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Effects of intraarticular treatment with stanozolol on synovial membrane and cartilage in an ovine model of osteoarthritis

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ABSTRACT

Aim of the study was to verify the clinical and morphological effects of intra-articular stanozolol or placebo treatment, lasting 3 and 9 months, in sheep in which a femoro-tibial osteo-arthritis (OA) were surgically induced (medial bilateral meniscectomy).

Twenty healthy sheep divided into four groups and two control animals group, after surgical medial bilateral meniscectomy, were weekly injected in femoral-tibial joint (FTJ) with stanozolol or placebo. Lameness evaluation was performed and synovial fluid was collected from all sheep at each treatment time. Necropsies were performed after 3 or 9 month as described in experimental design. Gross pathologies were described and specimen tissues collected from femoro-tibial articular joints were processed for routine histological examination.

The gross anatomy of the FTJ was well-preserved in stanozolol-treated sheep; this also applied to the histological features of articular cartilage. Joint aseptic inflammation and fibrosis were observed in placebo-treated sheep, associated with a different degree of severity of condylar and tibial plate cartilage degeneration.

Stanozolol intra-articular treatment reduces osteophytes formation and subchondral bone reaction and promotes articular cartilage regeneration.

1. Introduction

The pharmacological treatment of osteo-arthritis (OA) is an ongoing challenge in equine clinical practice. Many treatments have been proposed, including those tested in experimental and clinical trials; however, response to treatment is not always satisfactory (Goodrich and Nixon, 2006; Frisbie et al., 2009; Pearson et al., 2009).

A variety of compounds used for the treatment of OA, including steroidal and non-steroidal drugs, inhibitor of nitric oxide production (NO) (Saleri et al., 2004). NO may be responsible for cartilage destruction in OA (Blanco et al., 1995), by damaging chondrocyte function, inhibiting collagen and proteoglycan synthesis, activating metalloproteinase, decreasing the expression of IL-1 receptor antagonist, inhibiting chondrocyte proliferation, and inducing apoptotic death (Studer et al., 1999). Stanozolol is a synthetic derivative of testosterone; its properties include anabolic/androgenic activity (Zannetti, 2004), probably associated with its affinity for androgenic and, at lower doses, glucocorticoid receptors (Fernandez et al., 1994). Because of its strong anabolic effects, studies on the effects of stanozolol treatment on OA are not performed in human medicine (Belch et al., 1986; Ellis et al., 1994): the same promising results (Zannetti, 2004; Dondi et al., 2008; Adamama-Moraitou et al., 2009) has led to renewed interest in the use of the product in veterinary medicine for companion animals.

Systemic stanozolol is used in horses as an anabolic steroid and doping agent (Yamada et al., 2008; You et al., 2009). However, stanazolol reduces apoptosis in equine chondrocytes *in vitro*, by reducing the production of NO and stimulating IGF-1 production (Saleri et al., 2004).

Lateral (Amstrong et al., 1994) or medial (Oakley et al., 2004) sheep meniscectomy is considered the best widely used animal model to investigate possible therapeutic approaches for degenerative joint disease in human and companion animals. Meniscectomy induces lesions both in the articular cartilage and in subchondral bone with the characteristic features of OA: articular space narrowing, osteophyte formation, focal cartilage lesions and decreased histochemical staining for proteoglycan (Moskowitz and Goldberg, 1987; Moskowitz et al., 1979). The technique of bilateral medial meniscectomy described by Oakley et al. (2004) is considered the golden standard for experimental AO investigation. For these reasons this model has been considered ideal for the present study.

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The aim of this study was to verify the clinical and morphological effects of intra-articular treatment with stanozolol on surgically induced OA of the stifle joints of sheep after 3 and 9 months of treatment.

2. Materials and methods

The experiment was performed according to the legislative and ethical requirements on animal care. The study protocol followed the "Principles of good laboratory practice" of OECD (Organization for Economic CO-operation and Development) and was approved by the University of Bologna Ethics Committee (119-120/2004 C). The experiment took place in the spring 2005.

2.1. Treatment preparation

The stanozolol[1] suspension and placebo were produced according to the following protocol: the test product was produced by adding stanozolol to a sterile apyrogenic water suspension containing sodium chloride and phosphate salts as a buffering system; the placebo did not contain any stanozolol molecules. The syringes were filled of drug, or placebo, and capped in a sterile environment. Analytical controls of the product were carried out before use.

2.2. Animals and experimental design

Twenty, 1 year-old, female Bergamasca sheep, weighing 42.85 ± 3.7 kg, were used in the study.

Fifteen days before surgery, twenty sheep were housed in small inside stalls with controlled temperature (18 °C) and relative humidity (70%); they were given a regular feeding regimen and divided randomly into six groups: 4 groups of 4 animal each and two groups of 2 animal each. Sixteen animals underwent bilateral medial meniscectomy as described by Oakley et al. (2004). Sheep were subjected to blood tests (hematological and biochemical profile) to verify their health status and orthopedic examination was performed to exclude signs of lameness in the hind limbs. A radiographic examination of the right and left stifle was also performed to rule out arthritic alterations of the stifle before the start of the study.

