Chondrocyte Secretion of NO, ROS, GAG and PGE_2 in Surface and Middle Porcine Articular Cartilage Is Altered by Aqueous Seed Extract of Bambara Groundnut (*Vigna subterranea* L. Vedc.)

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Article

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Abstract: In Africa, Bambara groundnut (BG) (*Vigna subterranea* L. Verdc.) is used both as a food legume and a medicinal plant. Traditional healers used the seeds of Bambara groundnut for treating diarrhoea, impotency, colon cancer, nausea, vomiting, malignancies, and inflammations caused by osteoarthritis. In humans, the proinflammatory cytokine, interleukin-1β (IL-1β), plays a major role in the aetiology of osteoarthritis as it induces the release of catabolic factors that contribute to cartilage degradation. The aim of this study was to evaluate the protective effects of lyophilised aqueous extract of BG on porcine articular chondrocytes, following cell stimulation with proinflammatory cytokine IL-1β (10 ng/ml) and addition of different concentrations of BG aqueous seed extract (12.5, 50 and 200 µg/ml) or quercetin (12.5, 50 and 200 µg/ml) as antioxidant. Measurement of nitric oxide (NO), reactive oxygen species (ROS) and prostaglandins (PGE_2) using ELISA, fluorescent probe 2′-7′-dichlorodihydrofluorescin diacetate (DCFH-DA) and Griess reagent respectively showed that BG extract decreased the stable free radical DPPH in both the middle zone and surface zone chondrocyte cells, and inhibited the IL-1β-induced production of NO and ROS, which have harmful effects on porcine articular chondrocytes. However, the level of glycosaminoglycans (GAG) rose relative to the pure IL-1β treatment 72 h after the addition of BG crude extract or quercetin. These results suggest that BG crude extract has chondroprotective effect which can safeguard cells against inflammation during osteoarthritis and other inflammatory disorders.

Keywords: Anti-inflammation; Chondroprotective effect; Interleukin-1β; Osteoarthritis; Nitric oxide; Reactive oxygen species; Prostaglandins; Glycosaminoglycans

1. Introduction

Osteoarthritis (OA) is a progressive disease of the joint tissues, characterized by cartilage degradation [1]. Factors contributing to the development of OA often include age, obesity, inflammation and trauma [2,3]. However, available evidence suggests that inflammation plays a critical role in the development and progression of OA [4]. Proinflammatory cytokine IL-1β in particular is reported to be the cause of OA as it can upregulate inflammatory mediators such as ROS, NO and PGE_2 [5,6], which drive the inflammation process, leading to the progression of OA [7,8].

However, recent studies have shown that inhibition of these inflammatory mediators can alter the progression of OA [9]. For example, phytochemical compounds from plants, either acting alone or in synergy with other molecules have proven to be potent inhibitors of these inflammatory mediators [10,11]. Although there are already nonsteroidal anti-inflammatory drugs (NSAIDs) for treating OA, most of them are ineffective and only reduce pain without addressing the underlying the cause of OA [12].

Bambara groundnut (BG) is a neglected, underutilised food legume that has been used traditionally to treat various physiological disorders, including inflammation, which is a first step

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towards the development of OA [13]. Not surprisingly, chemicals analysis of edible BG grain has revealed the presence of antioxidant compounds such as flavonoids, tannins, anthocyanins, anthocyanidins and phenolic acids [14,15]. As a result, BG crude extract has been reported to inhibit bacterial growth, as well as exhibit antifungal activity against *Candida albicans* [16]. In another study, protein hydrolysate from BG also revealed antioxidant properties [17]. Furthermore, the presence of flavonoids, tannins and alkaloids has been detected in BG cultivars, with the alkaloids showing analgesic properties [18].

In a separate study [15], we showed that BG seed extract consists of free and glycosilated derivatives of flavonoids such as catechin, gallocatechin, quercetin, kaempherol, apigenin, daidzein, and phenolic acids like caffeic acid, benzoic and hydroxycinnamic acids, quinic and shikimic acids, as well as triterpenoid saponins, sphingolipids and fatty acids. The beneficial health-promoting properties of edible Bambara groundnut grain can be attributed to these phytochemicals, especially to the polyphenolics, which act as free radical scavengers and/or primary antioxidants in human nutrition and health. So far, however, no study has evaluated the bioprotective effect of BG seed extract on mammalian cells.

The aim of this study was to ascertain whether lyophilised crude extract of this legume can alter the production of NO, PGE_2, ROS and GAGs, key molecules released during chronic inflammatory events using an *in vitro* model based on porcine articular chondrocyte cultures stimulated with IL-1β.

