Repair of experimentally induced femoral chondral defect in a rabbit model using Lyophilized growth promoting factor extracted from horse blood platelets (LGF<sub>equina</sub>)

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**A B S T R A C T**

Lyophilized equine platelet derived growth factors (LGF) is a novel advanced platelet rich protein growth factor. It has been successfully applied in various fields of regenerative medicine to treat a variety of inflammatory and degenerative musculoskeletal conditions. Our study aimed to evaluate the efficacy of intraarticularly injected LGF for the remedy of articular cartilage injury, commonly characterized by progressive pain and loss of joint function in osteoarthritic rabbits. Full-thickness cylindrical cartilage defects were generated in both femoral condylar articular surfaces in twenty rabbits. The left joint of all animals was injected with the adjuvant as a self-control negative, while the right joint was injected by LGF. Four- and eight-weeks post-surgery, the femoral condyles were harvested, and assessed grossly, microscopically and immunohistochemically. Cytokines (TNF-α, IL-1β, PDGF and TGF-β1) contents of the chondral defects were quantified by ELISA as well as the gene expression of Col I and Col II via RT-qPCR. The LGF treated defects showed significant higher ICRS (International cartilage repair society) healing scores of cartilaginous regeneration with a significant higher histological healing score on using O’Driscoll histological scoring system. Additionally, LGF significantly lowered the levels of the pro-inflammatory cytokines TNF-α and IL-1β. It also significantly increased the anabolic and angiogenic growth factors (PDGF and TGF-β1), and significantly elevated the expression of chondrogenic-related marker genes; Col I and Col II. The current study reveals that LGF improves chondral healing and thus it can be a superior nominee as an adjunctive therapy to positively influence regeneration of chondral defects in osteoarthritic patients.

**Impact statement**

Lyophilized growth factor extracted from horse blood platelets (LGF) is a novel advanced platelet rich protein growth factor, which is a leucocyte free and fibrinogen free product. It was found that the use of LGF could efficiently promote the healing of induced femoral chondral defect. LGF could significantly elevate the expression of chondrogenic-related marker genes; Col I and Col II, significantly increase the anabolic and angiogenic growth factors, and significantly lower the levels of the pro-inflammatory cytokines. LGF could be a superior nominee as an adjunctive therapy to positively influence regeneration of chondral defects in osteoarthritic patients.

**Introduction**

Development of reliable approaches for regeneration and repair of injured articular cartilage, considered as an important target in the field of orthopedic research. The articular cartilage lubricates and absorbs the shock at the ends of long bones developing diarthrodial joints. Its composition is mainly hyaline cartilage containing an abundant extracellular matrix and devoid of blood, lymphatics, and nerve supplies that contribute to reduce the capability of cartilage repair [1,2].

Osteochondral defects (OCD) are a common condition caused by severe trauma, sports injuries, or physical diseases, leading to joint pain, deformity, and dysfunction. Joint injuries caused by trauma and sports accidents often progress into osteoarthritis (OA). So, OCD are considered as a significant cause of OA [3]. The articular cartilage damage and degeneration is the main feature of OA, which its pathogenesis and progress still the key for resolving this
joint problem. OA development is accelerated through increasing the mechanical stress on the articular cartilage via subchondral bone sclerosis. The tissue reparative process produced after damage, being devoid of the biochemical capability to express certain cartilage-specific molecules that resulted in decreased biomechanical strength compared with normal hyaline cartilage [4,5].

The clinical signs and radiographic alterations resulted from the articular cartilage defects showed advanced worsening in the last years. Therefore, cartilage defect management and repair assumed to be important to prevent the progression to consequent OA. The cartilage defects that reach the subchondral bone, has vascular access, can enhance defect recovery. However, small defects can be improved with fibrocartilage tissue stemming from the subchondral locale but large defects recovery is relatively difficult [6].

Articular cartilage defects treatment should be inexpensive and prevent progression of OA in the long term [7]. Several techniques have been directed to defect restoration like: periosteal arthroplasty, perichondral arthroplasty, autologous osteochondral transplantation, autologous chondrocyte transplantation, cancellous grafts and tendon autografts that are aimed to procedure a new chondral surface [8,9].