2.2.1. Group STAN3

Four sheeps (weighing 42.0 ± 1.4 kg) underwent meniscectomy and were treated, 7 days post-surgery (p.s.), with weekly bilateral FTJ (femoral-tibial joint) intra-articular administration of 1 mg of stanozolol in 0.4 ml of aqueous suspension for three months. Sheep were euthanized 3 months after the first treatment applied 6 days after meniscectomy.

2.2.2. Group MEN3

Four sheeps (weighing 42.0 ± 4.2 kg) underwent meniscectomy and were treated 7 days p.s., with weekly bilateral FTJ intra-articular administration of placebo in 0.4 ml solution for three months. The animals were euthanized 3 months after the first treatment applied 6 days after meniscectomy.

2.2.3. Group STAN9

Four sheeps (weighing 43.5 ± 0.7 kg) underwent meniscectomy and were treated 7 days after the surgery, with weekly bilateral FTJ intra-articular administration of a dose of 1 mg of stanozolol in 0.4 ml of aqueous suspension for three months and then every two weeks for the following six months. Sheep were euthanized 9 months after the first treatment applied 6 days after meniscectomy.

2.2.4. Group MEN9

Four sheeps (weighing 48.5 ± 2.1 kg) underwent meniscectomy and were treated 7 days after the surgery, with weekly bilateral FTJ intra-articular administration of placebo in 0.4 ml solution for three months; then every two weeks over the next six months, they were treated with placebo in 0.4 ml solution; the sheep were euthanized 9 months after the first treatment applied 6 days after meniscectomy.

2.2.5. Group CON1

Two sheeps (weighing 42.5 ± 2.1 kg) without meniscectomy, were treated bilateral FTJ intra-articular administration of 1 mg of stanozolol in 0.4 ml of aqueous suspension; one sheep CON(1–3) received the same treatment plan and timing of the STAN3 and was euthanized at the same time as this group (after 3 months of treatment); the other one CON(1–9) followed the same treatment plan and timing of the STAN9 and was euthanized at the same time as this group (9 months of treatment).

2.2.6. Group CON2

Two sheeps (weighting 41 ± 1.4 kg) without meniscectomy were treated bilateral FTJ intra-articular administration of placebo in 0.4 ml solution; one sheep CON(2–3) received the same treatment plan and timing of the MEN3 and was euthanized at the same time as this group (3 months of treatment); one CON(2–9) followed the same treatment plan and timing of the MEN9 and was euthanized at the same time as this group (9 months of treatment).

To induce OA, sixteen sheep were subjected to open bilateral medial meniscectomy under general anesthesia (premedication with xylazine[2] 0.1 mg/kg im and butorphanol[3] 0.05 mg/kg im; induction with ketamine[4] 2.2 mg/kg; maintenance with isoflurane[5] and 100% O_2) and dorsal recumbency.

Before the incision, a sample of synovial fluid was taken from all FTJ joints to determine normal baseline values. Post-surgery (p.s.) sheep, stabled individually, were treated with ampicillin[6] (20 mg/kg, IM, q 12 h) and gentamicin[7] (4 mg/kg, IM, q 24 h) and sheep flunixine meglumine[8] (1 mg/kg, IM, q 12 h) five days p.s. Any bandage was placed following surgery.

After a period of 30 days of confinement in the stall, the 20 sheep were forced to walk in a paddock 50 × 50 m. Handlers forced them to walk twice a day, at least for half-an-hour until the euthanasia for all animals.

Treatment with stanozolol or placebo began at day six p.s. For 3 months, the 20 sheep received weekly intra-articular injections. As described previously, after 3 months the sheep in groups MEN3, STAN3 and CON1 (1–3) and CON2 (2–3) were euthanized. The remaining sheep grouped in MEN9, STAN9 and CON1 (1–9) and CON2 (2–9) were treated every two weeks for a further 6 months and subsequently euthanized.

The dosage and interval times between injections were based on the results of an *in vitro* study on the effects of stanozolol on primary chondrocyte cultures (Saleri et al., 2004) and from a pharmacological study performed after intra-articular injection on sheep to assess tolerability and pharmaco-kinetics (unpublished data).

[1] Megaxilor, Bio 98, Milan, Italy.

[2] Nargesic, ACME, Cavriago (RE), Italy.

[3] Ketavet, Intervet, Aptilia (LT), Italy.

[4] IsoFlo, Abbot Laboratories Queenborought, Kent, United Kindom.

[5] Vatamplius, FATRO, Ozzano dell'Emila (BO), Italy.

[6] Aagent, FATRO, Ozzano dell'Emila (BO), Italy.

[7] Meflosyl, Fortdodge, Aprilia (LT), Italy.

[8] Tanax, Intervet International BV, Boxmeer, NL.

Table 1
Score used for reading the radiographs. From (Kellgren and Lawrence, 1957).

Score	Description
0	None. Absence of X-ray changes of osteoarthrosis
1	Doubtful OA. In presence of moderate osteophytes
2	Minimal OA. Osteoarthrosis was present though the minimal severity and definite osteophytes
3	Moderate OA
4	Severe. Multiple osteophytes, severe sclerosis and indefinite bone margins

Euthanasia was performed with injection of xylazine (0.1 mg/kg, IM); ketamine (3 mg/kg, IM) and Tanax®[9] (0.5 ml/kg, IV).