2. Results

2.1. Free Radical Scavenging Activities

In both the surface and middle zone, BG crude extract showed less ability to scavenge DPPH free radical compared to quercetin, the antioxidant (Figure 1). Furthermore, the mixtures with the lowest concentration of BG crude extract and quercetin were least effective in scavenging DPPH free radical than their counterparts with higher quercetin and BG crude extract levels (Figure 1).

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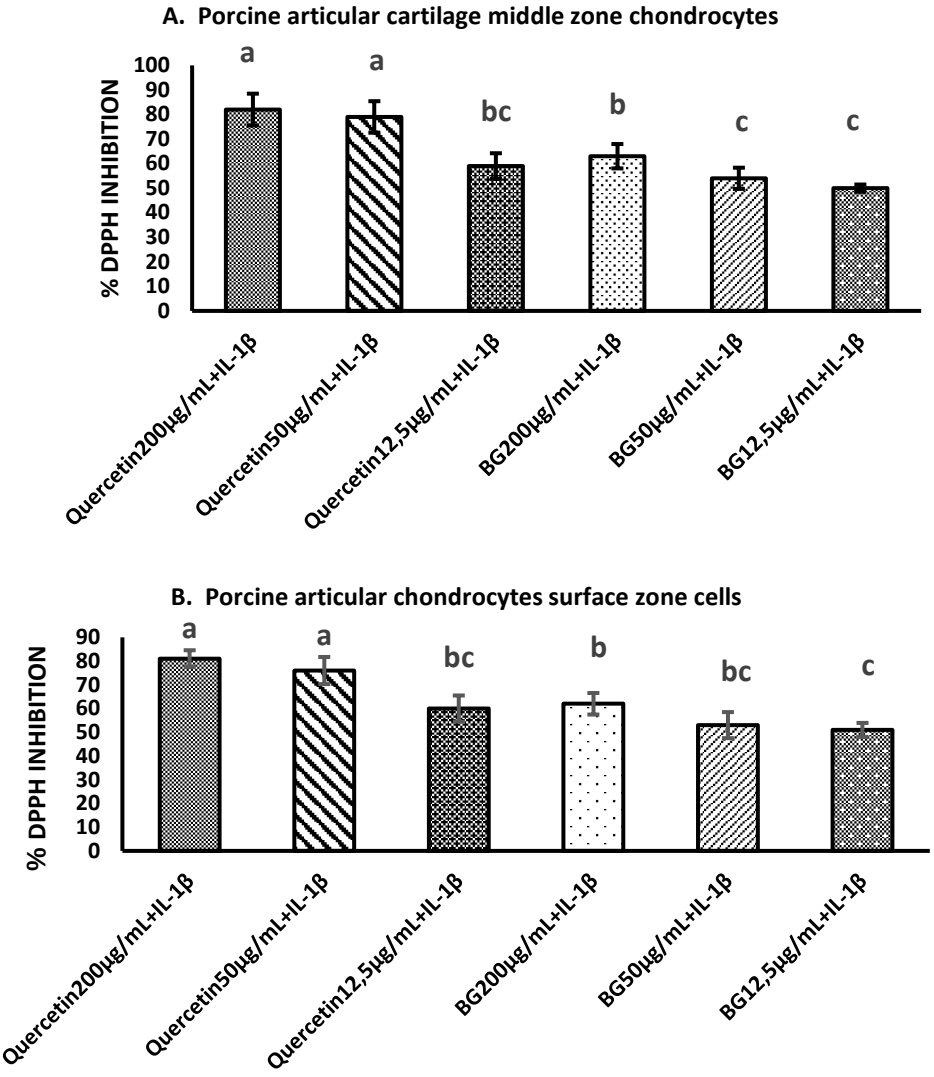

Figure 1. Percentage DPPH inhibition of BG extracts and Quercetin at 12.5, 50 and 200 µg/ml on the (A) surface zone and (B) middle zone of porcine articular chondrocytes. Values are expressed as % DPPH inhibition.

2.2. Effect of Quercetin and BG Crude Extract on ROS Production

Supplying cytokine IL-1β alone to chondrocytes in both surface and middle zone articular cartilage triggered an increase production of reactive oxygen species (ROS) when compared to all the other treatments (Figure 2 A, B). However, adding different concentrations of quercetin or BG crude extract significantly reduced the production of ROS by chondrocytes in both the surface and middle zones of porcine articular cartilage. The culturing of quercetin/IL-1β or BG/IL-1β combinations also revealed a significant concentration- dependent reduction in ROS levels relative to IL-1β treatment in both the surface and middle zone of articular chondrocytes (Figure 2 A, B). In both zones, the quercetin 200 g/ml + IL-1β showed the highest reduction in ROS production, just as the BG 200 g/ml + IL-1β also recorded the highest reduction in ROS production (Figure 2 A, B).