Nowadays, many reports are developed on less invasive regenerative techniques as intra-articular injections of stem cells, which contributed to the effective articular cartilage regeneration in animal models [10,11].

Platelets rich plasma (PRP) has been used as a possible treatment adjuvant for many inflammatory and degenerative disorders. Recently, optimizing the delivery methods that assist the platelets to degranulate their biological ingredients slowly. These ingredients may promote healing and improve OA symptoms for a longer duration [12].

Different commercial systems are available for PRP preparations. Wide variations in the final platelets concentrate obtained due to the differences in platelet technical details related to the centrifugation method and time as well as the integrity and quality of the platelets may be damaged [13].

Several researches have attempted to develop novel strategies to overcome the disadvantages of PRP processing and increase the wound healing process, among those strategies is the Lyophilized Equine Platelets Growth Factors (LGF), a novel, advanced, standardized and refined form of platelets growth factors based on the use of allogenic pathogen free platelets from horses instead of autologous platelets as a source of growth factors.

LGF is an allogenic platelet releasate that contains all the growth factors found in the alpha granules of platelets. Upon stimulation of platelets found in a platelet rich plasma preparation, the resulting releasate contains supra-physiological doses of growth factors including but not limited to, TGF-β (transforming growth factor beta), PDGF (platelet derived growth factor), EGF (epidermal growth factor), VEGF (vascular endothelial growth factor), and FGF (fibroblast growth factor). Those growth factors can be subjected to qualitative and quantitative measurements. Quantitative measurement of TGF-β1 was carried out in the fresh platelets releasate of LGF, and it was found that, the average concentration of TGF-β1 was (3827± 693pg/ml) which is 2–3 times the resting baseline levels in plasma. However, quantitative measurements of the other growth factors are in progress.

Generally, LGF have a much longer shelf-life of the final lyophilized growth factors when compared to the autologous PRP (12–18 months versus 4–8 h or even less) [14,15]. Recent research proved that the lyophilized growth factor has a significant improvement of induced wound healing in dogs [16].

The growth factors across mammalian species have a homology in the structure and functions: as in case of fetal bovine serum which stimulate human and other mammalian cell proliferation and biological production, for the reason that it has more growth factors [17]. Canine PRP has been used in the treatment of a feline contaminated cutaneous wound successfully, [18] human PRP induced chondrocyte apoptosis in rabbits and treatment with heterologous canine PRP accelerated the healing process in rabbits with surgically induced skin wounds [19].

To the authors’ knowledge, there is no literature study on the effect of equine platelets derived lyophilized growth factors on treatment of chondral defect. Therefore, the purpose of the present study was to assess the influence of the direct intra-articular injection of equine platelets derived lyophilized growth factors (LGF) in management of experimentally induced femoral chondral defect in rabbits.

Materials and methods

Preparation of LGF

LGF was produced according to a patented method (Code number: WO2018091713) developed by Dr. Hossam M Fahmy, Professor of Laboratory and Transfusion medicine, Ain Shams Medical School, Cairo, Egypt. PRP was collected by pheresis machine. The collected PRP was exposed to treatment with UV/Riboflavin by the Mirasol system (Terumo BCT, Lakewood, Colorado, USA), for pathogen inactivation and viral reduction. This was followed by in vitro stimulation of platelets by Calcium Chloride and Thrombin for the release of growth factors from the alpha granules. The released growth factors were purified and separated from the cellular debris and fibrin clot, then exposed to a second step, the Solvent/Detergent method for viral inactivation, followed by sterile filtration. Hence, any probable microbiological contamination assured to be eliminated. Following this, the purified sterile growth factors were dispensed in sterile vials under aseptic precautions. The concentration of growth factors in each vial is equivalent to that gained from 20 ml of whole blood of a normal donor which ensures the standardization of concentrations of growth factors and clinical effects, within the same batch and between different batches.

Ethical statement

The present study was carried out after the approval Institutional Animal Care and Use Committee of Cairo University (IACUC, CU/I/II/107/18).

Experimental animals

Twenty males New Zealand albino rabbits, 120–180 days old, weighing 2.8–3.2 kg were enrolled in the current study. They were obtained from CLAVCAP-VACSER, Cairo, Egypt. Animals were dwelled in separate cages, under controlled conditions (25 ± 2°C, 12:12 light: dark cycle, and 50% ± 20% relative humidity). They offered water ad libitum and were granted a standard pellet diet. Prior to onset of the experiment, rabbits were let one week for accommodating on lab conditions.