2.3. Assessment of clinical condition

Animals were observed on a daily basis to ensure that they were eating, drinking regularly and moving. The hind limbs were examined and palpated regularly and the range of motion was evaluated. Sheep in group CON1 (1–3), CON2 (2–3), MEN3, STAN3 were weighed at the third month; the sheep from groups CON1 (1–9), CON2 (2–9), MEN9, STAN9 were weighed at the third and the ninth month.

2.4. Lameness

Weekly or every other week, before the injection (stanozolol or placebo), sheep were checked for lameness and evaluated using the score proposed for large animal lameness (Ross, 2003) adapted for sheep.

The sheep were forced to walk first and then to run and the lameness score was determined by an expert veterinary orthopaedist (S.A.).

2.5. Radiological evaluation

All sheep underwent conventional radiological examinations pre-surgery for inclusion in the experimental protocol; this was repeated at the end of the third month for all of the groups, while groups CON1 (1–9), CON2 (2–9), MEN9 and STAN9 did the evaluation after nine months again.

Radiographs were performed with handle restrain of the sheep in two conventional projections for stifle joint.

Diagnostic image evaluation was performed by two expert orthopaedic veterinarians (A.S.; N.R.) blinded to the treatment group, according to the score proposed by Kellgren and Lawrence (1957) for radiographic assessment of OA severity (Table 1).

2.6. Synovial fluid

Synovial fluid (SF) was aseptically collected before each treatment time from all sheep restrained in lateral recumbency with a 22G needle from the medial or lateral femoro-patellar pouch. Immediately after synovial fluid collection, smears were performed for cytological examination. The smears (air-dried) were stained using the May-Grunwald-Giemsa method, examined with a Nikon Eclipse E800 microscope and images were captured by an Image Analyzer.[10,11]

The synovial fluid was also put in cryo-vials with the addition of EDTA. Then were centrifuged at 2000 rpm for 10 min and the supernatants were frozen at −20 °C until determination of the glycosaminoglycans (GAGs) concentration according with previously papers (Farndale et al., 1982, 1986).

2.7. Gross pathology, histopathology and tissue processing

Sheep's necropsies were performed immediately after pharmacological euthanasia. Stifle joints were examined and specimens from synovial membrane were fixed in 10% phosphate buffered formaldehyde (pH 7.4), whereas specimens of articular cartilage, from tibial and femoral condyles, were collected and simultaneously fixed-decalcified using a double step system. Specimens, 3–5 mm thick, were placed in Decalcifier-I® (Surgipath) overnight to fix and perform decalcification, using the decalcificant at a sample:volume ratio of 1:20. Overnight decalcification was performed at room temperature, with gentle agitation. Specimens were then directly transferred, without washing, into Decalcifier-II® (Surgipath) to complete demineralization. Soft and decalcified tissues were embedded in paraffin wax (56–58 °C, 08-7910 Bio-Optica, Italy) by using a Vacuum Tissue Processor (Renaissance Ventana). Five μm thick paraffin sections were obtained using a Rotary microtome Leica RM2155 and stained for histological routine methods (H&E, Masson's trichrome stain).

Histological sections were analysed using a Nikon Eclipse E800 microscope and images were captured by an Image Analyzer.[10,11]

To process the data obtained from the study of macroscopic lesions found in synovial joints (SJs) of sheep killed three or nine months after meniscectomy, the score of the International Journal of Cartilage Repair Society (ICRS), reported below, was used (Table 2).

2.8. GAGs determination in articular cartilage

At the end of the 3- and 9 month-experimental period, after removing the periarticular soft tissue, the stifle joints were opened and full-depth plugs of condyle articular cartilage were obtained by means of 6 mm punch. The cartilage plugs were promptly weighed (fresh weight) and then dehydrated at 60 °C for 20 h. The dehydrated cartilage disks were weighed (dried weight), placed into vials containing 1.0 ml of papain solution (50 mM Tris–HCl pH 8, 1 mM EDTA, 2 mM dithiothreitol and 0.3 mg/ml papain) and were incubated at 65 °C until complete tissue digestion. The reaction was stopped with 50 mM iodoacetic acid in 2.5 mM NaCl. A DMMB solution (2.5 ml) was added to 200 μl of each sample and to 200 μl of each standard (obtained as above mentioned); the absorbance was measured at 525 nm by using a spectrophotometer (Perkin Elmer Lambda 25UV/Vis). The GAG content was calculated from the standard curve obtained and was reported as mg of GAGs/mg of dried tissue.

2.9. Statistical analysis

Descriptive statistics were applied to analyze the clinical and non-parametric data.

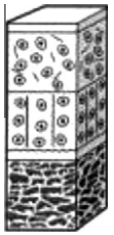
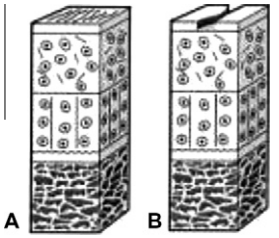
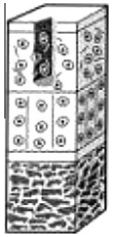
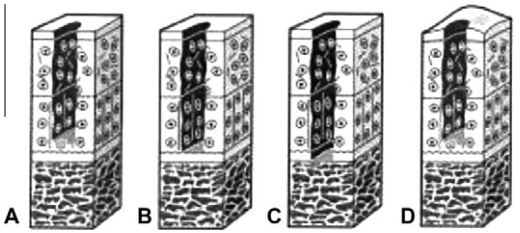
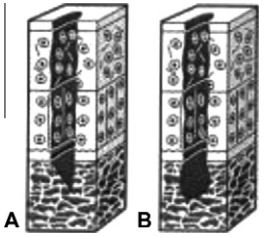
The data obtained from lameness evaluation, the results from radiological imagines evaluation and the body weight were analyzed with no-parametric test Mann Whitney with significance for $P < 0.05$ and high significance for $P < 0.01$.

