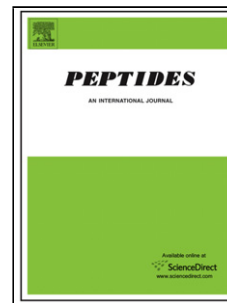


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THE GHRELIN PARADOX IN THE CONTROL OF EQUINE CHONDROCYTE FUNCTION: THE GOOD AND THE BAD.

Serena Ceriotti¹, Anna Lange Consiglio², Lavinia Casati³, Fausto Cremonesi^{2,4}, Valeria Sibilìa^{3*},
Francesco Ferrucci¹

¹ Department of Health, Animal Science and Food Safety, School of Veterinary Medicine, Università degli Studi di Milano, Italy

² Reproduction Unit, Large Animal Veterinary Hospital (Lodi), Università degli Studi di Milano, Italy

³ Department of Medical Biotechnology and Translational Medicine, Università degli Studi di Milano, Italy

⁴ Department of Veterinary Medicine, Università degli Studi di Milano, Italy

*Corresponding author: Valeria Sibilìa PhD

HIGHLIGHTS:

- Ghrelin exerts a dual action on chondrocyte viability.
- Low ghrelin concentration reduced chondrocyte viability.
- 10^{-7} mol/L ghrelin protects against LPS-induced cytotoxicity.
- The protective effect of ghrelin involves GHS-R1a.

ABSTRACT

Increasing evidence suggests a role for ghrelin in the control of articular inflammatory diseases like osteoarthritis (OA). In the present study we examined the ability of ghrelin to counteract LPS-induced necrosis and apoptosis of chondrocytes and the involvement of GH secretagogue receptor (GHS-R)1a in the protective action of ghrelin.

The effects of ghrelin (10^{-7} – 10^{-11} mol/L) on equine primary cultured chondrocytes viability and necrosis in basal conditions and under LPS treatment (100 ng/ml) were detected by using both acridine orange/propidium iodide staining and annexin-5/propidium iodide staining. The presence of GHS-R1a on chondrocytes was detected by Western Blot. The involvement of the GHS-R1a in the ghrelin effect against LPS-induced cytotoxicity was examined by pretreating chondrocytes with D-Lys3-GHRP-6, a specific GHS-R1a antagonist, and by using des-acyl ghrelin (DAG, 10^{-7} and 10^{-9} mol/L) which did not recognize the GHS-R 1a.

Low ghrelin concentrations reduced chondrocyte viability whereas 10^{-7} mol/L ghrelin protects against LPS-induced cellular damage. The protective effect of ghrelin depends on the interaction with the GHS-R1a since it is significantly reduced by D-Lys3-GHRP-6. The negative action of ghrelin involves caspase activation and could be due to an interaction with a GHS-R type different from the GHS-R1a recognized by both low ghrelin concentrations and DAG. DAG, in fact, induces a dose-dependent decrease in chondrocyte viability and exacerbates LPS-induced damage.

These data indicate that ghrelin protects chondrocytes against LPS-induced damage via interaction with GHS-R1a and suggest the potential utility of local GHS-R1a agonist administration to treat articular inflammatory diseases such as OA.

1. Introduction

Osteoarthritis (OA) is a chronic debilitating musculoskeletal disease characterized by progressive loss of joint function leading to pain, loss of mobility and functional limitations. The osteoarthritic joint is characterized by degeneration and loss of cartilage, synovial inflammation and sclerosis of peri-articular bone without an appropriate healing response [1] [2]. Chondrocytes are the only cellular elements of the cartilage and they are responsible for extracellular matrix homeostasis [3] [4]. Inappropriate mechanical joint stress damages matrix integrity and affects chondrocyte metabolism, reducing anabolic capacity to replace healthy matrix components, increasing the release of pro-inflammatory cytokines (IL-1 β , TNF- α), and stimulating apoptosis and necrosis [5]. Inflammatory mediators induce up-regulation of catabolic enzymes gene expression (matrix metalloproteinases, MMPs), facilitating cartilage matrix destruction [6]. Thus, the identification of new compounds capable to prevent chondrocyte apoptosis and inflammatory reaction could represent an interesting goal to treat OA.

Ghrelin, the natural ligand for the growth hormone secretagogue receptor (GHSR) [7], was proposed to have a role in controlling immunity and inflammation. Ghrelin and its target receptors are expressed by leukocytes such as neutrophils, lymphocytes, and macrophages [8]. Furthermore, ghrelin not only inhibits proinflammatory cytokines production such as TNF- α and IL-1 β [9] [10], but also constitutes a protective factor against the cytotoxic effects of TNF- α in adipocytes [11], LPS in hepatocytes [12] or oxidative stress both in cardiomyocytes [13] and osteoblasts [14]. Previous *in vivo* studies showed that ghrelin administration prevents the development of hyperalgesia and oedema induced by carrageenan in rats [15], it is able to reduce mRNA expression

of inducible forms of both nitric oxide and cyclooxygenase synthase activated by a noxious stimulus in the gastric mucosa [16] and reduce the duration of post-surgical early inflammatory response in man [17].

Interest into the possible use of ghrelin in the treatment of inflammatory diseases such as OA has also recently emerged. In patients with primary OA, synovial fluid ghrelin levels are decreased, being negatively related to inflammatory markers TNF- α and IL-6 and also inversely correlated with OA clinical severity [18]. Similarly, ghrelin levels demonstrated an independent and negative association with meniscus injury, cartilage damage, and clinical severity in patients with anterior cruciate ligament deficiency, suggesting that ghrelin might serve as a potential cartilage protective factor for post-traumatic OA [19]. In fact, in addition to immunomodulatory and anti-inflammatory properties, ghrelin is able to influence both bone cells and chondrocytes metabolism.