All animals were evaluated pre-operatively through routine physical examination, joint movement, lameness examination, range of motion and functional disability as well as for presence of pain on manipulation. Animals suffered from any signs of knee joint disorder were omitted. Animals were randomly allocated into two groups (10 per each) according to the time of euthanasia at 4 weeks or 8 weeks post injection.

Surgical procedures

All animals have had bilateral induction of femoral chondral defect. After general anesthesia, a lateral para-patellar skin was cut and the knee joint was exposed after the joint capsule was opened
and the patella was displaced laterally. A rounded trephine was used to induce a full-thickness cylindrical cartilage defect of 3 mm in diameter and 1 mm in depth near the patellar groove [20]. The left knee joint of all animals was intrarticularly injected with the adjuvant (distilled water and lidocaine) as a self-control negative, while the right joint was injected by the LGF after reconstitution by distilled water (2 ml) and lidocaine 2% (1 ml). The injection was performed once at one-week post-surgery.

Rabbits of each group were euthanized using intravenous injection of pentobarbital (120 mg/kg) according to observation period at 4- or 8-weeks post injection (ten animals at each period). The femoral condyles were harvested and examined macroscopically and photographed. Specimens of half of the defect area from each animal of each group (ten specimens for each group) were collected and fixed in 10% neutral buffer formalin for 72 h for routine histopathological examination, while the rest of the samples were kept frozen at −80 °C until further investigations.

**Determination of cytokines levels in the chondral defects**

**Preparation of tissue homogenate**

To get 20% w/v homogenate, 0.5 gm chondral tissue was collected from each defect area and homogenized with ice-cooled saline utilizing a homogenizer (Medical instruments, MPW-120, Poland). To get rid of cell debris, the obtained homogenate was centrifuged in a cooling axis (Laborzentrifugen, 2k15, Sigma, Germany) for 5 min at 4000 rpm at 4 °C. The aliquot obtained was kept at −80 °C for further biochemical investigation.

The proinflammatory cytokines; interleukin 1β (IL-1β), tumor necrosis factor-alpha TNF-α as well as the anabolic and angiogenic cytokines; Platelet-derived growth factor (PDGF), and transforming growth factor beta 1 (TGF-β1) were all determined in the obtained tissue homogenate using ELISA kits (Cusabio Biotech®, CSB-E11987s, CSB-E04640r and CSB-E04595r respectively, Germany) following the manufacturing instructions.

Quantitative real time (RT-qPCR)

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) was carried out to detect the gene expression of Col I and Col II depending on the following steps.

The first step is the extraction of RNA: The all-out RNA was segregated utilizing QiaGen tissue extraction Kit (Qiagen, USA) as indicated by instructions of manufacture. The second step is the synthesis of cDNA: The absolute RNA (0.5–2 μg) was employed for cDNA conversion utilizing high limit cDNA reverse transcription kit (Fermentas, USA). Following this, Real-time qPCR was carried out using SYBR Green I. Amplification and examination of Real-time qPCR were performed using an Applied Biosystem with software version 3.1 (StepOneTM, USA). The sequences of the primer sets used which were optimized at the annealing temperature are presented in Table 1. The amplification reactions were implemented in a 50 ml final volume, with thermal cycling conditions of 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 15 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C, and 10 min at 72 °C. βαta actin was used as a housekeeping gene for normalization of the cycle threshold (Ct) data. All represented samples from each group were assayed with three biological replicates. The internal controls were used to estimate the relative fold change with comparative Ct values of the designated genes as determined previously [21].

**Morphological and histopathological evaluation**

Digital photographs were directly taken after euthanasia clarifying the defect area followed by their evaluation and macroscopic scoring of the repair tissue using the international cartilage repair society (ICRS) score (Wayne, McDowell, Shields, & Tuan, 2005). In the later score, the defect repair degree as well as the integration to border zone and the macroscopic appearance was assessed. The total score ranged from 0 to 12 where; 12 exemplifying normal cartilage and 0 represented abnormal cartilage of severe degree.