The results obtained from the gross evaluation of synovial fluid analysis were compared among the groups using Fisher's exact test with significance at $P < 0.05$ and high significance for $P < 0.01$.

Statistical analysis was performed on gross pathology. Data were analyzed by Mann Whitney "*U*" test for different in medians: Due to the low number of sheep, and in order to achieve an acceptable power of the test, the analysis was performed for the six

[9] Sungate, ACME, Cavriago (RE), Italy.
[10] Nikon digital sight, Nikon Corporation, Calenzano (FI), Italy.
[11] Sigma Aldrich Srl, Milan, Italy.

Table 2
The score of the International Journal of Cartilage Repair Society (ICRS).

Grade	Description of macroscopic lesions of articular cartilage (From International Cartilage Repair Society (ICRS) http://www.cartilage.org/_files/contentmanagement/ICRS_evaluation.pdf)	
0	Normal cartilage	
1	Superficial abnormalities: soft indentation (A) and/or superficial fissures and cracks (B)	
2	Superficial fibrillation: fissures are extended down to 50% of cartilage thickness	
3	Deep fibrillation: fissures are extended down almost all/all the thickness of cartilage. (A) as well as down to calcified layer (B) and down to but not through the subchondral bone (C). Blisters are included in this Grade (D)	
4	Exposed bone (the cartilage is completely eroded and subchondral bone is exposed)	

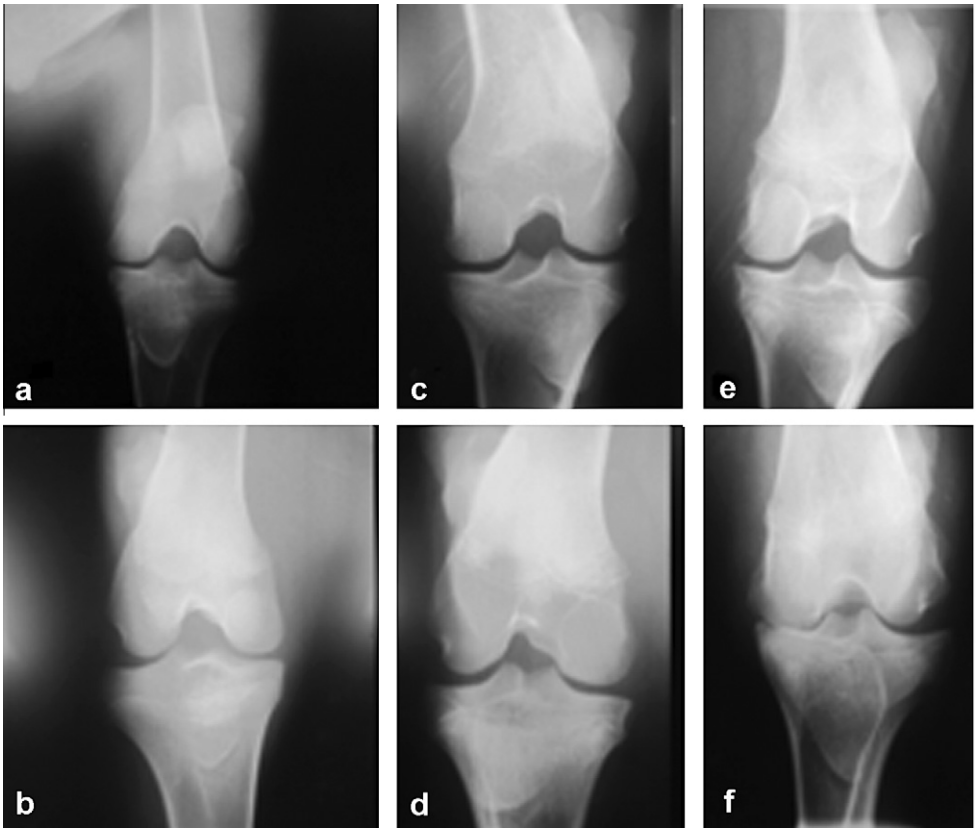


Fig. 1. Radiological images of the left FTJ, dorso-plantar view: (a) CON1–3; radiological score 0; (b) CON2–9 radiological score 0; (c) MEN3; (d) MEN9; (e) STAN 3; (f) STAN 9. All the images was detected at the end of the protocol before the euthanasia.

Table 3
Results of macroscopic evaluation at 3 months of the joints in all groups.

Macroscopic score 3 months after surgery											
Joint		STAN 3				MEN3				CON1	CON2
		Sheep	Sheep	Sheep	Sheep	Sheep	Sheep	Sheep	Sheep	Sheep	Sheep
Right limb	Femoral condyle	2	2	2	3	3	3	2	4	2	1
	Trochlea	3	3	3	4	4	4	4	4	3	3
	Tibial plateau	1	2	3	3	2	2	1	4	0	0
Left limb	Femoral condyle	2	3	3	3	3	4	3	3	2	0
	Trochlea	0	3	3	3	4	4	4	4	2	3
	Tibial plateau	1	2	3	2	2	3	1	2	0	0

groups on data cumulated (STAN3, MEN3, CON1 and CON2 at 3 months, STAN9, MEN9, CON1 and CON2 at 9 months).

