4. Materials and method

A total of 88 synovial fluid samples from healthy joints (n=18) and diseased joints (n=62) and tendon sheaths (n=8) were collected from a total of 74 horses between 2003 and 2005 at the Clinic for Horses, General Surgery and Radiology of the Faculty of Veterinary Medicine of the Freie Universität Berlin and analyzed for viscosity, pH, total protein concentration and total white blood count. Activity of the enzyme myeloperoxidase (MPO) in the synovia was measured at the Institute for Veterinary Biochemistry of the Faculty of Veterinary Medicine of the Freie Universität Berlin. The activity of MPO in the different diagnostic groups and its relationship to synovial parameters and clinical signs of orthopedic diseases was studied.

4.1. Materials

4.1.1. Control Group

The control group consisted of 18 samples taken from 16 horses of different ages, sexes and breeds (Tables 1, 2, 3 respectively). Samples were obtained from 10 carpal and 8 tarsocrural joints.

Criteria for selection of control animals were as follows (PERSSON 1971; SANDER 1990):

1- The horse presented no signs of general disease.
2- No factor causative of lameness could be attributed to the joint.
3- The joint showed normal mobility.
4- No swelling, tenderness or heat over the joint was noticed.
5- No thickness or lack of elasticity of the joint capsule was felt on palpation.
6- No signs of synovial effusion were apparent in the punctuated joint.
7- Synovia presented a normal appearance and physiological parameters after analysis.

4.1.2. Patients Groups

Synovial fluid samples were acquired from clinical cases presented at the Clinic for Horses, General Surgery and Radiology of the Freie Universität Berlin. General data of the horse's owner and the name, sex, breed, and anamnesic history of each animal was registered at the time of presentation in the clinic. The age of each horse was listed as recorded in the respective equine passport. The group consisted of 62 samples from diseased joints obtained

from 50 horses of different ages, sexes and breeds. Additionally, a total of 7 samples from the digital flexor tendon sheaths and 1 from the tendon sheath of the muscle extensor carpi radialis were also acquired from 8 horses. A detailed description of the distribution of ages, sexes, breeds and joint types in the different diagnostic groups is presented on tables 1, 2, 3, 4, respectively.

Table 1. Age of control horses and patients in years

Diagnostic group and number of samples	Total number of horses	Minimun	Maximun	Mean ± S.D.
Control (n= 18)	16	1	23	9 ± 8, 149
CA (n=28)	23	5	13	9 ± 2, 497
OCD (n=15)	10*	1	6	3, 2 ± 1, 740
OA (n=12)	11*	1	25	10, 727 ± 7, 115
OCD+ OA (n=4)	4	8	25	13, 5 ± 7, 767
SA (n= 3)	3	1	3	1, 667 ± 1, 155
TS (n= 8)	8	5	18	11, 625 ± 5, 041

Table 2. Distribution of sexes among the groups of controls and patients

Diagnostic group and number of samples	Total number of horses	Sex		
		Intact males	Geldings	Females
Control (n= 18)	16	5	5	6
CA (n=28)	23	-	12	11
OCD (n=15)	10*	3	3	4
OA (n=12)	11*	1	6	4
OCD+ OA (n=4)	4	-	3	1
SA (n= 3)	3	-	-	3
TS (n= 8)	8	-	4	4

*Two samples from a same horse were classified into different diagnostic groups (OA and OCD), in accordance to the criteria shown on table 7. The individual is shown in the group of OA and in the group of OCD.