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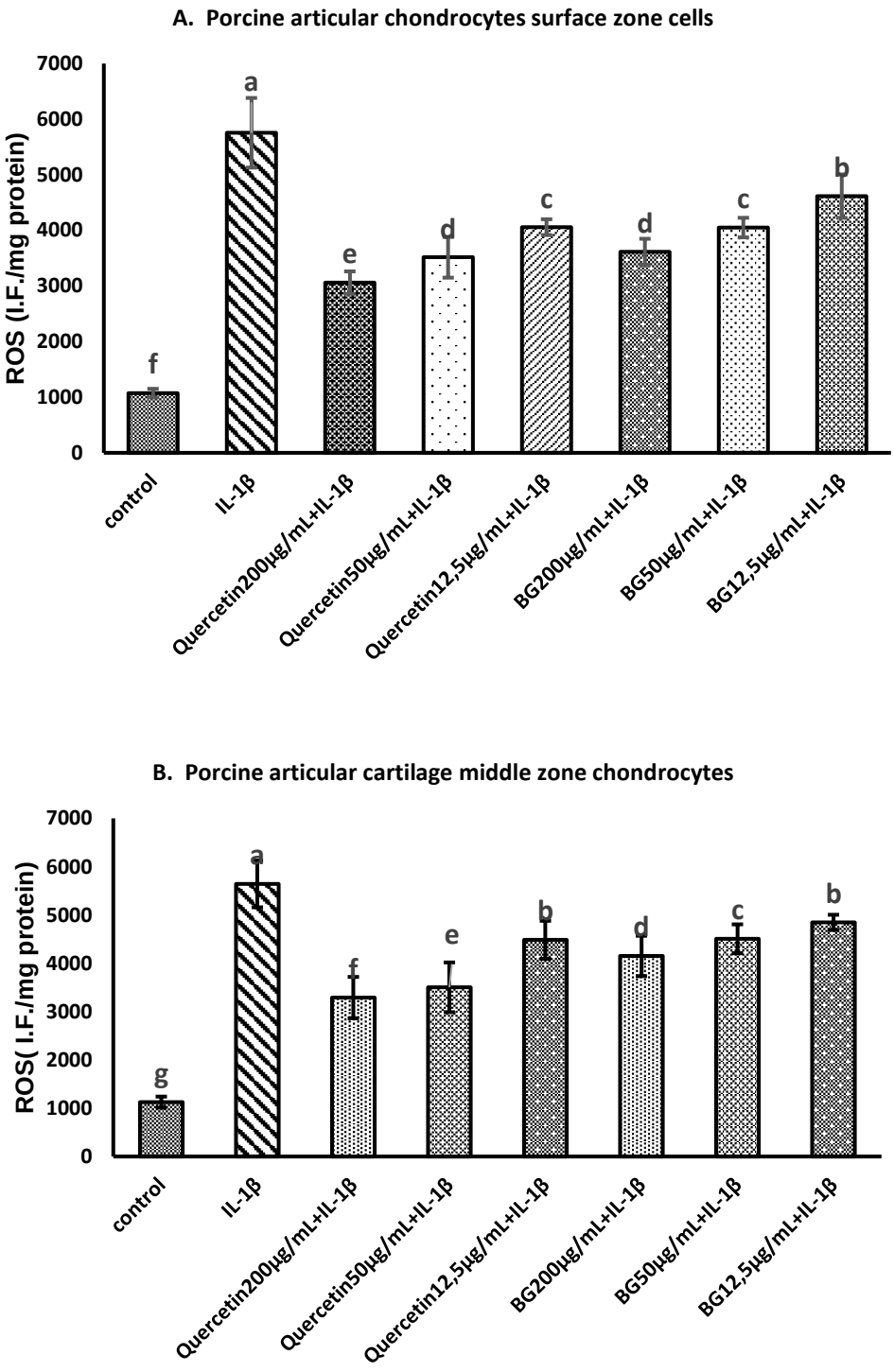

Figure 2. ROS production (means ± S.E.M.) in the culture medium from (A) surface zone and (B) middle zone of porcine articular chondrocytes 72 hrs after the addition of BG crude extract and Quercetin at 12.5, 50 and 200 μg/ml with IL-1β. Values are expressed as I.F. per mg protein.