3. Results

3.1. Clinical condition

During the first month p.s. 3 sheep (2 MEN3, 1 MEN9) showed episodes of difficulty in standing up and one sheep of STAN3 group showed p. s. signs of right radial nerve paresis. By the end of the first month these symptoms had disappeared in the three sheep. Some animals had a mild rise in body temperature (>38.5 °C) in the first days p. s. Moderate inflammation with serum-hemorrhagic subcutaneous swelling at the sutures occurred in three sheep: in the right joint of a MEN3 animal and in both legs of two MEN9 sheep. No other major variation in body conditions concerning local pain or swelling of the knees were detected. Two sheep in the MEN9 group suffered monolateral left medial patellar sub-luxation only in the first three weeks p.s.

3.2. Weight

The weight of sheep were measured pre-operatively and ante-mortem with an increase of weight three months after meniscectomy of 0.80% for the sheep of MEN3 group (42.4 ± 4.8 kg pre mortem) and 6.39% for the sheep of the group STAN3 (45.8 ± 3.8 kg pre mortem), and after nine months 21% increase for MEN9 (55.5 ± 6.8 kg pre mortem) and 32.7% for STAN9 (62.0 ± 4.6 kg pre mortem). In both CON group the mean increase of weight was 1.2%.

3.3. Lameness evaluation

Groups STAN3 and STAN9 showed a lower overall lameness score than groups MEN3 and MEN9. The groups were paired

(MEN3 *vs* STAN3 and MEN9 *vs* STAN9) to compare the results, with each group also having its control sheep (CON2–3 and CON1–3, CON2–9 and CON1–9). From the comparison of the scores between the paired groups, a lower grade of lameness was detected in the STAN groups. The difference between the STAN and MEN groups was statistically significant only at week 1 and 10 ($p < 0.05$) for the 3-months groups (STAN3 *vs* MEN3) while only at week 2 ($p < 0.05$) for the 9-months groups (STAN9 *vs* MEN9).

3.4. Radiological evaluation

The features of OA observed in the radiographic images (Fig. 1) had a slightly arthritic change after three months (radiological score MEN 3 1.75 ± 1.1 and STAN 3 1.4 ± 0.7), that was more consistent at nine months (radiological score MEN9 2.6 ± 1.2 and STAN 9 1.5 ± 0.7) in all meniscectomized sheep (16/20). Comparing the scores between the paired groups, the difference between MEN 9 and STAN 9 was statistically significant ($p < 0.05$).

3.5. Cytomorphology

Arthrocentesis was unsuccessful in many cases; consequently it was not possible to describe the cytology for every time-point in the experimental design. Over the first 3 months, of the 192 arthrocentesis procedures completed on the MEN3 and MEN9 groups, 87 synovial fluid samples were obtained; in the STAN3 and STAN9 groups 65/192 synovial fluid samples were collected. In the subsequent 6 months, of the 96 arthrocentesis procedures completed on the MEN9 and STAN9 groups, 23/96 and 21/96 synovial fluid samples were collected, respectively. In the cases for which synovial fluid smears were prepared, no pathological cellularity resembling inflammation or allergy patterns were observed in sheep from the STANs groups. The most common feature was mild exfoliation of synoviocytes and the occasional presence of erythrocytes, probably were related to the sample invasive collection technique. In the CON and MEN groups, the cytomorphological pattern was very similar to the STANs group.

3.6. Gross pathology

Necroscopies were performed and no evidence of gross pathologies were recorded in organs, apparatuses and systems contained in the neuro-cranial, thoracic and abdominal cavities.

FTJ lesion scores and descriptions are summarized in Table 3 and Table 4. A value from 0 to 4 of increasing grade of severity, depending on the depth and extent of the skeletal lesion, was assigned to each animal.

3.6.1. Three months

In sheep CON1–3 normal gross morphology was recorded in the FTJ cartilage, whereas a histological aseptic inflammation and fibrosis were observed in sheep CON2–3.

In group MEN3, synovial fibrosis, condylar and tibial plate cartilage degeneration were recorded at histological examination.

In group STAN3, condylar (Fig. 2(4)) and tibial plate cartilage degeneration were histologically recorded but less severe compared sheep in group MEN 3.

Grade 4 was frequently recognized in sheep group MEN 3 but only one sheep in the STAN3 group showed comparable lesion scores (Table 2).