Table 3. Distribution of the different breeds in the groups of controls and patients

Diagnostic group and number of samples	Total Number of horses	Breed					
		Warmblood	Standardbred	Pony	Thoroughbred	Arabian	Quarter Horse
Control (n= 18)	16	9	3	1	2	-	1
CA (n=28)	23	20	1	1	1	-	-
OCD (n=15)	10*	10*	-	-	-	-	-
OA (n=12)	11*	8*	-	1	-	2	-
OCD+ OA (n=4)	4	3	-	1	-	-	-
SA (n= 3)	3	3	-	-	-	-	-
TS (n= 8)	8	6	2	-	-	-	-

Table 4. Distribution of the different joint types in the groups of controls and patients

Diagnostic group and number of samples	Total Number of Horse	Joint				
		Distal Interphalangeal (coffin joint)	Metacarpo/ Metatarso phalangeal (Fetlock joint)	Carpal †	Tarsocrural (Hock joint)	Femoropatellar (Knee joint)
Control (n=18)	16	-	-	10	8	-
CA (n=28)	23*	19	6	2	1	-
OCD (n=15)	10*	-	5	-	10	-
OA (n=12)	11	-	2	4	2	4
SA (n=3)	3	-	1	-	2	-
OCD+ OA (n=4)	4	1	2	-	1	-

† Radiocarpal and intercarpal joints were pooled.

*Two samples from a same horse were classified into different diagnostic groups (OA and OCD), in accordance to the criteria shown on table 7. The individual is shown in the group of OA and in the group of OCD.

4.2. Method

4.2.1. Diagnosis of lameness

A complete clinical examination was carried out. The presence of lameness was assessed at the walk and at the trot in a straight line and in a circle at both hands on a hard surface. The degree of lameness was classified into 5 categories: doubtful, mild, moderate, severe and non-weight-bearing lameness (Table 5). A general flexion test was performed for 1 minute (STASHAK 1987; VERSCHOOTEN and VERBEECK 1997) on the affected limb and the response was assessed immediately thereafter at the trot. Perineural blocks with lydocain hydrochloride 2% with adrenalin 1:200 000* using a 23-gauge x 1¼-inch needle** were carried out under strict aseptic technique in order to localize the affected joint (GERWECK et al. 1994). Ten minutes thereafter, the response to the anesthetic block was assessed at the trot.

Table 5. Criteria to the grading of lameness

Degree of Lameness	Description
Doubtful	Lameness is not observed at the walk and it is barely observed at the trot.
Mild	Lameness is not observed at the walk but it is obvious at the trot.
Moderate	Lameness is obvious at both at the walk and at the trot.
Severe	The horse tends to stand with the dorsal angle of the hoof of the diseased limb and for a short period of time.
Non-weight-bearing lameness.	Non-weight-bearing lameness. The horse stands on 3 legs

4.2.2. Radiographic examination

A complete radiographic examination of the affected joint was performed with an X-ray machine Philips Super 100 CP. Screen X-ray films RetinA x-ray XOD TM *** were used. Rare-earth intensifying screens of the type Trimax T2 – 100 † were used for the distal interphalangeal, metacarpo/metatarsophalangeal, and tibiocrural joints. The type Trimax T6 – 300 † was preferred for the carpal and femoropatellar joints. The radiographic views performed for each joint type are detailed on table 6.

* Xylocain ® AstraZeneca - GmbH Wedel, Germany

** BRAUN Vetcare - Melsungen AG - Melsungen, Germany

*** Fototechnische Werke GmbH - Berlin-Köpenick, Germany

† Company 3M GmbH - Neuss, Germany

Table 6. Radiographic views performed for each joint type

Joint	Number of radiographic projections	Description of radiographic projections
Distal interphalangeal joint (Coffin joint)	4	• Dorsopalmar/plantar • Lateromedial • Flexed dorsolateral-palmaro/plantaromedial oblique • Flexed dorsomedial-palmaro/plantarolateral oblique
Metacarpo/metatarsophalangeal joint (Fetlock joint)	5	• Dorsopalmar/plantar • Lateromedial • Flexed lateromedial • Dorsolateral-palmaro/plantaromedial oblique • Dorsomedial-palmaro/plantarolateral oblique
Carpal joints	5	• Dorsopalmar • Lateromedial • Flexed lateromedial • Dorsolateral-palmaromedial oblique • Dorsomedial-palmarolateral oblique
Tarsocrural joint (Hock joint)	4	• Dorsoplantar • Lateromedial • Dorsolateral-plantaromedial oblique • Dorsomedial-plantarolateral oblique
Femoropatellar joint (knee joint)	2	• Lateromedial • Caudocranial

4.2.3. Diagnostic classification criteria

Samples were classified into different diagnostic groups according to criteria based on the anamnesic data, clinical and radiological examination and synovial fluid analysis as shown on table 7.