Culturing chondrocyte cells from surface and middle zone of articular cartilage with a pure solution of cytokine IL-1β alone induced a significantly huge increase in NO production relative to all the other treatments (Figure 3A, B). The three concentrations of quercetin prepared with IL-1β, and the three levels of BG crude extract mixed with IL-1β showed a significantly decreased production of NO when incubated with chondrocyte cells. This response was concentration-dependent. Whether with quercetin or BG, the 200 μg/mL+ IL-1β produced the least NO, followed by 50 μg/mL+ IL-1β, and then 12.5 μg/mL+ IL-1β (Figure 3A, B). Furthermore, the 200 μg/mL and 50

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μg/mL concentrations of BG crude extract were more potent in inhibiting NO production than same levels of quercetin prepared with IL-1β (Figure 3A, B).

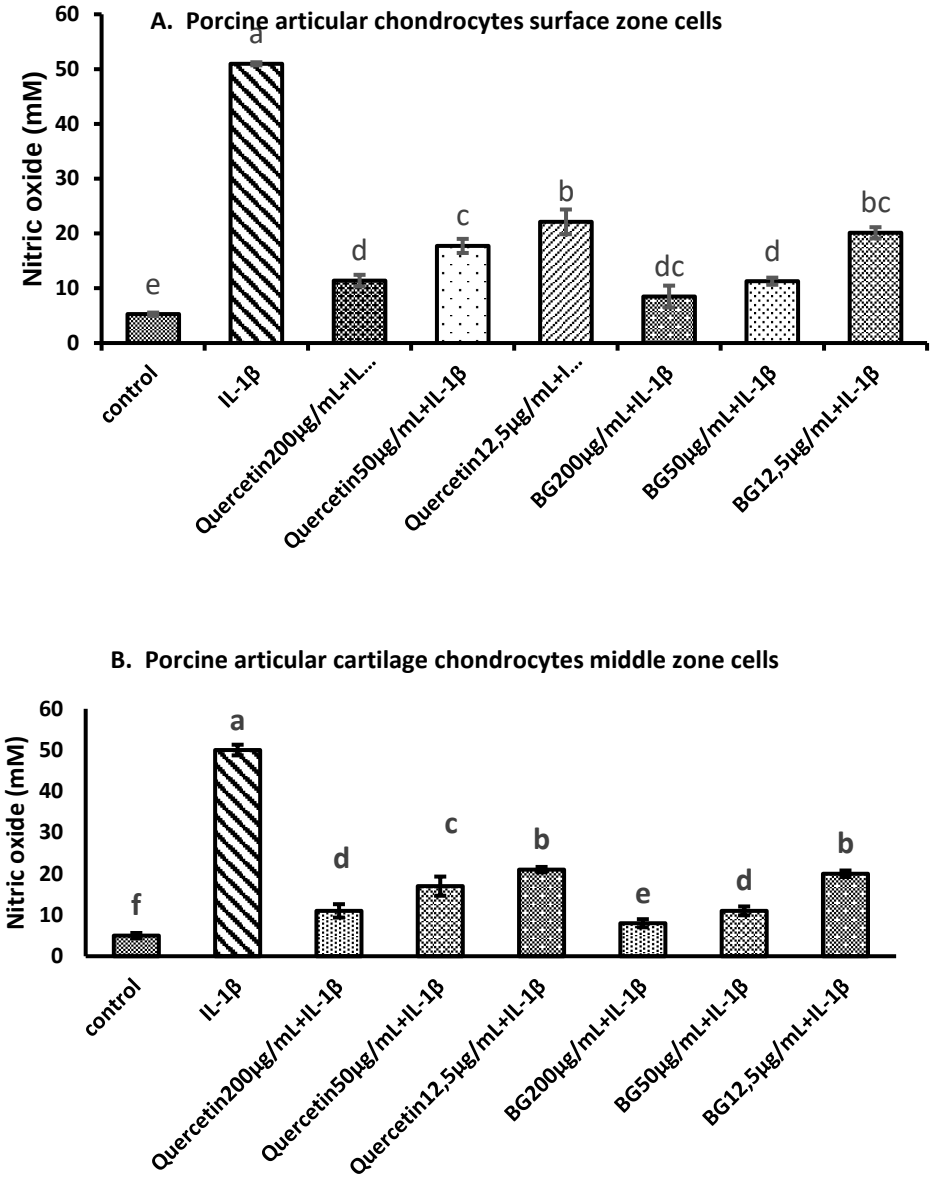

Figure 3. NO production (means ± S.E.M.) in the culture medium from the A) surface zone and B) middle zone of porcine articular chondrocytes 72 hrs after the addition of BG crude extract and Quercetin at 12.5, 50 and 200 μg/mL with IL-1β. Values are expressed as μM.