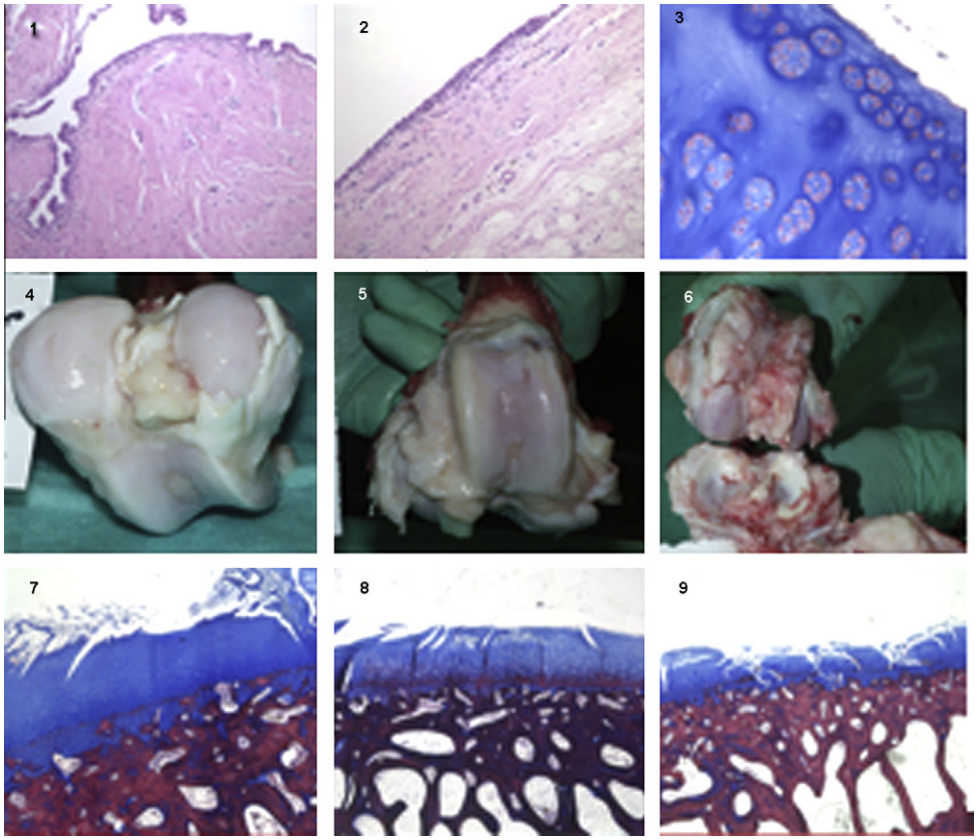


Fig. 2. 1 – Hyperplasia of synoviocytes: polipoides proliferations. (sheep CON3); 2 – Hyperplasia of synoviocytes: regular distribution. (sheep STAN3); 3 –Proliferation of chondrocytes (sheep STAN3); 4 – second level lesion (femoral condyles sheep STAN3); 5 – third level lesion (trochlea sheep MEN9); 6 – fourth level lesion (left joint sheep MEN9); 7 – first level lesion (sheep STAN3); 8 – third level lesion (sheep MEN9); 9 – fourth level lesion (sheep MEN3).

Table 4
Results of macroscopic evaluation at 9 months of the joints in all groups.

Macroscopic score 9 months after surgery											
Joint		STAN 9				MEN9				CON1	CON2
		Sheep	Sheep	Sheep	Sheep	Sheep	Sheep	Sheep	Sheep	Sheep	Sheep
Right limb	Femoral condyle	2	2	2	3	3	4	3	4	1	1
	Trochlea	4	4	4	4	4	4	4	4	2	3
	Tibial plateau	3	3	3	3	3	4	4	4	0	2
Left limb	Femoral condyle	2	3	3	3	4	4	2	4	1	1
	Trochlea	4	4	4	4	4	4	4	4	2	3
	Tibial plateau	2	3	3	2	3	4	4	3	0	1

Table 5
Statistics analysis of the score of gross pathology in 3 months groups.

3 months					
Two sample test record					
Group	Count	Mean	S.D.	Power	Prob level
MEN 3 vs CONS	24	3.08	1.02	0.99	0.00
	12	1.33	1.30		
STAN 3 vs CONS	24	2.45	0.88	0.84	0.01
	12	1.33	1.30		
STAN 3 vs MEN 3	24	2.45	0.18	0.60	0.02
	24	3.08	0.2		

Table 6
Statistics analysis of the score of gross pathology in 9 months groups.

9 months					
Two sample test record					
Group	Count	Mean	S.D.	Power	Prob level
CONS vs MEN 9	12	1.41	0.99	1.00	0.00
	24	3.700	0.55		
CONS vs STAN 9	12	1.41	0.99	0.99	0.00
	24	3.08	0.75		
MEN vs STAN 9	24	3.70	0.55	0.88	0.00
	24	3.08	0.77		

Statistical analysis performed between groups MEN3, STAN3 and CONs is summarized in Table 5 and has shown: STAN3 vs. MEN3 $p = 0.02$; STAN3 vs CON1/2–3 $p = 0.01$ and MEN3 *vs* CON1/2–3 $p = 0.00$.

3.6.2. Nine months

In sheep CON1–9 normal FTJ gross morphology was recorded in the stifle cartilage and synovial capsule whereas cartilage degeneration was recorded in sheep CON2–9.

In sheep of group MEN9, synovial fibrosis, condylar and tibial plate cartilage degeneration were histologically observed. Gross pathology of lateral condyle of the femur also showed clear structural deformation of the surface profile with lateral tibio-femoral anatomical incongruence (Fig. 2(6)).

Less severe, but identical lesions were recorded in sheep of group STAN9.

Gross pathology of FTJs, developed during nine months following meniscectomy, were evaluated in each sheep.