Table 7. Classification criteria of samples in diagnostic groups based on anamnesic data, clinical and radiographic examination and analysis of synovia

Diagnostic group	Classification criteria
Chronic arthritis (CA)	History of chronic joint disease (> 5 weeks) Lameness Positive response to flexion test Positive response perineural anesthesia No radiographic findings
Osteoarthritis (OA)	History of chronic joint disease (> 5 weeks) Positive response to flexion test Positive radiographic findings: Either thinning of joint space and/or; subchondral bone sclerosis and/or; periarticular marginal osteophytes/enthesiophytes
Osteochondrosis dissecans (OCD)	Osteochondritic fragment in convex articular surfaces in young horses
Osteochondrosis dissecans + secondary osteoarthritis (OCD + osteoarthritis)	Lameness Positive response to flexion test Positive response to perineural anesthesia OCD in adult horses with secondary osteoarthritic radiographic findings
Septic arthritis (SA)	Lameness Joint effusion Penetrating wound into the joint High white blood cell count in synovia
Tenosynovitis (TS)	History of chronic disease (> 5 weeks) Synovial tendon sheath effusion Ultrasonographic signs of tenosynovitis

Synovial fluid was collected during clinical diagnostic procedures or at the time of surgery. In the first case, most collection of synovia was followed by the intraarticular/intrathecal administration of medication.

Out of a total of 15 samples from the group with OCD, ten came from joints that showed no signs of disease. In these cases, the diagnosis was based on the roentgenographic evaluation and synovia was collected during surgery performed for removal of the osteochondritic fragments.

Horses with septic arthritis in the present study had penetrating wounds into the joint. Hence, no lameness examination was carried out. The affected joint was immediately bandaged and the horse was prepared for surgery to perform a joint drainage. The synovial fluid sample was taken during surgery.

4.2.4. Analysis of synovial fluid

Arthrocentesis: Synovia was collected from diarthrodial joints with an 18-gauge x 1 ½ -inch needle* and from tendon sheaths with a 21-gauge x 2-inch needle*, following a strict aseptic technique. After punctuation, synovia began to flow through the needle. A 2 ml sterile vacutainer containing calcium balanced heparin (50 I.U./ml)** was inserted on the finger-stabilized needle hub and the synovia was carefully aspirated. Restraint methods (e.g. nose twitch) or mild tranquilization with detomidine hydrochloride *** (20 µg/kg of body weight) or romifidine hydrochloride **** (0, 04 mg/kg of body weight) were used when necessary. All samples were analyzed immediately after collection for pH, viscosity, total white blood cell count and total protein concentration.

pH: The pH was measured with an automatic blood gas system. †

Viscosity: Viscosity was estimated by observing the length of the strand of synovia that forms on the end of the syringe before it drips (HERTSCH 1983). Samples were categorized into three groups:

Category of viscosity	**Length of strand**
Normal ††	> 3 cm
Decreased	< 3 cm
Severely decreased	Non-stringing fluid

Protein: total protein (g/dl) was determined using a refractometer ††† (KAWCAK et al. 1997; DIMOCK et al. 2000; McILWRAITH et al. 2001).

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* BRAUN Vetcare – Melsungen AG - Melsungen, Germany
** Monovet ® Sarstedt Aktiengesellschaft & Co. – Nümbrecht, Germany
*** Domosedan ® Pfizer GmbH – Karlsruhe, Germany
**** Sedivet ® Boehringer Ingelheim, Vetmedica GmbH - Ingelheim, Germany
† AVL 995 S. AVL List GmbH - Graz, Austria
†† Hertsch (1983)
††† HRM 18 – A. Krüss Optronic GmbH - Hamburg, Germany

Total white blood cell count (TWBC): TWBC was determined with an automated cell coulter ‡ (ATILOLA et al. 1986; FRISBIE et al. 1999; DIRKS 2000). In order to ensure a uniform resuspension of the cellular content of the sample, each sample was gently shaken before measuring (McCARTY 1997). Results were given by the coulter in cells/l. The metrical unit was mathematically adapted and expressed in cells/µl in the present work.

