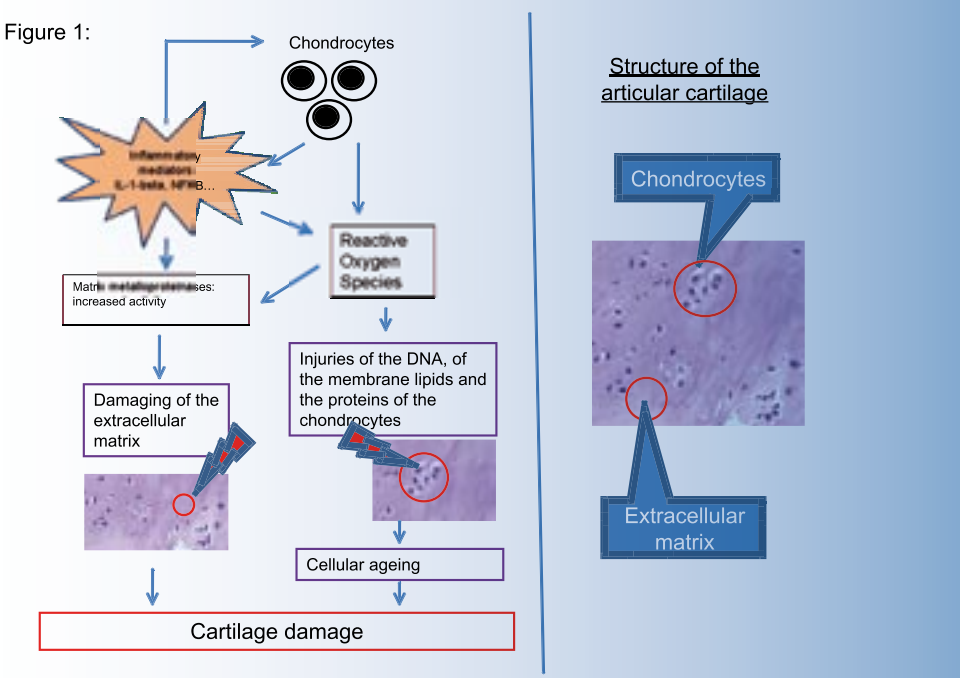
EFFECTS OF DIETARY EXTRACTS ON CHONDROCYTE PROLIFERATION, A SPRINGBOARD TO TISSUE RECONSTRUCTION

Marie Daix[1], Brieuc de Moffarts[2], Samuel Schulsse[1], Nathalie Kirschvink[1]
([1] Animal Physiology, Department of Veterinary Medicine, University of Namur, Belgium) ([2] Pavesco AG-TWYDIL®, Switzerland)

Figure 1:



INTRODUCTION

Osteoarticular pathologies represent a major cause of lameness in the equine species. They are also the first cause of performance regression. These diseases are characterised by signs of lesions on the articular cartilage of horses. The lesions can affect the chondrocytes (unique cells in the cartilage) or the extracellular matrix. This extracellular matrix is a supporting structure produced by the chondrocytes in order to furnish the cartilage with the resistance properties needed to absorb shocks occurring during locomotion. The pathologic process that leads to cartilage lesions is complex (Fig. 1). Many factors are accordingly involved in this destruction of the cartilage. The major ones seem to be oxidative stress, enzymatic stress and inflammatory mediators; their mutual influence leads to a serious cause of lesions of the cartilage.

Oxidative stress is defined as excessive accumulation of free radicals following oxygen metabolism. The production of free radicals then exceeds the defence capacities of the metabolism, which ends up in damage to the cartilage cells [1] and particularly in inducing a cellular ageing that can cause osteoarthritis [2]. It also appears that the reactive oxygen species (ROS) are involved in the triggering of the cascade leading to inflammatory reaction and to enzymatic stress [1].

Actually inflammatory reaction amplifies the process and the increasing cascade leads to a higher activity of the proteinases (notably the matrix metalloproteinases) which damage the extracellular

matrix around the chondrocytes [3]. This phenomenon is called "enzymatic stress".

The result of this chain of occurrences is a weakening of the cells and of the extracellular matrix in the cartilage, which compromises its principal functions: mobility and absorption.

The common treatment of articular troubles is based on the administration of steroid or non-steroid anti-inflammatory drugs which enable a "breaking" of this chain reaction. These drugs can hardly be used in a context of competition since they produce a doping effect, and moreover, they can induce significant side effects.

Another important aspect is the reconstruction of the cartilage after the pathologic stage. The cartilage extracellular matrix produced by the chondrocytes must restore itself on the injured spots, which may take some time, particularly when the chondrocytes have been affected by cellular damage. This applies to most tissues, i.e. tissue repair or remodelling is crucial for a good restoration of the functions of the affected organ. Every tissue experiences daily aggression and injuries, and its cells are exposed to different weakening factors. This process would lead to a diminishing functional efficiency if tissue remodelling did not balance it out. Thus, this repair enables a "regeneration" of the tissue and a balance between tissue damage and tissue reconstruction. Under pathologic conditions, inflammatory reaction, oxidative stress, and very often enzymatic stress disturb this balance, which may possibly lead to excessive tissue damage.

