braunstein E, *et al.* Quadriceps strength in women with radiographically progressive osteoarthritis of the knee and those with stable radiographic changes. J Rheumatol 1999;26: 2431–7.

5. Brandt K, Heilman D, Slemenda C, Katz B, Mazzuca S, Braunstein E, *et al.* A comparison of lower extremity muscle strength, obesity, and depression scores in elderly subjects with knee pain with and without radiographic evidence of knee osteoarthritis. J Rheumatol 2000;27:1937–46.

6. Ikeda S, Tsumura H, Torisu T. Age-related quadriceps-dominant muscle atrophy and incident radiographic knee osteoarthritis. J Orthop Sci 2005;10:121–6.

7. Mikesky A, Mazzuca S, Brandt K, Perkins S, Damush T, Lane K. Effects of strength training on the incidence and progression of knee osteoarthritis. Arthritis Rheum 2006;55:690–9.

8. Eyigor S. A comparison of muscle training methods in patients with knee osteoarthritis. Clin Rheumatol 2004;23:109–15.

9. Mohammadi F, Taghizadeh S, Ghaffarinejad F, Khorrami M, Sobhani S. Proprioception, dynamic balance and maximal quadriceps strength in females with knee osteoarthritis and normal control subjects. Int J Rheum Dis 2008;11:39–44.

10. Longino D, Frank C, Leonard T, Vaz M, Herzog W. Proposed model of botulinum toxin-induced muscle weakness in the rabbit. J Orthop Res 2005;23:1411–8.

11. Hayes K, Song J, Dunlop D, Cahue S, Sharma L. The quadriceps/hamstring ratio and protection against patello-femoral osteoarthritis progression. Arthritis Rheum 2002; 46:S142.

12. Slemenda C, Brandt K, Heilman D, Mazzuca S, Braunstein E, Katz B, *et al.* Quadriceps weakness and osteoarthritis of the knee. Ann Intern Med 1997;127:97.

13. Marks R, Kumar S, Semple J, Percy J. Quadriceps femoris activation in healthy women with genu varum and women with osteoarthritis and genu varum. J Electromyogr Kinesiol 1994; 4:153–60.

14. Longino D, Frank C, Herzog W. Acute botulinum toxin-induced muscle weakness in the anterior cruciate ligament-deficient rabbit. J Orthop Res 2005;23:1404–10.

15. Mills P, Wang Y, Cicuttini F, Stoffel K, Stachowiak G, Podsiadlo P, *et al.* Tibio-femoral cartilage defects 3–5 years following arthroscopic partial medial meniscectomy. Osteoarthritis Cartilage 2008;16:1526–31.

16. Ericsson YB, Roos EM, Dahlberg L. Muscle strength, functional performance, and self-reported outcomes four years after arthroscopic partial meniscectomy in middle-aged patients. Arthritis Rheum 2006;55:946–52.

17. Lohmander L, Englund P, Dahl L, Roos E. The long-term consequence of anterior cruciate ligament and meniscus injuries – osteoarthritis. Am J Sports Med 2007;35:1756–69.

18. Thorstensson C, Petersson I, Jacobsson L, Boegard T, Roos E. Reduced functional performance in the lower extremity predicted radiographic knee osteoarthritis five years later. Ann Rheum Dis 2004;63:402–7.

19. Segal NA, Torner JC, Felson D, Niu J, Sharma L, Lewis CE, *et al.* Effect of thigh strength on incident radiographic and symptomatic knee osteoarthritis in a longitudinal cohort. Arthritis Care Res 2009;61:1210–7.

20. Amin S, Baker K, Niu J, Clancy M, Goggins J, Guermazi A, *et al.* Quadriceps strength and the risk of cartilage loss and symptom progression in knee osteoarthritis. Arthritis Rheum 2009;60:189–98.

21. Sharma L, Dunlop DD, Song J, Hayes KW. Quadriceps strength and osteoarthritis progression in maligned and lax knees. Ann Intern Med 2003;138:613–9.

22. Keefe F, Blumenthal J, Baucom D, Affleck G, Waugh R, Caldwell D, *et al.* Effects of spouse-assisted coping skills training and exercise training in patients with osteoarthritic knee pain: a randomized controlled study. Pain 2004;110: 539–49.

23. Diracoglu D, Aydin R, Baskent A, Celik A. Effects of kinesthesia and balance exercises in knee osteoarthritis. J Clin Rheumatol 2005;11:303–10.

24. Jan M, Lin J, Liau J, Lin Y, Lin D. Investigation of clinical effects of high- and low-resistance training for patients with knee osteoarthritis: a randomized control-led trial. Phys Ther 2008;88:427–36.

25. Rosemffet M, Schneeberger E, Citera G, Sgobba M, Laiz C, Schmulevich H, *et al.* Effects of functional electrostimulation on pain, muscular strength, and functional capacity in patients with osteoarthritis of the knee. J Clin Rheumatol 2004;10: 246–9.

26. O'Reilly SC, Muir KR, Doherty M. Effectiveness of home exercise on pain and disability from osteoarthritis of the knee: a randomised controlled trial. Ann Rheum Dis 1999; 58:15–9.

27. Thomas KS, Muir KR, Doherty M, Jones AC, O'Reilly SC, Bassey EJ, *et al.* Home based exercise programme for knee pain and knee osteoarthritis: randomised controlled trial. Br Med J 2002;325:752–5.

28. Baker KR, Nelson ME, Felson DT, Layne JE, Sarno R, Roubenoff R. The efficacy of home based progressive strength training in older adults with knee osteoarthritis: a randomized controlled trial. J Rheumatol 2001;28:1655–65.