It has been reported that osteoblastic cells express ghrelin receptors and ghrelin stimulates bone formation, by inducing osteoblast proliferation, differentiation with enhanced deposition and mineralization of bone matrix [20] [21]. As far as the effects of ghrelin on chondrocytes are concerned, ghrelin mRNA is expressed in human, rat and mouse chondrocytes. Furthermore, ghrelin regulates chondrocyte metabolism and seems to modulate the synthesis of eicosanoids in the cartilage [22].

More recently, in 2009, Gomez and colleagues reported the presence of ghrelin *O*-acyltransferase (GOAT), the enzyme required for the *n*-octanoyl modification of ghrelin in both murine and human chondrocytes [23]. This chemical modification is necessary for ghrelin binding to the GHS-R1a as des-acyl ghrelin (DAG) is a weak ligand of this GHSR. On the other hand, both ghrelin and DAG have been shown to bind to GHS-R1a independent common sites, mediating similar signals to inhibit cellular apoptosis or inflammatory pain [24] [14] [25]. Thus, in most cases, anti-inflammatory and protective effect of des-acyl ghrelin remains debated.

In the present study, we examined, *in vitro*, the effects of increasing ghrelin concentrations on chondrocyte viability, necrosis and apoptosis in basal conditions and the possible ability of ghrelin

to counteract chondrocyte necrosis and apoptosis induced by administration of LPS, as a model of inflammatory stress. According to the results obtained, we studied the involvement of caspase activation in the detrimental effects of low ghrelin concentrations on basal cell viability and the involvement of GHS-R1a in the effect of the highest ghrelin concentration used (10^{-7} mol/L) against LPS cytotoxicity. The presence of GHS-R1a in cultured chondrocytes was investigated by Western Blot analysis. Then, we evaluated its involvement in ghrelin protective action on LPS-challenged chondrocytes, by using D-Lys3-GHRP-6, a specific GHS-R1a antagonist. Eventually, to investigate possible involvement of GHSR types other than GHS-R1a, we examined the effects of DAG, which does not bind the GHS-R1a, on basal chondrocyte viability and LPS-induced apoptosis/necrosis. All the experiments were performed on equine primary cultured chondrocytes.

2. Materials and methods

2.1 Drugs

Ghrelin and DAG were synthesized by conventional solid phase synthesis and purified to at least 98% purity by HPLC by Neosystem (Strasbourg, France). D-Lys3-GHRP-6 was purchased from Bachem AG (Budendorf, Switzerland). All compounds were dissolved in the culture medium, carefully preserving sterile conditions. *E. Coli* LPS (serotype 0111:B4) was purchased from Sigma (St Louis, CA, USA). The pan-caspase inhibitor carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethyl-ketone (Z-VAD-FMK, InvivoGen, Roma, Italy) was solved in dimethyl sulfoxide.

2.2 Cartilage sources

A total number of 6 joints from 6 different adult horses slaughtered for purposes other than the research were used as cartilage source. The study was approved by the animal ethical committee of the University of Milano and carried out in accordance with national ethical guidelines. As previously reported [26-28], cartilage samples were collected from load-bearing joints (metacarpophalangeal joints), according to sterile surgical procedures. Briefly, a transversal incision was made along the dorsal aspect of the fetlock, involving the skin, the *subcutis*, the common

extensor tendon and the joint capsule. The articular surfaces of third metacarpal bone, first phalangeal bone and sesamoid bones were exposed by dislocating the joint and resecting joint collateral ligaments. The articular surfaces were scraped to remove the most superficial layer of the articular cartilage, then rinsed with saline (NaCl 0.9%) and polyphosphate buffered saline (PBS, Euroclone, Milano, Italy) to remove residual synovial fluid. Cartilage fragments of about 1 x 1 cm² were sliced from the subchondral bone, deposited in a sterile plate and covered with sterile PBS solution. The total amount of cartilage collected from each joint varied from 3.5 to 5 g.

2.3 Chondrocyte culture

Cartilage specimens were finely chopped, transferred to a flask, and rinsed with PBS by centrifugation at 250 x g for 5 minutes, at room temperature. The exceeding PBS solution was removed and 10 ml of digestive medium/g of cartilage were added. The digestive medium was composed by minimal essential medium (Euroclone, Milano, Italy), 1 mg/ml collagenase, 20 µg/ml DNase, 5 mmol/L CaCl₂ (Sigma-Aldrich, Milano, Italy), 0.25 µg/mL amphotericin, 100 UI/mL penicillin-100 µg/mL streptomycin (Euroclone, Milano, Italy). Digestion was obtained by overnight incubation (16 hours) at 38 °C and the chondrocyte suspension was filtered with an 80 µm filter (Merck Millipore, Milano, Italy), added with PBS solution and centrifuged at 250 x g for 10 minutes. Cells were cultured in a high glucose Dulbecco's modified Eagle medium (Euroclone, Milano, Italy) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% glutamine, 1% insulin-transferrin-selenium and 10 ng/ml of epidermal growth factor (Sigma-Aldrich, Milano, Italy). Passage 0 (P0) chondrocytes were seeded into tissue culture plates at 500 000 cells/1 ml-well to allow cells to adhere to the plates and to proliferate as a monolayer culture. Cultures were maintained until passage 3 (P3) and chondrocytes were characterized throughout the passages with microscopic evaluation (Olympus BX51 microscope) and RT-PCR analysis. For each passage (P1, P2, P3), shares of more than 1.000.000 cells were cryopreserved in liquid nitrogen. All experiments were performed on P1 chondrocytes, before confluence occurs, to minimize risk of dedifferentiation [29]. On the day of experiments, P1 shares were thawed, mixed as "pool from

different animals”, seeded into tissue culture plates at 30.000 cells/1ml-well (24 well-plates) and maintained in the culture medium.