In this regard, nutraceuticals or functional foods, which are therapeutic or preventative dietary supplements, have a threefold use: they possess an anti-inflammatory effect with minimal side effects on the one hand; on the other hand, few of them considered to be doping substances, and finally, since some of them are constituents of the extracellular matrix, they are able to stimulate tissue respiration.

OBJECTIVES OF THE PRESENT RESEARCH

The aim of this research is to investigate the effect of dietary extracts on the proliferation of equine chondrocytes *in vitro*.

A sample of articular cartilage has been taken from horse limbs from a slaughterhouse. The fetlock articulations were opened and cartilage samples were taken using a scalpel; then they were cut into pieces and subjected to enzymatic digestion in order to isolate the chondrocytes.
The chondrocytes were then cultivated for 7 days in 12-well plates in a growth medium with or without (for control purposes) addition of dietary extracts based on glucosamine/chondroitin sulphate

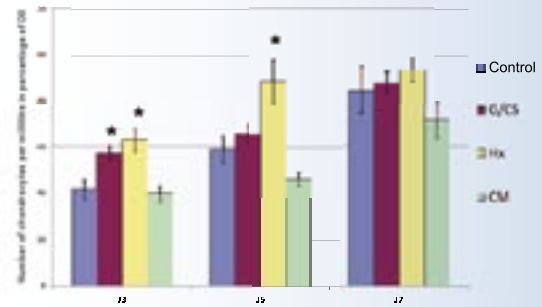
(G/CS), hydroxylapatite (Hx) or on a complex mineral (CM) made of carbonate, phosphate and calcium gluconate and sea salts. The chondrocyte proliferation was evaluated by counting the cells using trypan blue every second day. Each well was cultivated and measured in quadruplicate.

OUTCOME OF THE RESEARCH

The results of the cellular counts show that the Hx extract has a stimulating effect on the chondrocyte proliferation on the 3rd and the 5th day of cultivation (Fig. 2). Concerning the G/CS extract, it shows a stimulating effect on the 3rd day. By the 7th day, there is no further noticeable difference between the cultures with addi-

Figure 2:

Equine chondrocyte proliferation *in vitro* with different dietary extracts

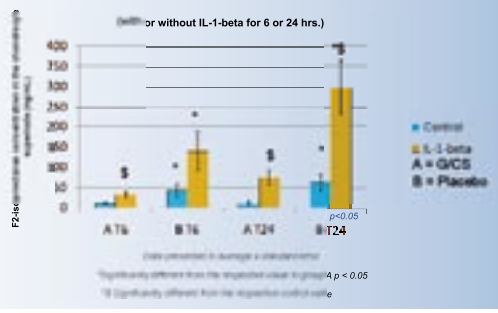


G/CS = glucosamine / chondroitin sulphate
Hx = Hydroxylapatite
CM = complex mineral

** significantly different from the control sample p<0.05*

Figure 3:

***ex vivo* F2-isoprostanes in the chondrocyte supernatant coming from horses first orally supplied with the feed complement**



tion of feed supplement and the control cultures. These very interesting results underline the possible effect of the extracts tested on tissue repair; an effect produced by stimulating proliferation of the chondrocytes.

Results from a previous research about chondrocyte cultures coming from horses that had been orally provided with feed supplements show that the C/CS complement induces a decrease of the F2-isoprotanes (markers of oxidative stress) in the chondrocyte supernate, and it remains so even after a 6 or 24-hour stimulation with 1-β interleukins, which was intended to simulate an *ex-vivo* inflammation of the joints (Fig. 3). Consequently, it seems that the complement given orally to the horses provides the chondrocytes with some protection against oxidative stress.

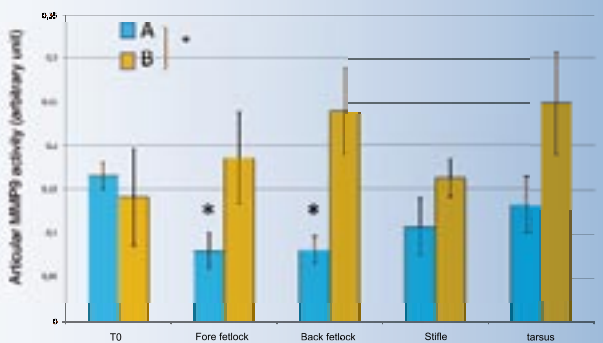
In addition, a modulating effect had been observed for the G/CS complement regarding its influence on oxidative stress after 6 weeks of oral use of this supplement [5]. Indeed, a decrease of the MMP9 activity (type 9 matrix metalloproteinase; Fig. 4; the MMP9 being the enzyme involved in the damaging of the cartilage) was observed together with a beginning increase of the MMP2 activity (type 2 matrix metalloproteinase; the MMP2 being the enzyme that is apparently more involved in the repair than in the destruction of the cartilage) [4]. This effect on the MMP2 has also been observed in a subsequent study *in vitro* during which the chondrocytes, which came from horses that had been orally supplied with the feed supplement, were cultivated and subjected to 1-β interleukins to provoke an inflammatory stimulation in them (Fig. 5).