**Histological and immunohistochemical studies**

The collected specimens were decalcified in 10% EDTA at 37 °C for 1 month, then exposed to a routine histological processing for getting histological sections of 6–12μm thickness. For general histological evaluation, the obtained sections were stained with hematoxylin and eosin (H&E) and for cartilaginous matrix distribution evaluation, Toluidine blue stain was used [22]. The intensity of toluidine blue staining denoting cartilaginous matrix intensity was quantified and expressed as optical density in 5 microscopic fields using Image analysis software (Image J, 1.46a, NIH, USA).

The O’Driscoll scale for grading the histological cartilage repair [23] was used to grade and evaluate the regenerated tissue. The grading scale depends on evaluation of ten different parameters, and the overall score ranged from 0 to 24 whereas, the higher scores denoting better histological repair of articular cartilage. The later parameters included: the morphology of the predominant tissue, matrix staining, the surface regularity with the surrounding, structural integrity, thickness of newly-formed cartilage, binding to adjacent cartilage, chondrocyte clustering, cellular status, inflammation, and degenerative changes in the adjacent cartilage. Immuno-histochemical analysis of NF-κB p65 and MMP2 protein expression. Immuno-histochemical analyses were implemented following the procedures mentioned by Abdelhameed et al. [24]. Briefly, the sections were deparaffinized, rehydrated, and exposed to antigenic retrieval of the epitopes by preheating the tissue sections with 10 mM citrate buffer, pH 6.0 for 10 min in a microwave oven. Then the sections were incubated with rabbit anti NF-κB p65 monoclonal anti-rat as well as with rabbit anti MMP2 polyclonal antibody (Abcam, Cambridge, UK), (at dilutions of 1:100 and 1: 50 respectively) in a humidified chamber overnight. To block the endogenous peroxidase activity, the tissue sections were incubated with hydrogen peroxide blocking solution. Then the slides were incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) for 10 min, followed by counterstaining with Mayer's hematoxylin and then mounted. The positive brown area denoting the expression of each marker was quantified using Image analysis software (Image J, 1.46a, NIH, USA) by measuring the area percentage in 7 high power microscopic fields.

**Statistical analysis**

The statistical software: SPSS 25.0 was used for data analysis and all data were presented as means ±SE. Comparisons between groups was carried out using one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. Values were considered statistically significant at P<0.05. Kruskal wallis H test was used for comparing the frequency data for nonparametric analysis followed by the Mann-Whitney U test. The nonparametric data were presented as median.

**Table 1**
The forward and reverse primers’ oligonucleotide sequences.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Forward primer 5’-</th>
<th>Reverse primer 5’-</th>
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<tbody>
<tr>
<td>Col II</td>
<td>TGGCCCTCCCTGATTGACG</td>
<td>GAGGGCCCTGACCCAGTGTT</td>
</tr>
<tr>
<td>Col I</td>
<td>AGGCTGACCTGTCGATTGCC</td>
<td>ATGGGCCGGATGTCGGTGATGG</td>
</tr>
<tr>
<td>βαta actin</td>
<td>TGTGTCGGACCTTCACAACC</td>
<td>CGCTCATTGCCCGATAGTGAT</td>
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</tbody>
</table>
Results

Physical examination

All experimental animals were healthy post-surgery and continued along the experimental period. The animals exhibited mild lameness on both limbs during motion and trotting with moderate pain during manipulation. One-week post injection, the animals displayed a normal left limb function and activity slight lameness was observed on trotting with normal range of motion and mild pain on passive movement.

Effect of LGF on chondral contents of the pro-inflammatory, anabolic, and angiogenic cytokines

The LGF treatment induced significant (p < 0.05) decrement in the defects’ contents of pro-inflammatory cytokines; IL-1β and TNF-α as well as significantly elevated the anabolic and angiogenic growth factors in the defects including; TGF-β1 and PDGF levels at the two experimental periods compared with the control untreated group at the same periods (Fig. 1).

Effect of LGF on collagen type I and II gene expression

The RT-qPCR analysis showed that the chondrogenic-related marker genes, including both collagen type I (Col I) and collagen type II (Col II) were significantly (p < 0.05) up-regulated in LGF treated chondral defects compared to the control group, with an obvious increase after 8 weeks (Table 2).