2.4 Viability and apoptosis assay

Cell viability and apoptosis were assessed using a combination of acridine orange (AO) and propidium iodide (PI) staining. Briefly, reagent solutions were prepared by solving 3 mg of PI in 1 ml of absolute ethanol and 5 mg of AO in 1 ml of ethanol and stored at 4 °C. Working solution was obtained by mixing 1 µl of AO solution, 1 µl of PI solution and 1 ml PBS. After dilution of cells with working solution, the examination under fluorescence light microscopy (Olympus BX51 microscope) at a magnification of 40x was performed immediately. AO dye was excited at 460 nm while the emission wavelength was set at 650 nm. PI was excited at 535 nm while the emission wavelength was set at 617 nm. According to literature [30], cell staining and morphology were considered to define viable cells, apoptotic cells and necrotic cells.

The Annexin V-fluorescein isothiocyanate (FITC) and PI apoptosis detection kit (Santa-Cruz Biothecnology, Inc.) was used to detect the early and late apoptotic cells. According to the manufacturer’s instructions, the cells on culture plates were collected and subsequently washed with pre-cold PBS for 3 times. The pellets were resuspended in 1 x Assay Buffer at a concentration of 1×10^6 cells /ml. 100 µl of cell sample was bathed in 0.5 µg/ml Annexin V FITC and in 0.5 µg/ml PI solution for 15 min, and then rinsed with PBS. The samples were analysed by fluorescence microscopy using a dual filter set for FICTC and Rhodamine. The fluorescent images of cells in different groups were captured and examined under fluorescence light microscopy (Olympus BX51 microscope) at a magnification of 40x. Double negative cells were defined as normal viable, Annexin V+/PI- as early apoptotic, Annexin V+/PI+ cells in later stages of apoptosis and Annexin V-/PI+ as necrotic ones.

2.5 RNA extraction and RT-PCR analysis

Total chondrocyte RNA was extracted using total RNA mini Kit, according to manufacturer's protocol (Biorad, Milano, Italy). RNA concentration was measured using a Nanodrop Spectrophotometer (ThermoFisher Scientific, Milano, Italy). Total RNA was retrotranscribed using iScript RT supermix (Biorad, Milano, Italy), according to manufacturer's protocol. For each sample, 240 ng were retrotranscribed. To characterize chondrocytes, the aggrecan (ACAN) and collagen type II-a1 (COL2A1) gene expression was investigated using specific primers designed for *Equus caballus* [31]. As house-keeping gene, GAPDH was used. PCR was carried out on 50 ng of cDNA using Zymo Taq Premix PCR kit for 40 cycles. The PCR products were resolved on 2% agarose gel.

2.5 Western Blot Method

Cells were collected in protein buffer with protease inhibitors (NaCl 150 mmol/L, Tris-HCl 50 mmol/L, 1% Triton X-100 and COMPLETE, Roche, Milano). Lysates were centrifuged at 10,000 g for 10 min at 4 °C and protein concentration were determined with the BCA assay. The extracts were electrophoresed on 15% SDS/PAGE. As positive control a protein extract from HUVEC (human endothelial cells) was used [32]. All protein gels were electro-transferred to 0.2 µm pore nitrocellulose membranes by wet procedure (1 h for 350 constant mA and 30 min 400 constant mA, room temperature). After transfer, membranes were systematically stained with Ponceau S dye to verify the correct loading and transfer quality of proteins. Membranes were blocked and then probed overnight at 4° C with the primary specific antibodies and processed, as described by the manufacturer, with fluorescent secondary antibodies (WesternDot™ 625 from Western Blot Kits, Thermo-Fisher Scientific, Milano). Fluorescent signals were detected using a VERSADOC system (Bio-Rad Italia, Milano, Italy, see reference [33]). The primary antibody employed for Western was a monoclonal antibody against GHS-R1a mAb isolated and characterized as previously reported [34].

2.7 Experimental procedure

We initially assessed ghrelin effects on basal chondrocyte viability and apoptosis. For this purpose, chondrocytes were pretreated with increasing ghrelin (10^{-11} - 10^{-7} mol/L) concentrations, 24 hours before viability and apoptosis assessment.

To study whether or not the detrimental effect of 10^{-11} mol/L ghrelin involves an activation of caspase pathway, we pretreated cells (24 h before) with an irreversible broad-spectrum caspase inhibitor (Z-VAD-FMK, 50 μ mol/L) that has been extensively used in programmed cell death inhibition studies [35]. Cell viability and apoptosis were evaluated 24 h after ghrelin treatment.

On the basis of the results obtained in basal conditions, we choose ghrelin 10^{-7} mol/L and 10^{-11} mol/L to study the effects of ghrelin in conditions of LPS-induced inflammation. LPS challenge (100 ng/ml) was induced 24 hours after ghrelin treatment on the basis of previous reports [36] and preliminary challenge studies (data not shown) showing that, at this concentration, LPS was able to induce a reproducible and reversible inflammatory stress. Cell viability and apoptosis were evaluated 24 hours after LPS challenge. To characterize the ghrelin receptor involved in the protective action of ghrelin against LPS-induced cytotoxicity, we treated cells 1) with D-Lys3-GHRP-6 (10^{-6} mol/L), a specific GHS-R1a antagonist, together with ghrelin and 2) with DAG, the unacylated ghrelin form that does not bind GHS-R1a. D-Lys3-GHRP-6 was added 30 minutes before ghrelin (10^{-7} mol/L). The effects increasing DAG concentrations (10^{-9} and 10^{-7} mol/L) on chondrocyte viability and apoptosis were assessed at basal conditions and under LPS-induced inflammatory. In all the experiments, non-treated chondrocytes were used as control group. In LPS challenge experiments, chondrocytes treated only with LPS were also maintained.