The lesions observed in FTJ of sheep 9 months after meniscectomy were more severe in sheep of group MEN 9 compared to sheep of group STAN 9, despite highlighting a general framework for extremely serious lesions (MEN9).

Lesions of the tibial plateau were less severe, recording rather low scores in all groups; cartilage fibrillation was the most frequently observed histological feature, and was more severe in sheep of group MEN.

The femoral trochlea of sheep of MEN9 and STAN 9 groups was the anatomical surface that suffered the worst damage after surgery. Moreover, the most severe lesions were seen in FTJs in sheep of group MEN 9. In particular, large areas affected by erosion involving the entire cartilage thickness reaching the subchondral bone, phenomena of intense vertical fibrillation and osteochondrophyte formation were observed (Fig. 2(5)). In reference to the latter data, a better score was given to animals of STAN9 group.

Statistical analysis performed between the groups MEN9, STAN9 and CON1–2 9 months is summarized in Table 6 and has shown: STAN9 *vs* MEN9 $p = 0.00$; STAN9 *vs* CON1/2–9 $p = 0.00$ and MEN9 *vs* CON1/2–9 $p = 0.00$.

3.7. Histopathology

For the sheep in group CON1–3 months, microscopic lesions were not observed in the synovial membrane or in articular cartilage of FTJ, whereas in sheep CON2–3 an aseptic inflammation and fibrosis were observed.

In sheep CON1/2–9 months a mild synovial membrane aseptic chronic inflammation as well as focal cartilage degeneration were observed. These lesions are induced by the numerous intrarticular sampling collection as indicated in experimental design.

In the sheep of group MEN3, synovial aseptic inflammation was observed in association with synovial fibrosis. In groups MEN3 and STAN3, slight synoviocytes hyperplasia was recorded. In group MEN3, the synovial membrane showed an irregular profile with invaginations and the corion was fibrotic. Synoviocytes showed hyperplasic features characterized by proliferation of clusters arranged in polypoid shape (Fig. 2(1)).

In sheep of STAN3 (Fig. 2(2)) and STAN9 groups, the synovial membrane showed a straight profile and synoviocytes hyperplasia which covered regularly the surface of synovial membrane.

Fibrillation of cartilage and thinning of the femoro-tibial articular cartilage plates accompanied by chondrocytes hyperplasia, were observed in sheep of groups STAN3 (Fig. 2(7)) MEN9 (Fig. 2(8)) and MEN3 (Fig. 2(9)).

In sheep of group STAN3 chondrocytes regeneration, arranged in clusters were very prominent (Fig. 2(3)).

3.8. GAGs determination in the synovial fluid

The analysis of the synovial fluid for the determination of GAGs during the first 3 months highlighted a variable and irregular course in groups MEN3 and STAN3. Regarding group MEN3, a reduction for GAGs was observed during the first week post-surgery. This was followed by erratic changes in the 3rd week. From 4 to 12 weeks the course was more constant and characterized by values of 5 μg/100 μl that progressively decreased up to 2 μg/100 μl at 22 weeks. Regarding groups STAN3 and STAN9, the

course was fluctuated considerably until 9 weeks: peaks at 6 and 9 weeks were observed together with a strong decrease at 8 weeks. Between 10 and 23 weeks, the curve was comparable to that observed in group MEN9 a trend to decrease of the GAGs amount. However, the trend was similar in both groups and the major difference was observed at 6 and 11 weeks. Statistical analyses was not applied because of the limited numbers of sample.

3.9. GAGs determination in articular cartilage

Any significant differences were observed in the cartilage GAGs content among considered groups nor at three months neither nor at nine months of treatments.

4. Discussion

The morphological and histological changes in cartilage, subchondral bone and surrounding articular tissues observed in the ovine joints following meniscectomy are considered a good representation of induced OA.

When the study was designed, it was essential to produce a complete "expert," overview including the clinical report, to present to the National Pharmacological Authority; the study aimed to collect data useful to license a trademark for the drug. The whole process required five years and it was not possible to publish partial data throughout this period. Of the animal models presented in literature, the bilateral medial meniscectomy followed by forced walking for different periods, appeared to be most appropriate for the purposes of the research. The creation of a bilateral lesion in the knees avoided the protective effect sustained by the sound hind limb and the controlled, progressive effect of the instability. The joint damage was expressed to a greater degree on the medial side because of the meniscal removal, accompanied by the mechanical stress prevalence of medial weight bearing; it promoted the progress of the features of OA in the short term. The medial articular surfaces had the most severe degenerative changes with development of chondro-osteophytes not clearly evident at radiological examination (lateral view), while the lateral compartment of the joint, after a period of rapid modifications, stabilized with less progressive and more linear changes. For the different characteristics of pathological changes inside the joint obtained in the short and long term, the model proved to be ideal for studying the progression of the pathology and outlined the capability of the structure-modifying drugs for OA that could have rapid onset of action and a long-lasting effect.

Using this model the counteracting effects of the stanozolol on the degenerative changes were observed with a series of clinical and morphological evaluations 3 and 9 months p.s. Moreover sheep demonstrated to be good patients and ideal candidates for this type of experimental work, because of their docile nature and pliant behavior, despite the complex and demanding study protocol.