2.3. Glycosaminoglycan Secretion by Chondrocytes Incubated with IL-1β, Quercetin and BG Crude Extract

Incubating chondrocyte cells from the surface and middle zones of porcine articular cartilage with IL-1β alone significantly inhibited the production and secretion of GAG into the culture filtrate (Figure 4A, B). However, the different quercetin concentrations prepared with IL-1β, and different BG crude extract levels prepared with IL-1β revealed greater GAG exudation relative to IL-1β control when cultured with chondrocyte cells from the two zones of porcine articular cartilage (Figure 4A, B). The zero control released more GAG than all the treatments. Thus, the inhibition of GAG synthesis by cytokine IL-1β was reduced by the presence of BG crude extract and quercetin in the culture medium.

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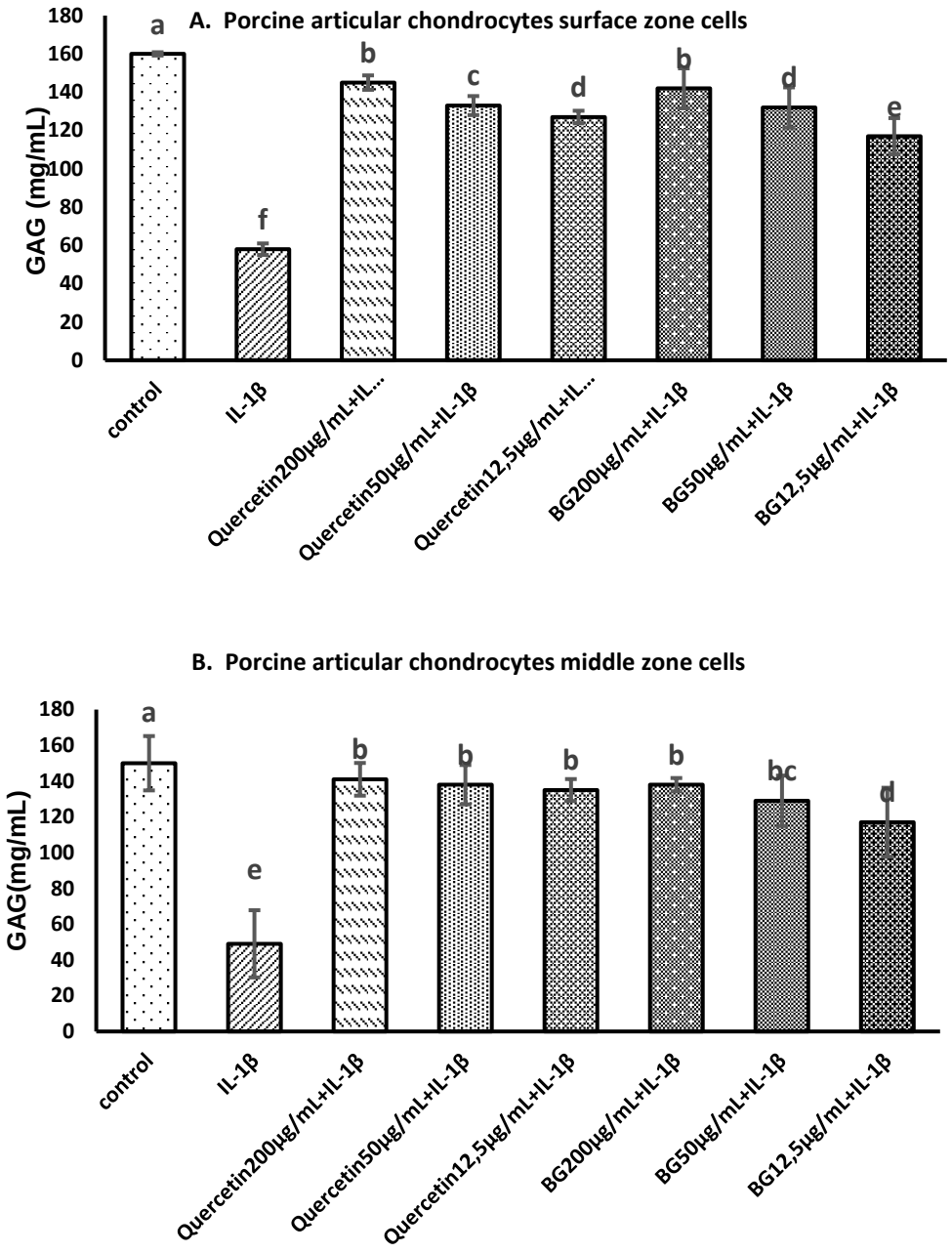