The presence of GHS-R1a was detected by Western Blot analysis on P1-P3 passages on chondrocytes primary cultures.

2. 8 Statistical analysis

Statistical analysis was performed using the statistics package GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). All experiments were replicated at least four times. In each experiment, for every experimental condition (control or treated), populations of viable, apoptotic and necrotic chondrocytes were counted and expressed as relative percentages. Mean values and SEM of the four repetitions were calculated and compared among different experimental conditions using one-way ANOVA with Bonferroni post-hoc test or Student *t* test, when appropriate. Level of statistical significance was set for $p < 0.05$.

3. Results

3.1 Chondrocyte characterization

Chondrocytes were characterized using morphological evaluation and RT-PCR analysis. Characterization was made after isolation (P0) and for every culture passage (P1, P2, P3).

At microscopic evaluation, cultured cells showed a rounded morphology and a moderately slow proliferation rate, that are typical features of chondrocyte-like cells. Typical chondrocyte phenotype was preserved throughout culture passages (Fig. 1A and 1B). Furthermore, RT-PCR showed correspondence between the bands obtained and the amplicon length expected for ACAN (175bp), COL2A1 (201 bp) and GAPDH (178 bp), for all cell culture passages (Fig. 1C).

3.2 Effects of ghrelin on basal viability and apoptosis

Figure 2A shows an example of cell viability and apoptosis assessment with combined AO/PI staining. Under fluorescence microscopy, viable cells showed normal nuclei staining with green chromatin; apoptotic cells showed condensed or fragmented chromatin (green or orange) and budding of cell membrane; necrotic cells had similar normal nuclei staining as viable cells except the chromatin was orange or red instead of green. As shown in Fig. 2 B ghrelin exerts different effects on basal viability, apoptosis and necrosis depending on the concentrations used. At the highest concentration (10^{-7} mol/L) used, the effects of ghrelin were comparable to those detected in

control chondrocytes, whereas at low concentrations, ghrelin exerted a significant detrimental effect on chondrocytes that reached a maximum action at 10^{-11} mol/L.

The cytotoxic action of 10^{-11} mol/L ghrelin, on basal chondrocytes was confirmed by the results obtained with Annexin V apoptosis assay. As shown in Fig. 3, ghrelin significantly increased both apoptotic and necrotic cells as compared with control group.

We then examined the involvement of caspase activation in ghrelin negative effects on chondrocytes by testing the ability of a pan-caspase inhibitor (Z-VAD FMK) to prevent ghrelin action. As shown in Fig. 4 Z-VAD FMK, per se, did not significantly modify cell viability, apoptosis and necrosis, but significantly reduced ghrelin-induced cytotoxicity (Fig. 4)

Both a low (10^{-11} mol/L) and a high (10^{-7} mol/L) concentrations were chosen for further experiments.

3.3 Ghrelin effects on LPS- induced cytotoxicity on chondrocytes

The effects of different ghrelin concentrations on chondrocytes exposed to LPS treatment are shown in Fig. 5. As expected, LPS treatment significantly decreased chondrocyte viability, and increased both apoptotic and necrotic cell percentages. Pre-treatment with 10^{-7} mol/L ghrelin prevented the detrimental effects of LPS, since under ghrelin treatment, cell viability, apoptosis and necrosis did not significantly differ from those detected in controls. However, pre-treatment with 10^{-11} M ghrelin induced a significant increase of LPS-induced cytotoxicity.

3.4 GHS-R1a involvement in ghrelin protective effect on chondrocytes

We first studied the presence of GHS-R1a on chondrocytes by performing Western Blotting analysis. As shown in Fig. 6, we have detected GHS-R1a protein expression for all cellular passages (P1-P3). In the upper part of the figure it has been reported the ponceau staining to verify the correct loading of the total protein, whereas in the lower part of the figure the GHS-R1a band is

clearly showed both in the positive control (HUVEC, lane 1) and in the chondrocyte extracts at the different culture passages (lane 2, 3 and 4).

Pre-treatment with D-Lys3-GHRP-6, a specific GHS-R1a antagonist, completely prevented the protective action of 10^{-7} mol/L ghrelin against LPS-induced chondrocytes cytotoxicity but failed to modify the negative effects of 10^{-11} mol/L ghrelin (Fig. 7). Overall these data indicate that GHS-R1a is involved in ghrelin protective action against LPS cytotoxicity and suggest the presence of a GHSR different from GHS-R1a that could mediate the pro-apoptotic action of ghrelin.

This assumption is in line with the results obtained with DAG, which does not bind the GHS-R1a. As shown in Fig. 8A, increasing DAG concentrations (10^{-7} and 10^{-9} mol/L) exert a significant reduction of basal chondrocyte viability and increase both necrosis and apoptosis with a maximum effect at the highest concentration used.

When we examined the effects of pre-treatment with DAG on chondrocytes treated with LPS, we found that DAG not only did not prevent LPS activity on chondrocyte viability and apoptosis, but at the highest concentration (10^{-7} mol/L) used significantly worsened LPS-induced cytotoxicity (Fig. 8 B).

4. Discussion

In the present study, the ghrelin effects on basal viability of equine *in vitro* cultured chondrocytes and the ghrelin ability to counteract chondrocyte necrosis and apoptosis induced by an inflammatory *stimulus*, such as LPS treatment, were examined. Furthermore, whether the GHS-R1a is involved in mediating ghrelin effects on chondrocytes was investigated.

Our results showed that ghrelin has a dual effect on equine chondrocyte viability either in basal condition or in the presence of LPS-induced cytotoxicity. Ghrelin, in fact, at 10^{-7} mol/L prevents both chondrocyte necrosis and apoptosis induced by LPS whereas at low concentrations (10^{-11} and 10^{-9} mol/M) increases necrosis and apoptosis in basal condition and worsened LPS-induced cellular damage.