Pathological evaluation

Macroscopically, at the time of euthanasia, all animals were healthy; additionally no signs of osteoarthritis or infection were noticed at any joint. The condylar samples were collected and assessed. At 4 weeks (Figs. 2A-D), the left joints showed no signs of regeneration, a thin transparent layer was observed covering the defect area under which the subchondral bone was clearly seen as a reddish brown area. At the same period, the right joint defects was filled by a normal cartilage like- tissue looked alike the surrounding with regaining its glistening look while the defects margins were still obvious.

At 8 weeks (Figs. 2E-H) the left joints articular surfaces revealed the defect area occupied with a tough tissue that lacked the shiny appearance of the normal cartilaginous tissue. On the other side, the right joints articular surfaces showed the defect area occupied with a smooth glistening tissue that had a bluish coloration similar to the surrounding cartilaginous tissue.

The International Cartilage Repair Society (ICRS) scoring for macroscopic evaluation of cartilage repair revealed significant (P<0.05) higher healing scores for LGF-treated defects at both 4 and 8 weeks post-surgery compared with those of control ones (Fig. 2I).

Microscopic examination of control non-treated chondral defects of the left joints four weeks post-surgery revealed that; the defect was filled by fibrous tissue containing few fibroblasts especially at the top area of the defect and large blood vessels (Figs. 3A and B) filling the center of the defect. Whilst, examination of LGF-treated chondral defects four weeks after surgery revealed regular fibrous tissue filling the defect area (fibrocartilage) with numerous fibroblasts particularly at the surface area and some blood vessels (angiogenesis) at the base (Fig. 3C). The surface of the repair tissue (mainly fibrocartilage) was smooth with appearance of scattered areas or plates of cartilaginous matrix at the base of the defect area (Fig. 3D). Some defects showed healed cartilaginous tissue but with very low intensity cartilaginous matrix (Fig. 3E) and scattered chondrocyte-like cells, some of which appeared necrotic and others appeared apoptotic.
Table 2
The relative expression of Col I and Col II genes in the control and LGF treated groups.

<table>
<thead>
<tr>
<th>Groups Parameters</th>
<th>Control group after 4 weeks</th>
<th>LGF-treated group after 4 weeks</th>
<th>Control group after 8 week</th>
<th>LGF-treated group after 8 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col I</td>
<td>1.7980 ± 0.08794*</td>
<td>4.7560 ± 0.14250b</td>
<td>2.6000 ± 0.07975*</td>
<td>6.6620 ± 0.11989d</td>
</tr>
<tr>
<td>Col II</td>
<td>1.5420 ± 0.05774*</td>
<td>7.1560 ± 0.12127b</td>
<td>3.0220 ± 0.14894*</td>
<td>8.6340 ± 0.08841d</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SE. Data were analyzed by using one way ANOVA followed by Tukey post-hoc test. a, b, c and d were significant differences at P < 0.05.

Fig. 2. Macroscopic Photography of the harvested condyles of both control and LGF-injected defects (Red dotted circle). (A and B) control defects at 4 weeks; the subchondral bone was obviously seen as a reddish-brown area under this layer covered the defect area. (C and D) LGF injected defects at 4 weeks; the defects are filled by a tissue alike the surrounding normal cartilage while the defects margins were still obvious. (E and F) control defects at 8 weeks; the defect area filled with a tough tissue that lacked the shiny appearance. (G and H) LGF injected defects at 8 weeks; the defect areas filled with a smooth glistening tissue that has a blush coloration similar to the surrounding cartilage. (I) The mean International Cartilage Repair Society (ICRS) macroscopic scores for control non-treated and LGF-treated chondral defects at 4 and 8 weeks post-operation. Data are presented as median using Kruskal-Wallis test followed by the Mann-Whitney U test. Significant difference was considered at P<0.05. Data were analyzed by using one way ANOVA. a, b, c and d were significant differences at P < 0.05.