Figure 4. GAG release (means ± S.E.M.) in the culture medium from A) the surface zone and B) the middle zone of porcine articular chondrocytes 72 hrs after the addition BG crude extract and Quercetin at 12.5, 50 and 200 µg/ml with IL-1β.

2.4. Prostaglandins E_2 (PGE_2) Production by Chondrocytes Cultured with IL-1β, Quercetin and BG Crude Extract

Prostaglandin E_2 (PGE_2) production and release was significantly triggered by the addition of IL-1β (10 ng/mL) to cultured chondrocyte cells harvested from the surface and the middle zone of porcine articular cartilage (Figure 5 A, B). The stimulated production of PGE_2 by IL-1β was significantly reduced with the addition of quercetin or BG crude extract to the culture medium, and this decrease in PGE_2 was concentration-dependent.

The effect of both quercetin and BG crude extract in decreasing PGE_2 production was more marked in chondrocyte cells from the middle zone relative to surface zone (Figure 5 A, B).

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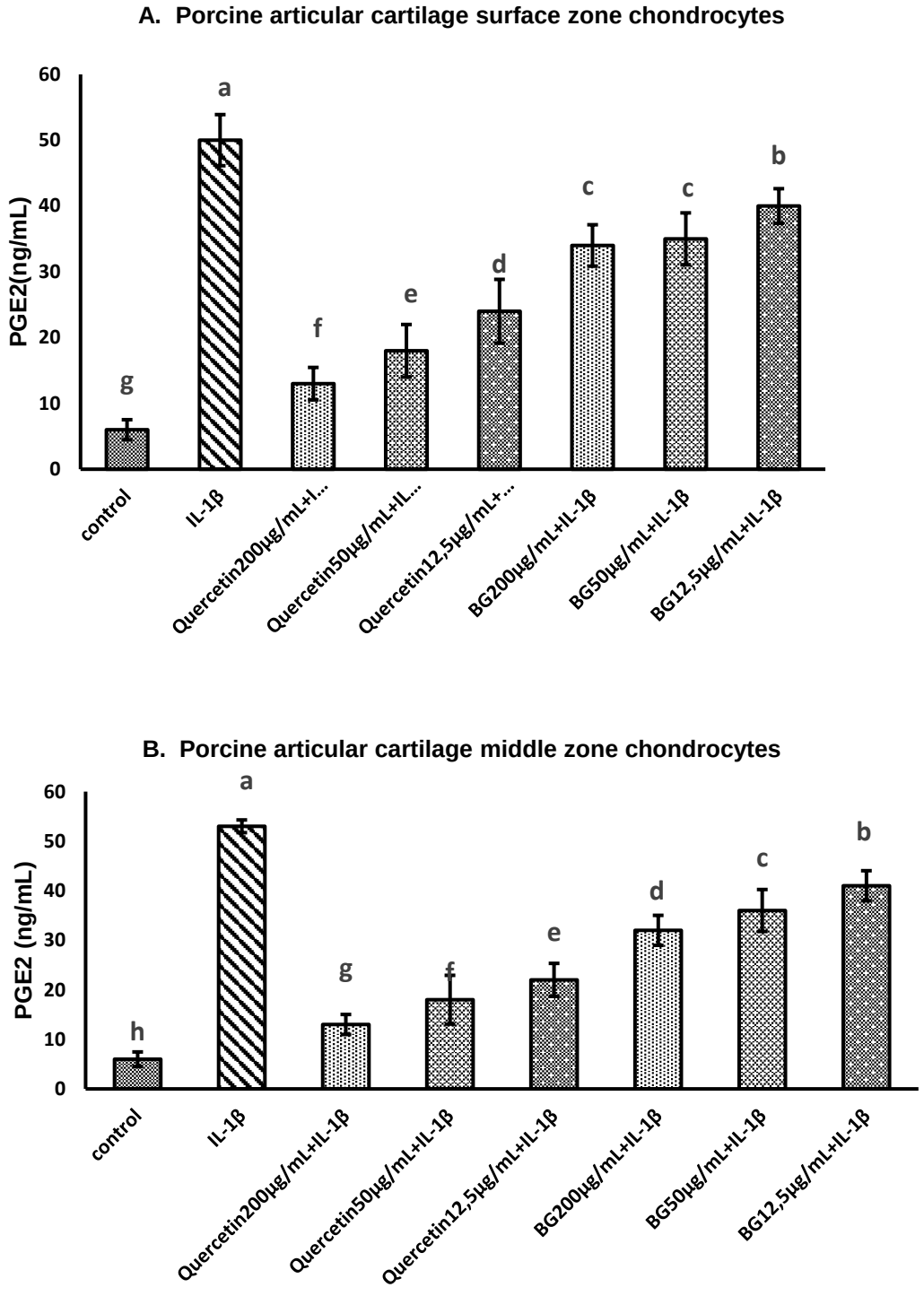