Further studies will be required to clarify the molecular pathways involved in the detrimental effects of 10^{-11} mol/L ghrelin on chondrocytes. A common feature of apoptotic pathways is caspase activation [37] and previous studies have shown that caspase inhibitors are effective at limiting chondrocyte apoptosis in vitro [38] and preserve chondrocyte function in vivo [39].

We can suggest that the detrimental effects of ghrelin could be due to an activation of the caspase family members, since the general caspase inhibitor Z-VAD FMK prevents cell death induced by the peptide.

The protective effect exerted by ghrelin depends on the interaction with the GHS-R1a, since pre-treatment with D-Lys3-GHRP-6, a specific GHS-R1a antagonist, significantly prevented ghrelin beneficial effects on LPS-induced cytotoxicity. It is possible that the negative effects of ghrelin on chondrocyte viability could be due to an interaction of the peptide with a GHSR type or sub-type, different from the GHS-R1a. Indeed, it has been reported the presence of both low and high affinity receptors for ghrelin on rat and human chondrocytes which appear to be distinct from GHS-R1a [22]. The ghrelin receptor involved in the negative action of ghrelin is recognized both by ghrelin at low concentration and by DAG. This assumption is supported by the present data showing that D-Lys3-GHRP-6 did not prevent the negative effects of 10^{-11} mol/L ghrelin and by the evidence that DAG, which did not bind to GHS-R1a, shares with low ghrelin concentrations a toxic action on basal chondrocyte viability exacerbates LPS-induced inflammatory damage.

Our results are in agreement with previous studies suggesting the existence of different GHSR types or subtypes, yet unidentified, with differential affinities for the peptides that could account for the different actions of ghrelin on viability and apoptosis depending on the cell type used [40].

DAG, in fact, did not displace the binding of radiolabeled ghrelin in the hypothalamus and pituitary, and had no endocrine activities in humans [7]. However, both ghrelin and DAG recognize a common binding site on H9C2 cardiomyocytes which does not express GHS-R1a [24]. Moreover, it has been reported that DAG shares with ghrelin a stimulatory action on MC3T3E1 cell viability

[14] and bone formation via mitogen-activated protein kinase (MAPK)/phosphoinositide 3-kinase (PI3K) pathways in the absence of GHS-R1a [21, 41-43].

Recent evidence has suggested a role for ghrelin in the control of chondrocyte activity and cartilage metabolism. Ghrelin, in fact, is physiologically expressed in the growth plate where it is involved in cartilage maturation and hypertrophy, clearing the way for osteoblastic proliferation and bone formation [22]. As showed by Gomez et al. [23], growth plate cartilage concentration of ghrelin is correlated to GOAT expression by chondrocytes, suggesting that, high ghrelin levels could promote chondrocyte hypertrophy and metabolic activity, through a GHS-R1a-mediated pathway. Ghrelin is able to increase in a dose dependent manner the chondrocyte cAMP production, with a related increase of matrix components such as hyaluronate and sulfate proteoglycan synthesis [22]. During cartilage degeneration, GOAT expression is likely to decrease, leading to lower ghrelin and higher DAG concentrations, thus favoring the effects mediated by GHSR other than 1a and resulting in chondrocyte apoptosis and, at the same time, osteoblast invasion and proliferation.

Our *in vitro* study showed, for the first time, that ghrelin is able to prevent chondrocyte apoptosis and necrosis induced by LPS challenge. It is well known that chondrocyte death represents an important component in the pathogenesis of OA and *in vitro* studies are considered the first step to investigate potential therapeutic compounds targeting cell death mechanisms [3, 44, 45]. We phenotypically differentiated whether cell death was due to apoptosis or necrosis. Both these events have been reported to occur in OA cartilage [44] and they are both significantly induced in our experiment by LPS administration. Although LPS-induced damage reproduces septic joint inflammation, it has been also extensively used for *in vitro* and *in vivo* models of chronic non-septic inflammation, especially for OA, as a preliminary assay to discriminate potential therapeutic compounds [26, 27, 36, 46-48]. Interestingly, serum and synovial fluid LPS levels have been recently correlated with the abundance of activated macrophages in the joint capsule and synovium and clinical severity of knee OA in humans [49]. In OA, an increase in chondrocyte apoptosis is primarily caused by sustained mechanical injury and associated degradation of articular cartilage

matrix, especially loss of glycosaminoglycans (GAG) and type II collagen denaturation [38, 50]. Usually, if any cell dies through apoptosis, the integrity of cytoplasmic membranes is maintained and dead cells are removed through phagocytosis. However, chondrocytes are isolated by an extracellular matrix and the terminal phase of apoptosis is not engulfed by phagocytes, therefore a transition to a secondary necrosis ensues, and cells are eliminated by disruption [51]. Chondrocyte secondary necrosis initiates non-infectious inflammatory responses through PRRs (pattern recognition receptors) expressed in the OA cartilage synovium, causing complement activation, increase in nitric oxide (NO), reactive oxygen species (ROS) and associated oxidative stress, impaired mitochondrial function and induction of MMPs and aggrecanes, with further enhancement of chondrocyte apoptosis and progression of OA. Therefore, in OA, apoptosis and secondary necrosis belong to the same inflammatory and degenerative process.