Considering all these results, it seems that the G/CS supplement is able to modulate oxidative and enzymatic stress and to stimulate chondrocyte proliferation. Thus, this approach could help at two levels in the handling of articular pathologies: it could help decrease the cartilage destruction and stimulate its reconstruction.

It would be interesting also to study the effect of these extracts on the tendons. In fact, tendon damage is most frequent and accounts for severe lameness and for a more or less extended interruption of training. In cases of tendon damage, observation reveals that the difficult tissue repair is the biggest problem. Actually a tendon contains fewer cells than the extracellular matrix, while exactly the same cells deliver the constituents needed by the extracellular matrix composing most of the tendons. In many cases, tendon healing is long and the tendon never fully regains its original performance [6]. Accordingly it seems interesting to investigate the effect of the extracts on the test on tendinocyte proliferation. If the stimulation effect observed with the chondrocytes is confirmed for the tendinocytes, it would be a major outcome for the handling of tendinous regeneration.

Figure 4:

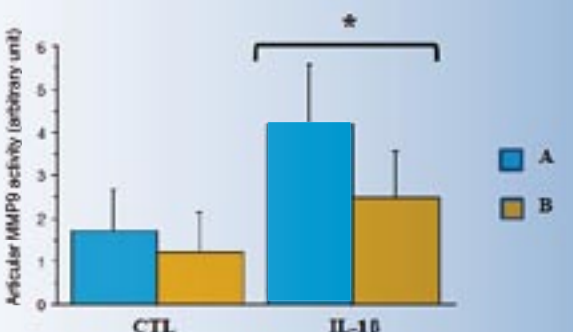
Articular MMP9 activity *in vivo* after 6 weeks oral supply



T0 = Before oral supply
T6= After 6 weeks oral supply
** Significant differences between group A and B, p<0.05*

Figure 5:

MMP-2 activity *ex vivo* in the chondrocyte supernate coming from horses orally supplied with the feed complement (with or without inflammatory stimulant (IL-1-ß))



CTL = control = without inflammatory stimulant IL-1-β = 1-beta interleukin = with inflammatory stimulant
** Significantly different p<0.05*

CONCLUSIONS AND PROSPECTS

The extracts tested here shows signs of a modulating effect on oxidative stress and on enzymatic stress. In addition, its apparent effect on chondrocyte proliferation could stand for a stimulation of the metabolism which is probably beneficial to the tissue respiration. The same effects have also been observed in tests of *ex-vivo* inflammatory stimulation, which suggests a preventative effect. Thus, not only are these molecules seen to be very promising in the management and the prevention of joint problems, but it would also be interesting to investigate their effects on tendons as well since tissue repair is the key to the horse's healing in cases of tendon damage.

REFERENCES

1. VALKO, M., LEIBFRITZ, D., MONCOL, J., CRONIN, M. T., MAZUR, M., and TELSER, J. (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int. J Biochem. Cell Biol. **39**, 44-84.

2. YUDOH, K., NGUYEN, T., NAKAMURA, H., HONGO-MASUKO, K., KATO, T., and NISHIOKA, K. (2005) Potential involvement of oxidative stress in cartilage senescence and development of osteoarthritis: oxidative stress induces chondrocyte telomere instability and downregulation of chondrocyte function. Arthritis Res. Ther. **7**, R380-R391.

3. NAGASE, H., VISSE, R., and MURPHY, G. (2006) Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc. Res. **69**, 562-573.

4. GEPSTEIN, A, SHAPIRO, S, ARBEL, G, LAHAT, N, and LIVNE, E. (2002) Expression of matrix metalloproteinases in articular cartilage of temporomandibular and knee joints of mice during growth, maturation, and aging. Arthritis & Rheumatism **46**, 3240-3250.

5. DAIX , M., BASTIN, JF. and KIRSCHVINK, N., Evaluation of an oral supplement enriched with glucosamine and chondroïtine sulphate on the joint enzymatic balance in young horses.(2006/2007) HPH High Performance Horses, 4-8.

6. RICHARDSON, L.E., DUDHIA, J., CLEGG, P.D. and SMITH R. (2007). Stem cells in veterinary medicine - attempts at regenerating equine tendon after injury. Trends in Biotechnology, **25**, 409-416.