At eight weeks post-surgery, the joint surface of the control non-treated defects was still concave, and the gap area was filled with fibrous tissue which appeared more impacted and regular (Figs. 3F and G). The LGF-treated chondral defects showed advanced healing process, the defects were fully filled with cartilage tissue that was well-incorporated with the normal surrounding cartilage. Some of the repaired cartilage presented with intense clustering of the chondrocytes in their lacunae at the native edges of the defect (Fig. 3H) particularly near the surface of the defect with decreased intensity of cartilaginous matrix in some areas of the defect. While most of the defects showed well-organized cartilaginous tissue in which the cells were arranged in columns originating from the deeper part of the defect toward the surface with a conspicuous difference in tinctorial intensity of the cartilaginous matrix (Figs. 3I and J). The surface of those defects was smooth. While the O’Driscoll histological scoring system for the histological observations revealed significant higher healing scores for the LGF-treated defects than that observed in the corresponding control defects (Figs. 3K).

The toluidine blue staining revealed that; 4 weeks post-surgery, there was scarce cartilaginous matrix in the control non-treated group (Fig. 4A) and considerably more in the LGF-treated group (Fig. 4B). However, eight weeks post-surgery, the control group showed scattered areas of cartilaginous matrix at the base parts of the gag (Fig. 4C), while much more cartilage matrix, sometimes with variable degrees of intensity was observed in the LGF treated group with apparent color uniformity particularly at the basal parts (Fig. 4D and E). Quantitative analysis of the intensity of the positive blue color of cartilaginous matrix expressed by the optical density at 5 different microscopic fields revealed significant increase in the cartilaginous matrices in the LGF-treated groups particularly at 8 weeks post-surgery (Fig. 4F).
The immunohistochemical evaluation revealed that the LGF treated groups showed significant decrement in both MMP2 and NF-κB p65 immune-expression in the defect areas compared to the control groups particularly at 8 weeks post treatment (Fig 5) as assisted by image analysis software of the area percent of each marker expression in all groups.

Discussion

The knee joint is mostly affected by osteoarthritis (OA) which is an advanced loss of joint role and progressive pain as a result of gradual corrosion of articular cartilage. Repair of articular cartilage is still a challenge in musculoskeletal surgery in spite of several surgical procedures that have been developed in the last era and showed inadequate success. Current approaches for the chondral defect therapy have recently commingled the use of regenerative medicine and biologics that mediate the process of inflammation. Articular cartilage health and strength depends on a balanced biological environment of anabolic and catabolic factors.

In orthopedics, PRP was first to be appropriate popular and its clinical use was focused on both hard tissues as bone and soft tissues as muscle and tendon, this is because the concentrated platelets contain numerous factors which are chemoattractive, proliferative, and angiogenic such as; PDGF, VEGF, TGF-β1 and EGF.
Fig. 4. Representative toluidine blue stained sections showing: (A) scarce cartilaginous matrix in the control non-treated defect (B) and increased matrix in the LGF-treated defect at 4 weeks post-surgery. (C-E) At 8 weeks post-surgery: (C) scattered areas of cartilaginous matrix at the base parts of the defect in the control non-treated defect, (D and E) more condensed cartilage matrix with apparent color uniformity at the basal parts in the LGF treated defects. (F) Quantitative analysis of the intensity of the positive blue color of cartilaginous matrix revealing significant increase in the cartilaginous matrix in the LGF-treated groups particularly at 8 weeks post-surgery (image analysis software, ImageJ, 1.46a, NIH, USA). Values are expressed as mean ± SE. Data were analyzed by using one way ANOVA followed by Tukey post-hoc test. a, b, c and d were significant differences at P < 0.05.

PRP controls the catabolic and inflammatory milieu through a locally applied concentrate of growth factors, platelets, and leukocytes [25]. Different methods were used for PRP obtaining, as using commercially available systems, or noncommercial double-centrifugation systems. Hematological and biochemical characteristics vary markedly among different methods used in the field to obtain PRP, such differences could have an influence on the clinical results [26]. LGF is a leucocyte free product (patented) because of the multiple steps taken in preparation and Lyophilization. Recent scientific research has shown the controversial role of white blood
cells in a PRP preparation may play. PRP rich in white blood cells is able to promote the healing process, by eliminating the potential microbiological pathogens and stimulating the release of growth factors. Yet, and on the other hand, a big number of leukocytes in a platelet rich suspension may exert an inhibitory effect [27].

Several research papers have proposed to exclude leukocytes from PRP preparations used in management of bone defects, chronic tendon injury and osteoarthritis [28]. However, the ability of the PRP to influence the biological response of different cells as endothelial cells and fibroblasts in vitro does not rely on the presence of leukocytes [29].