The present results indicate that ghrelin exerts a protective effects against chondrocyte LPS-induced both apoptosis and secondary necrosis. Further studies will be required to identify the intracellular mechanisms involved in such protective action. We can only postulate that ghrelin could inhibit mitogen-activated protein kinases/inducible NO synthases (MAPKs/iNOS) and promote protein kinases B/endothelial NO synthases (Akt/eNOS) pathways, directly counteracting LPS-induced apoptosis as previously reported in hepatocytes [12]. It is possible that the decrease in chondrocyte necrosis and apoptosis observed in this study may result, at least partly, from the overall reduction of chondrocyte oxidative stress, promoted by ghrelin administration. This hypothesis is in line with the data of Caminos and colleagues (2005), showing that ghrelin significantly down-regulate chondrocyte expression of several genes encoding for key enzymes of the RedOx cellular system. Ghrelin could prevent chondrocyte apoptosis also by counteracting cartilage matrix degradation and limiting the non-infectious inflammatory response. Ghrelin, in fact, down-regulate fatty acid uptake in chondrocytes thus limiting precursor availability and reducing prostaglandin and/or leukotrienes synthesis and enhances synthesis of matrix components such as sulfated proteoglycan and hyaluronate [22]. Consistent with these notions, recent clinical studies showed that decreased

synovial fluid ghrelin levels in knee joints of OA patients is directly related with the severity of the disease, indicating that local application of ghrelin could represent a potential therapeutic intervention for knee OA [18, 19].

In this study we showed that ghrelin acylation and interaction with GHS-R1a are essential to achieve its protective effect, since DAG have deleterious effects on chondrocytes viability and specific GHS-R1a antagonism results in a loss of ghrelin beneficial effect against LPS damage. The majority of previous studies investigated ghrelin anti-inflammatory properties without differentiating between ghrelin and DAG but considering both forms as equally regulated and realistically expressed by total ghrelin levels. However, it been shown that experimental systemic inflammation in rat results in a temporarily changed acyl/des-acyl ghrelin ratio, pointing towards the relevance of measuring both forms of ghrelin [52]. Further studies demonstrated that the acylation status affects the anti-inflammatory and anti-degenerative properties of ghrelin in some specific conditions, such as obesity-related inflammation and Parkinson's neurodegeneration [53].

Our *in vitro* model used equine primary chondrocytes and this is the first study that investigates ghrelin effects on cell viability and apoptosis in this species. The equine model represents a widely accepted and suitable animal model for OA, particularly to investigate new potential therapeutic options, for many reasons. Spontaneous OA frequently occurs in horses and the challenges and expectations that exist regarding early diagnosis and the development of effective treatments are similar to humans [54]. Therefore, a lot of studies describing physiopathology, molecular mechanisms and joint histopathology of naturally-occurring OA are performed using the equine model [55]. Equine and human joints share important anatomic similarities, since articular cartilage in the stifle of the horse is the most similar of any domestic species to the thickness of human knee cartilage and has similar cellular structure, biochemical makeup, and biomechanical properties [56-58]. Furthermore, some compounds, such as corticosteroids, carprofen and glucosamine used to treat OA have been tested on equine cultured chondrocytes [26, 27, 36, 59].

In the present study we showed for the first time the presence of the GHS-R1a in primary equine chondrocytes by Western Blot analysis. Previous studies, in fact, failed to identify the GHS-R1a in mouse and human chondrocytes cell lines [22]. This discrepancy could be to the different experimental conditions (binding studies versus Western Blot analysis) and/or to the use of immortalized cell lines versus primary chondrocyte cultures.

In conclusion, our study indicates that ghrelin exerts a dual effect on primary chondrocytes depending on the GHS-R activated by the peptide and that the protective action of ghrelin against LPS- cytotoxicity is mediated by its interaction with GHS-R1a.

Even if further in vivo studies are needed to test the ability of ghrelin to regulate chondrocyte function, these results suggest that the local application of selective GHS-R1a agonists could be considered as an attractive potential preventive/therapeutic agent in conditions of articular degenerative inflammatory diseases such as OA.

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6. Figures

FIGURE 1 Morphological characterization of equine chondrocytes at cell culture passages P0 (A) and P1 (B). Images were taken at phase contrast microscope at 20X magnification. Gene expression characterization for chondrocytes specific markers (collagen -*Col2a1*- and aggrecan -*Acan*-; C) The figure shows the correspondence between the bands obtained and the amplicon length expected for *Acan* (175bp), *Col2a1* (201 bp) and the reference gene *Gapdh* (178 bp) for all cell culture passages

considered. **The black arrow indicates the height corresponding to 200 bp. From the left, for each gene, each cell culture generation (from P0 to P3) was loaded into the gel. The first series of bands is referred to Acan (left), the second to Col2a1 (middle), the last series to Gapdh (right).** As a marker, a DNA ladder 100bp (Promega, Milan, Italy) was loaded twice. All loaded samples show the bands corresponding to the amplicon length.

FIGURE 2 Effects of increasing ghrelin concentrations on chondrocytes viability, apoptosis and necrosis in basal conditions. Cells were incubated with ghrelin for 24 hours and cell viability, apoptosis and necrosis were detected by Acridine Orange/ Propidium Iodide staining (AO/PI). Cell numbers were counted on fluorescence microscope (magnification 40X). **Panel A:** representative images of viable cell with preserved chromatin (green), necrotic (orange/red nuclei) and apoptotic cell (green fragmented chromatin and budding membrane). **Panel B:** quantification of viability, necrosis and apoptosis. Values are the means + SEM of two counting fields (100 cells/each) of four independent experiments. * $p < 0.05$; *** $p < 0.001$ vs control group.