Figure 5. PGE_2 production (means ± S.E.M.) in the culture medium from A) the surface zone and B) the middle zone of porcine articular chondrocytes 72 hrs after the addition of BG crude extract and Quercetin at 12.5, 50 and 200 μg/mL with IL-1β.

3. Discussion

Osteoarthritis is a joint disease generally characterized by the simultaneous presence of degenerative and oxidative/inflammatory processes. It is often the result of an imbalance between reparative and destructive processes in the articular cartilage emanating from the effects of free radicals (ROS and NO), inflammatory cytokines (e.g. IL and TNF-alpha) and MMPs [19].

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These inflammatory cytokines play a major role in the development of OA [20], but the proinflammatory cytokine IL-1β in particular is responsible for changes in the joint tissues during OA [21].

Although IL-1β plays an important homeostatic function under normal conditions such as the regulation of feeding, sleep and temperature [22], overproduction of cytokine IL-1β is linked to pathophysiological changes commonly associated with different stages of disease such as rheumatoid arthritis, neuropathic pain, inflammatory bowel disease, OA, vascular disease, multiple sclerosis and Alzheimer's disease [23].

Additionally, IL-1β is also known to induce the production of NO, a potent molecule that can react rapidly with superoxide anions (O_2) to form peroxynitrite ($OONO^-$), a strong oxidant [24], hydrogen peroxide (H_2O_2) and hydroxyl radicals which are harmful to cells. Therefore, stimulation of chondrocytes with IL-1β can lead to the production of ROS, NO, PGE_2 and MMPs [25]. Because inflammation plays a central role in OA development and progression, targeting inflammation at the onset of OA could be an appropriate therapeutic strategy [26].

The aim of this study was to assess the effect of BG crude extract on free radical scavenging ability, IL-1β-induced release of GAG, and on the production of NO, PGE_2 and ROS in IL-1β-induced porcine articular chondrocytes. In essence, the study assessed the ability of BG crude extract to reduce the negative effects of cytokine IL-1β on cell proliferation, free radical scavenging, as well as the production of harmful inflammatory mediators such as NO, ROS, and PGE_2.

In this study, both BG crude extract and quercetin antioxidant were able to scavenge DPPH free radicals, a finding consistent with the report by Chinnapun [14] which showed that raw and processed BG seed extract could scavenge free radicals. Compared to quercetin however the BG crude extract was relatively moderate in scavenging free radicals (Figure 1 A, B). Nevertheless, the ability of BG crude extract to scavenge free radicals implies the presence of antioxidants in the extracts that quenched these free radicals.

For an effective therapy of OA, it is essential to reduce elevated catabolism while increasing anabolism. This can be done by inhibiting pro-inflammatory cytokines which are usually abundant in osteoarthritic joints [27]. Thus, inhibiting the production and release of inflammatory cytokines and mediators such as ROS, NO, and PGE_2 ought to reduce the progression of OA [28]. In this study, the BG crude extract significantly inhibited IL-1β- induced NO, ROS, and PGE_2 production by chondrocytes when compared to the known activity of quercetin antioxidant (Figures 2, 3 and 4).

Our results are consistent with the findings of previous studies which showed that phytochemicals in food legumes can play an important role in the oxidative and inflammatory processes leading to OA progression [29].

The catabolic effect of NO can inhibit proteoglycan synthesis while stimulating chondrocyte production of proenzymes that get converted into active enzymes such as MMPs which are known to destroy cartilage matrix in arthritic diseases [30,31,32].

In this study, cells cultured with only cytokine IL-1β produced high levels of NO in contrast to the very low NO production by chondrocytes in the zero-control treatment, primarily due to the activity of constitutive nitric oxide synthetase (cNOS). The addition of BG crude extract to cytokine IL-1β- induced porcine chondrocytes (IL-1β + BG) markedly reduced the level of NO production when compared to IL-1β alone, or IL-1β + quercetin in both zones (Figure 3A, B).