In addition to the absence of white blood cells, LGF preparation is fibrinogen free as well. Although fibrin gel resulting from the interaction between fibrinogen found in the alpha granules and the PRP activator, thrombin and/or calcium chloride, may be useful as a carrier for growth factors that is released under control, the formed fibrin gel minimizes the volume of the final product, hinder the easy injection of PRP, and is not favored in many clinical situations [30]. PRP from horses are a well-established modality for management of wound healing as well as various degenerative and traumatic injuries of the musculoskeletal system. [31] Standardizing the amount of growth factors in each vial to the equivalent to those coming from platelets found in 20 ml of whole blood. Having a Fibrinogen depleted, water soluble product for easy administration, with no gel formation was formed. This is particularly significant for intra-articular injections.

The current study aimed to evaluate the impact of intraarticular injection of equine platelets derived LGF on regeneration of cartilage. The primary results showed the safety of the LGF injection with no adverse reaction or signs of infection accompanied by improvement of the clinical signs over the study period when compared to the control joints that received the adjuvant only and this agreed with previous studies [25,32], which stated that PRP treatment led to clinical improvement of the osteoarthritic joints over time which was attributed to the growth factors and cytokines released led to generating a regenerative microenvironment and blocking the degenerative pathways of the cartilage [33]. Furthermore, in the current study LGF offered platelets of xenogenic pathogen free source instead of autologous platelets as a source for growth factors.

The key parameters for evaluation of cartilage repair are both macroscopic and histological evaluations, particularly the microscopic appraisal is deemed as the gold principle to characterize the type of repair tissue. In the current study, ICRS macroscopic grading which is validated and reliable [34] was used to grade the repair tissue. In animal studies, macroscopic evaluation is a familiar merit for cartilage repair because the whole joint is accessible, providing the first impressions of the quality of the repair tissue.

However, histological assessment is ideal for rating the success of different procedures of cartilage repair, in the current study we used the O’Driscoll scoring system, which is advised for histological analysis of cartilage repair in animal studies [35]. Both scoring systems revealed that the surface of the cartilaginous defect in the control untreated groups was less smooth than the LGF treated ones, additionally, the LGF treated groups developed more abundant cartilaginous extracellular matrix and showed good degree of cartilaginous tissue renewing. The increased collagen type I and II genes expression in our work, was reflected and demonstrated by the increased histochemical staining for ECM using toluidine blue, which indicated that the neocartilage in the LGF groups has the characteristics of hyaline cartilage, denoting its favorable effects on the regeneration of cartilage.

In tissue repair, the elucidation of some functions of growth factors has led up to the deduction that their progressive expression in a controlled manner could be important subsequent to surgical interferences and in musculoskeletal disorders treatment, like bone fractures and joint disorders. The treated joints showed restoration of the articular surface compared to the control ones similar to previous studies of Abdallah et al. and Mokbel et al. [36,37] who confirming the encouraging effects of growth factors derived from PRP on preventing articular cartilage corrosion, motivating the regeneration process, and prompt the migration as well as differentiation of stem cell faster.

Our results showed that the intra articular injection of LGF significantly decreased the defect contents of TNF-α and IL-1β and significantly increased the defect homogenate contents of PDGF and TGF-β1, which agreed with Wojdasiewicz et al. [38] who stated that two inflammatory cytokines are incorporated in the disturbed environment in osteoarthritis, which are tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β). Additionally, TNF-α and IL-1β were found at upraised levels in tissues and fluids affected by OA [39]. However, LGF counteracted erosion of the cartilage by curbing the catabolic cytokines; IL-1β and TNF-α.