FIGURE 3 Effects of 10^{-11} mol/L ghrelin on chondrocytes apoptosis and necrosis in basal conditions. Cells were incubated with ghrelin for 24 hours and cell apoptosis and necrosis were detected by Annexin V-FICT/ Propidium Iodide staining (AnV/PI). Cells in early apoptosis stain AnV⁺/PI⁻; cells in later stages of apoptosis stain AnV⁺/PI⁺; necrotic cells stain AnV⁻/PI⁺. Cell numbers were counted on fluorescence microscope (magnification 40X). Values are the means + SEM of two counting fields (100 cells/each) of four independent experiments. * $p < 0.05$; vs control group

FIGURE 4. Effects of pretreatment with the pan-caspase inhibitor (Z-VAD-FMK) on ghrelin-induced cytotoxicity on equine chondrocytes in basal conditions. Cells were pre-treated with Z-VAD-FMK, (50 μ mol/L) 24h before ghrelin (10^{-11} mol/L). Cell viability, necrosis and apoptosis were evaluated by AO/PI staining 24 hours after ghrelin. Cell numbers were counted on fluorescence microscope (magnification 40X) Values are the mean \pm SEM of two counting fields

(100 cells/each) of four independent experiments. * $p < 0.05$, *** $p < 0.001$ vs control group; ° $p < 0.05$, °° $p < 0.01$, vs ghrelin group

FIGURE 5 Effects of different ghrelin concentrations on LPS-induced cytotoxicity on equine chondrocytes. Cells were pre-treated with ghrelin 24 hours before LPS treatment (100 ng/ml). Cell viability, necrosis and apoptosis were evaluated by AO/PI staining 24 hours after LPS. Values are the mean \pm SEM of two counting fields (100 cells/each) of four independent experiments. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$ vs control group. °°° $p < 0.001$ vs LPS group.

FIGURE 6 Identification of the GHS-R1a on equine chondrocytes. GHSR1a protein expression was detected in P1, P2, P3 by WESTERN BLOTTING. HUVEC represents the positive control. The upper part shows the ponceau staining to verify the correct loading of the total protein. In the lower part the GHSR1a band is clearly showed both in the positive control (HUVEC, lane 1) and in the chondrocyte extracts at different culture passages (lane 2, 3 and 4).

FIGURE 7 Effects of pretreatment with the GHSR1a antagonist (D-Lys3-GHRP6) on the ghrelin effects on LPS-induced cytotoxicity on equine chondrocytes. Cells were pre-treated with D-Lys3-GHRP6 (10^{-6} mol/L, 30 min before) and ghrelin (10^{-11} and 10^{-7} mol/L) for 24 hours before LPS treatment (100 ng/ml). Cell viability, necrosis and apoptosis were evaluated by AO/PI staining 24 hours after LPS. Values are the mean \pm SEM of two counting fields (100 cells/each) of four independent experiments. ^a $p < 0.05$, ^b $p < 0.001$ vs control group; ^c $p < 0.05$, ^d $p < 0.01$, ^e $p < 0.001$ vs LPS group; ^f $p < 0.001$ vs ghrelin 10^{-7} M+LPS.

FIGURE 8 Effects of different DAG concentrations on chondrocytes viability, apoptosis and necrosis in basal conditions (**A**) and on LPS-induced cytotoxicity (**B**). Cells were pre-treated with DAG 24 hours before or not LPS treatment (100ng/ml). Cell viability, necrosis and apoptosis were evaluated by AO/PI staining 24 hours after LPS stimuli. Values are the mean \pm SEM of two

counting fields (100 cells/each) of four independent experiments. ** $p < 0.01$, *** $p < 0.001$ vs control group; $^{\circ\circ\circ}p < 0.001$ vs LPS group.

Fig.1

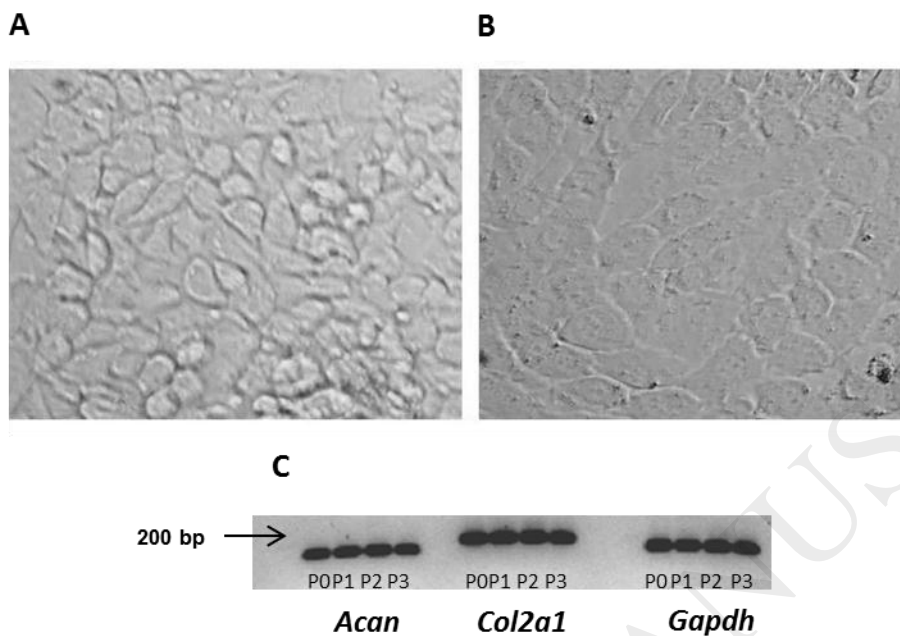
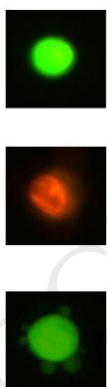