29. Lynch G, Schertzer J, Ryall J. Anabolic agents for improving muscle regeneration and function after injury. Clin Exp Rheumatol Physiol 2008;35:852–8.

30. Pap G, Machner A, Awiszus F. Strength and voluntary activation of the quadriceps femoris muscle at different severities of osteoarthritic knee joint damage. J Orthop Res 2004;22: 96–103.

31. Fink B, Egl M, Singer J, Fuerst M, Bubenheim M, Neuen-Jacob E. Morphologic changes in the vastis medialis muscle in patients with osteoarthritis of the knee. Arthritis Rheum 2007;56: 3626–33.

32. Nakamura T, Suzuki K. Changes in osteoarthritis of the hip and knee. Nippon Seikeigeka Gakkai Zasshi 1992;66:467–75.

33. Ryall J, Lynch G. The potential and the pitfalls of beta-adrenoceptor agonists for the management of skeletal muscle wasting. Pharmacol Ther 2008;120:219–32.

34. Maltin CA, Hay SM, Delday MI, Lobley GE, Reeds PJ. The action of the beta-agonist clenbuterol on protein-metabolism in innervated and denervated phasic muscles. Biochem J 1989; 261:965–71.

35. Herrera NM, Zimmerman AN, Dykstra DD, Thompson LDV. Clenbuterol in the prevention of muscle atrophy: a study of hindlimb-unweighted rats. Arch Phys Med Rehabil 2001;82: 930–4.

36. Stevens L, Firinga C, Gohlsch R, Bastide B, Mounier Y, Pette D. Effects of unweighting and clenbuterol on myosin light and heavy chains in fast and slow muscles of rat. Am J Physiol Cell Physiol 2000;279:C1558–63.

37. Carbó N, López-Soriano J, Tarragó T, González O, Llovera M, López-Soriano FJ, *et al.* Comparative effects of [beta]2-adrenergic agonists on muscle waste associated with tumour growth. Cancer Lett 1997;115:113–8.

38. Ricart-Firinga C, Stevens L, Canu M, Nemirovskaya T, Mounier Y. Effects of beta(2)-agonist clenbuterol on biochemical and contractile properties of unloaded soleus fibers of rat. Am J Physiol Cell Physiol 2000;278:C582–8.

39. Janusz M, Bendele A, Brown K, Taiwo Y, Hsieh L, Heimeyer S. Induction of osteoarthritis in the rat by surgical tear of the meniscus: inhibition of joint damage by a matrix metalloproteinase inhibitor. Osteoarthritis Cartilage 2002;10: 785–91.

40. Ivanavicius S, Ball A, Heapy C, Westwood F, Murray F, Read S. Structural pathology in a rodent model of osteoarthritis is associated with neuropathic pain: increased expression of ATF-3 and pharmacological characterisation. Pain 2007;128: 272–82.

41. Bradford MM. Rapid and sensitive method for quantification of microgram quantities of protein utilizing principal of protein-dye binding. Anal Biochem 1976;72:248–54.

42. Mizunoya W, Wakamatsu J, Tatsumi R, Ikeuchi Y. Protocol for high-resolution separation of rodent myosin heavy chain iso-forms in a mini-gel electrophoresis system. Anal Biochem 2008;377:111–3.

43. Talmadge RJ, Roy RR. Electrophoretic separation of skeletal muscle myosin heavy-chain isoforms. J Appl Phys 1993;75: 2337–40.

44. Lynch G, Ryall J. Role of beta-adrenoceptor signaling in skeletal muscle: implications for muscle wasting and disease. Physiol Rev 2008;88:729–67.

45. Adams G, Haddad F, Baldwink. Interaction of chronic creatine depletion and muscle unloading – effects on postural and locomotor muscles. J Appl Phys 1994;77:1198–205.

46. Gardiner P, Favron M, Corriveau P. Histochemical and contractile responses of rat medial gastronemius to 2 weeks of complete disuse. Can J Physiol Pharmacol 1992;70:1075–81.

47. Rothwell N, Stock M. Effect of selective beta-2-adrenergic agonist (clenbuterol) on energy-balance and body composition in normal and protein-deficient rats. Biosci Rep 1987;7:933–40.

48. Rothwell N, Stock M, Sudera D. Changes in tissue blood-flow and beta receptor density of skeletal-muscle in rats treated with the beta-2-adrenoceptor agonist clenbuterol. Br J Phar-macol 1987;90:601–7.

49. Arden N, Nevitt M. Osteoarthritis: epidemiology. Best Pract Res Clin Rheumatol 2006;20:3–25.

50. Bendele A, Hulman J. Effects of body-weight restriction on the development and progression of spontaneous osteoarthritis in guinea-pigs. Arthritis Rheum 1991;34:1180–4.

51. Hortobagyi T, Westerkamp L, Beam S, Moody J, Garry J, Holbert D, et al. Altered hamstring-quadriceps muscle balance in patients with knee osteoarthritis. Clin Biomech 2005;20: 97–104.

52. Schroeder E, Terk M, Sattler F. Androgen therapy improves muscle mass and strength but not muscle quality: results from two studies. Am J Physiol Endocrinol Metab 2003;285:E16–24.

53. Kalichman L, Zhang Y, Niu J, Goggins J, Gale D, Zhu Y, et al. The association between patellar alignment on magnetic reso-nance imaging and radiographic manifestations of knee oste-oarthritis. Arthritis Res Ther 2007;9.