Although OA is classified as a non-inflammatory disease, inflammation is implicated in many symptoms and in OA progression. Additionally, Large cartilage defects often fail to heal spontaneously and may result in progressive deterioration and eventually osteoarthritis [40]. In chondral defect, interleukin-1 beta (IL-1β) and TNF-α are the cytokines that are primarily implicated in cartilage matrix breakdown and associated with the subsequent inflammation in OA and are known to be investigated in induced chondral defects [41]. These cytokines induce chondrocytes to produce matrix metalloproteinases MMPs that in turn are responsible for cartilage matrix degradation. These cartilage matrix breakdown products in synovial fluid are thought to increase synovial inflammation [42], creating a positive feed-forward loop that increases inflammation and cartilage breakdown. For the previous reason of implication of the pro-inflammatory cytokines (IL-1β and TNF-α) in cartilage breakdown, we investigated whether our product LGF can decrease the expression of those pro-inflammatory cytokines and hence in part curbs the progression to OA or not, and paralleled if the LGF could elevate the anabolic and angiogenic growth factors sharing in cartilage repair. Thence, inhibition of these cytokines may limit inflammation and matrix degradation. constitute an effective OA therapy.

The elevated levels of IL-1β and TNF-α in the cartilage defects act as participating factors in the degradation of cartilage by impacting the chondrocytes expression so inhibiting the synthesis of type-II collagen and aggrecan thus altering the cartilage structural proteins, [41] as well as by promoting matrix metalloproteinases (MMPs) synthesis, which all are considered harmful to cartilage [42]. Moreover, the elevated levels of IL-1β and TNF-α induced turmoil of the chondrogenic progenitor cells (CPCs) and induced chondrocyte death which aids in more rapid aging and joint degeneration [43,44]. Additionally, reactive oxygen species (ROS) was found to be generated by IL-1β and directly damage the articular cartilage [45].

The use of LGF promoted factors associated with cartilage matrix synthesis by elevated the levels of PDGF and TGF-β1 which agreed with Boswell et al. [46]. The later factors play a key role in signaling the cartilage healing cascade, as those growth factors in platelets would stimulate the growth of chondrocyte cell in cartilage, so in that way, healing the cartilage defects which agreed with what was mentioned by Woodell-May et al. [47] has significant concentrations of anabolic and angiogenic growth factors such as TGF-1, PDGF-AB, PDGF-BB, and EGF.

Our current work revealed increased gene expression of Col I and II in LGF treated group that comes in agreement with Xie et al. [25] who found that PRP produced an increase in the protein levels and mRNA for aggrecan and type-II collagen, in addition to mini-
mizing the IL-1β-induced inhibition of type-II collagen and agree can [48].

A significant decrease in the expression levels of both MMP2 and NF-κB p65 was observed in the LGF treated groups particularly at 8 weeks period, which agreed with Conca et al. [49] who mentioned that an upregulation of MMP expression occurs during the development of OA, from chondrocyte binding to TNF-α and IL-1β through NF-κB signal pathways. It was reported that NF-κB significantly contributes to cartilage degradation by stimulating an elevation in unwelcome factors levels and hindering the process of cartilage repair via altered expression of inflammatory cytokines, enzymes, and transcription factors. Hence, once activated by TNF-α and IL-1β, NF-κB becomes a positive regulator for the pro-inflammatory cytokines expression, in particular, TNF-α, IL-1β, and IL-6 [50].

Thus, suppression of NF-κB will reduce the impact of degenerative factors by inhibiting the intra-articular signaling of TNF-α and IL-1 which in turn reduces the increase of aggreganases and MMPs, while in the same time minimizing the downregulation effect of collagen type-II in chondrocytes [51,52]. So, in general, the growth factors in PRP are supposed to play a major role as anti-inflammatory effect via inhibiting NF-κB activity [53].

Furthermore, PRP indirectly affects the activity of NF-κB, [54] and it may decrease the increased levels of nitric oxide Farrell (NO), [55] which share in cartilage degeneration by, curbing collagen synthesis triggering chondrocyte apoptosis and boosting production of MMPs [56]. Similarly, both of an inhibitor for NF-κB and TGF-β decrease NO production within chondrocytes, but upon testing with releasate of PRP, only the activation of NF-κB was counteracted, while the production of NO was unaltered [57].

In conclusion, LGF offers an alternative to the conventionally prepared PRP in promoting articular cartilage regeneration and inhibiting the degeneration of chondrocytes and the articular surface holding the advantage of standardization of the amount of growth factors, a long shelf life in the lyophilized form, having a fibrinogen depleted, water soluble form ready to use for easy administration, with no gel formation and a xenogeneic biomaterial. This is particularly significant for intra-articular injections.

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Author contributions


Conflict of interest

The authors have declared that no conflict of interests exists between anyone of the authors.

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