Fig.1

Panel A



Panel B

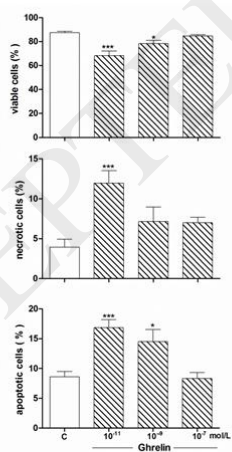


Fig. 3

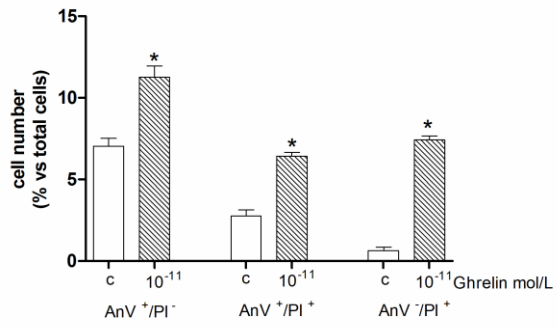


Fig. 4

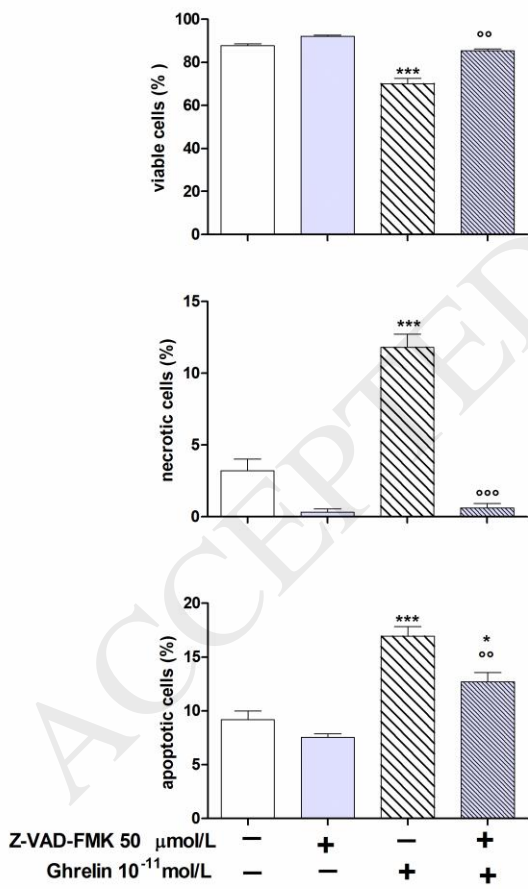


Fig.5

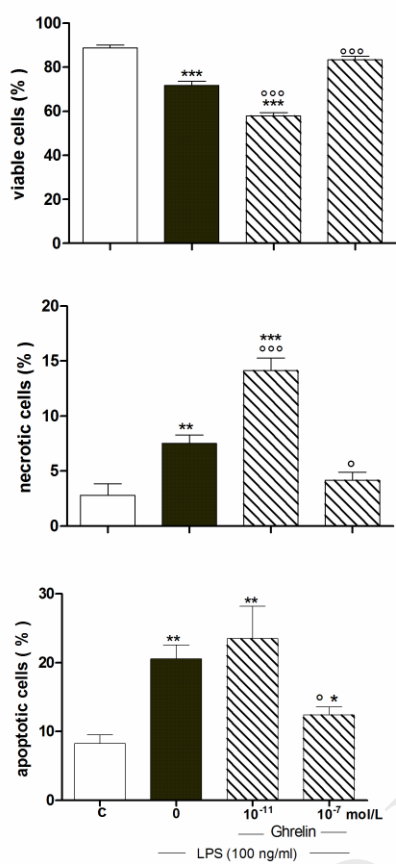


Fig. 6

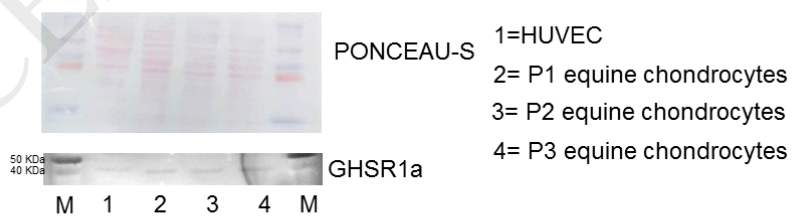


Fig. 7

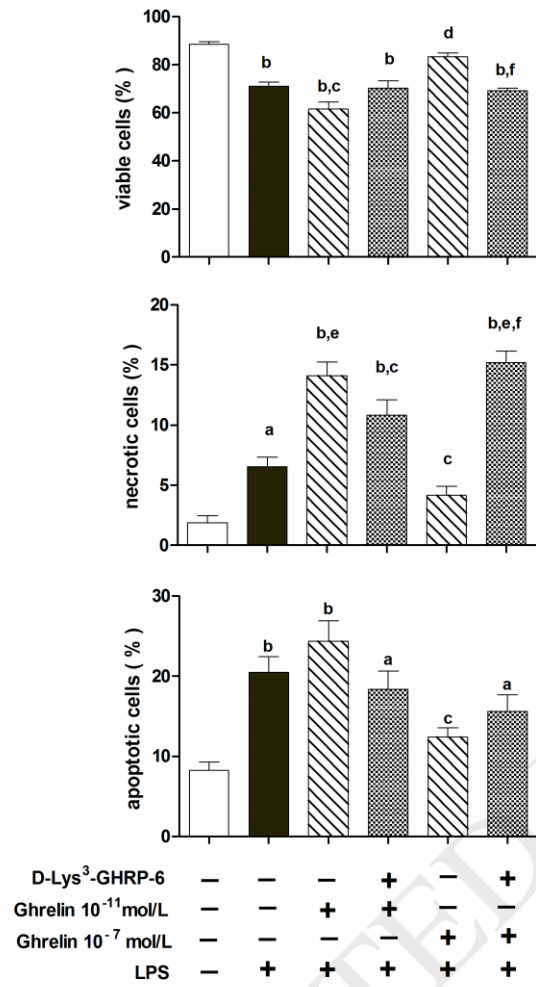


Fig.8