4.2.5. Measurement of the activity of MPO

Activity of the enzyme MPO in synovia was assessed with a new MPO activity assay developed at the Institute of Veterinary Biochemistry of the Faculty of Veterinary Medicine of the Freie Universität Berlin (FIETZ et al. 2004). All measurements presented here were performed by Simone Fietz or coworkers of that institute and kindly provided based on a current scientific collaboration.

The activity of MPO in the present work was assessed with a kinetic study performed with an enzyme activity assay based on the peroxidative activity of MPO on o-diananisidine and utilizing the inhibitor 4-Aminobenzoic acid hydrazide (ABAH) in the cell-free supernatant of synovia (FIETZ et al. 2004). The activity of MPO was expressed in mU/µl (FIETZ, S.- personal communication from November 2005).

‡ Automated Cell Coulter T 840 - Bedfordshire, United Kingdom. The coulter principle is based on the property of cells to behave as isolators. Cells are classified according to their number and size. In the coulter, an electrical tension exists in an opening. Cells shortly interrupt this electrical tension as they are made to pass through this narrow opening. Electrodes placed at both sides of this opening register the difference in electrical tension produced by the passage of cells and send this information to the central computer where it is analyzed and interpreted. The size of the cells is calculated in femtoliters (fl) and only cells with a size greater than 35 fl are considered as leukocytes. Three different measuring periods are automatically carried out by the coulter for each sample. At least two of these three values must coincide. Otherwise, no valid value is reported. This is the case when these individual values greatly varied between each other or when the total actual amount of leucocytes in the samples is below the background threshold (200 cells/µl). The latest possibility was assumed as more likely in the cases where no valid leukocyte number was obtained. For these samples, an arbitrary value of 100 cells/µl was assumed for the effects of the statistical analysis of the current work.

4.2.6. Statistical analysis

Data for synovial fluid parameters were analyzed using the statistical program SPSS 12 for Windows. Unless otherwise indicated, metrics were analyzed, processed and presented as the arithmetical mean value ± standard deviation (S. D.), median, maximum, minimum and 25^{th} and 75^{th} percentiles for each group.

Each group consisted of different types of joints. On occasions, one joint per horse was punctuated while sometimes synovia was obtained from two joints of the same animal. Each individual sample was considered as an independent statistical unit, whether or not the samples were obtained from the same horse or from different horses.

Graphic representation of synovial results for pH, total protein, total white blood cell count and MPO-Activity were performed in the form of box-plots for groups with 6 or more observations. For groups with 5 or less observations, a dispersion diagram was preferred and values were jittered (8%). In a box plot graphic representation, the box stretches from the lower hinge (25^{th} percentile) to the upper hinge (75^{th} percentile). The median is shown as a line across the box. Hence, ¼ of the distribution of the observations is between this line and the top of the box and ¼ of the distribution of the observations is between this line and the bottom of the box. Arithmetical mean values are not shown in the graphics of the present work. The "H-spread" is defined as the difference between the hinges and a "step" is defined as 1.5 times the H-spread. Inner fences are one step beyond the hinges. Outer fences are 2 steps beyond the hinges. The largest value below the upper inner fence and the smaller value above the lower inner fence are the two adjacent values. A line connecting the upper adjacent value is drawn. Another line is drawn from the lower hinge to the lower adjacent value. Scores located between the inner and outer fences are indicated by an "o". Any score located beyond the outer fences are indicated by an "✶".

pH, total protein concentration and total white blood cell count of each sample were graphically represented with the correspondent activity of MPO with a dispersion diagram to assess the relationship between these variables. The MPO-activity and the synovial viscosity where graphically represented with a box plot diagram in order to assess the relationship between these two variables.

The results expressed in the present work are to be interpreted as preliminary explorative data and may not be representative samples of the whole equine population.