The different gain in body weight observed between animals treated with stanozolol and placebo is not thought to be related to the anabolic effect of the drug. The dosage of 1 mg intraarticularly of stanozolol is considerably lower than the normal values for the anabolic effect indicated in literature – about 10 mg/kg once a week in man (Small et al., 1984). In a previous clinical work on 15 dogs systemically treated for tracheal collapse with a dose of 0.3 mg/kg, IM, q 24 h of stanozolol, no changes to the sexual characteristics or muscular hypertrophy typical of the anabolic/androgenic side effects were detected (Dondi et al., 2002; Adamama-Moraitou et al., 2009).

In both CON groups the weight increase was poor; it was suggested that the weight gain observed in the sheep of group STANs could be due to better soundness that lead to better body conditions, more movement and hierarchical predominance of these animals for feed.

It was also suggested that positive effects of stanozolol on cartilage could be attributed to an anti-dystrophic effect, different to the "classical", more well-known anabolic action (Fernandez et al., 1994).

This was explained because stanozolol, unlike natural androgenic molecules, may interact with the Type I androgenic receptors (AR) that also include receptors for glucocorticoids, mineralcorticoids and progesterone. Stanozolol has high affinity for these receptors and at low dosages is able to block glucocorticoid receptors, hindering progesterone activity and the catabolic effects of glucocorticoids, before showing the anabolic effect and activating the AR (Wright et al., 1989). In fact side effects associated to the systemic administration of stanozolol at high dosages or for prolonged periods cannot be excluded.

Accuracy in lameness evaluation was difficult because of the impossibility to lead the animals and because of their sudden and irregular movements. Despite this the AAEP scale adapted quite well to the study needs. Lameness was inserted in the protocol as one of the parameters that could produce a clinical overview on the effects of the treatment. The data of lameness evaluation between groups STAN9 and MEN9 was not statistically significant. However the apparent results observed of mean data lameness score could suggest a positive response regarding the efficacy of the stanozolol treatment. It should be mentioned that two of the sheep in the group MEN9 had patellar sub-luxation that recovered after some weeks, even if it is considered to have little influence in pathological OA development, as observed in a canine osteoarthritic and arthritic joint study (Hegemann et al., 2002).

The results from radiological examination had to be compared with the morphological ones reported in the gross pathology. In the radiographs taken 3 months p.s. some limited features of OA were observed, with no significance between the groups (MEN3 and STAN3) as well as in the others (MEN9 and STAN9). Findings at 9 months (MEN9 and STAN9) showed statistical significance, probably due to the slower progression of the pathology in the animals in STAN9. These results were also supported by the gross pathology as well as histopathology features.

Histological examination of the articular cartilage highlighted synoviocytes hyperplasia and clusters of regenerative chondrocytes in sheep of group STAN3 *vs* groups MEN3 and CONs.

Reactive synovial regular hyperplasia was more evident in groups STAN3 and STAN9 than in groups MEN3 and MEN9. This hyperplastic response persists and is partly due to adaptation lesions from the persistent damaging element (thinning of articular cartilage).

Gross and histopathological investigations demonstrated tolerability of the stanozolol molecule in the articular environment but also highlighted its pharmacological activity by inducing synovial membrane regular hyperplasia and articular cartilage regeneration. Of particular relevance was the fact that chondro-synoviocytes hyperplasic-reactive phenomena were observed in a validated experimental model that produced serious acute and chronic joint injuries, because of the constant traumas induced by the alteration of joint mechanics.

The synovial fluid sampling was not possible at every visit. In the first months we witnessed a decreased number of samples in the STAN groups compared to number from the MEN ones. It was suggested that the reduced inflammatory phase in STAN groups could have resulted in less fluid forming in the joint.

Synovial fluid cytologic data allowed observation of inflammatory cells (neutrophils and lymphocytes) and/or cells derived from synovial membrane (synoviocytes) without showing features of adverse cellular reaction.

Concerning the efficacy of the stanozolol and its mechanism of action, one study (Falanga et al., 1998) demonstrated that stanozolol was able to induce fibroblasts to increased collagen production in a dose-dependent pathway by means of TGF-b1 synthesis. The TGF-b1 effects are complex, variable, and cell line-dependent but ultimately increase cellular matrix production (Wright et al., 1989).

One of the main effects of stanozolol on *in vitro*-cultured chondrocytes is a significant reduction of NO production and stimulation of autocrin secretion of IGF-I (Saleri et al., 2004).

Differently to a previous report (Oakley et al., 2004) that focused on the assessment of bone mineral density, cartilage and subchondral bone thickness, the present study concentrated largely on the effects of the stanozolol on the synovial lining and cartilage.

The morphological changes associated with induced OA, such as osteophytes and cartilage and subchondral bone reaction could represent a physiological response with adaptive remodeling to abnormally distributed mechanical stresses (Hwa et al., 2001) or a different pathway of cytokine expression.

5. Conclusions and clinical relevance

The use of stanozolol in this model of OA in sheep did not produce any joint reaction even after repeated injections. Compared to the controls, it reduced the formation of osteophytes and subchondral bone reaction, in consideration of the morphological changes associated with induced OA. The features observed in articular cartilage demonstrate a valid and organized chondral matrix articular regeneration with a normal morphological structure. The results obtained with this pilot experimental study allow us to transfer the experience to clinical trials in the target veterinary species – such as horse and dog – affected by the spontaneous osteoarthritis.

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