Our results also show that the production of GAG in porcine articular chondrocyte culture supplemented with BG extract was enhanced, especially at 50 ug/mL in both surface and middle zones for day 3 and 7 in a similar pattern to aggrecan core protein mRNA expression, which suggests enhanced chondrogenic ability. As shown in Figure 4 A and B, the cytokine IL-1β significantly reduced the production of GAG compared to the zero control, and when quercetin or BG crude extract was added to IL-1β. This clearly shows that BG crude extract inhibited the activities of IL-1β, and this effect of BG extract can be attributed to the presence of flavonoids in the BG seed extract. Cardile et al. [33] have in fact shown that the presence of flavonoid molecules was able to prevent the inhibition of GAG synthesis in chondrocytes, hence the restored proteoglycan synthesis.

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With OA, PGE_2 production is associated with synovial inflammation. It has also been shown that PGE_2 can inhibit chondrocyte growth and upregulate IL-1β biosynthesis [34,35]. As shown in Figure 5, relatively less PGE_2 was produced in the zero control cells without IL-1β. However, cells treated with IL-1β alone showed a much higher production of PGE_2. But adding quercetin or BG crude extract to the medium significantly reduced the IL-1β-induced production of PGE_2 in a concentration-dependent manner, which indicates their positive effect in controlling the activity of PGE_2. Reactive oxygen species are also known to be involved in the initiation and progression of OA [36, 37]. In this study, the zero-control culture without IL-1β produced less than the cells treated with only IL-1β.

However, adding BG crude extract reduced the production of ROS compared to the treatment with IL-1β alone. In fact, the ROS-reducing activity of BG crude extract was comparable to quercetin (a known standard antioxidant), suggesting that the BG crude extract can be used as an anti-inflammatory, antioxidant agent.

Phenolic compounds such as phenolic acids, flavonols, flavones, isoflavones, anthocyanins, and condensed tannins, have been identified in BG seeds [15]. Therefore, the activity of the BG crude extract could be due to these phenolic components.

A number of grain legumes have exhibited various traits that are potentially protective of chondrocyte cells. For example, germinated and fermented mung bean seeds were found to be a potent inhibitor of NO production at 2.5 and 5.0 μg/mL [38], while adzuki bean ethanol extract suppressed the release of PGE_2 and NO in macrophage cells [39]. Furthermore Zia-Ul-Haq *et al.*[37], studied crude methanolic extracts of black gram, green gram, soybean, and lentil for anti-inflammatory activity and found 73.9%, 79.8%, 92.2%, and 74.5% inhibition respectively at a 20 mg/mL extract concentration. Also, Park *et al.*[40] used *in vitro* and *in vivo* experimental models to study the anti-inflammatory potential of a butanol fraction of red bean ethanolic extract, and found NO production in LPS-stimulated macrophages. Furthermore, Singh *et al.* [30] also found that common bean phenolic extract had high antioxidant and high anti-inflammatory activity. Taken together, these studies suggest that exploring food legumes as a source of remedy for OA has a very high chance of success.

In conclusion, the activity exhibited by BG crude extract *in vitro* suggests the possibility of developing novel attractive anti-inflammatory and antidegenerative compounds, devoid of significant cytotoxic effects, that can be used to block cartilage destruction during the inflammatory process *in vivo*. The mechanisms of cartilage degradation are multifactorial, and it is essential to tackle this orthopaedic pathology with treatments, which not only protect the cartilage against degenerative damage by stimulating its intrinsic repair capacity of chondroprotection, but also neutralize the inflammatory and destructive potential of the mediators involved in oxidative-inflammatory stress, such as ROS, NO, $PG\text{-}E_2$, inflammatory cytokines and proteolytic enzymes.

4. Materials and Methods

4.1. Plant Material

Bambara groundnut seeds were sourced from the experimental fields of Biological Nitrogen Fixation Laboratory, Tshwane University of Technology, Pretoria South Africa. The seeds were washed with water to remove any dust. Thereafter, the seeds were air-dried at room temperature for two days. The dried material was ground in a Wiley mill grinder (Polymix PX-MFC 90D, LASEC, Cape Town, RSA) and stored in the dark until extraction. Each ground sample was extracted separately by shaking the powder (30 g) for six hours with 300 ml distilled water on a Form Scientific orbital shaker (Labtech, Midrand, RSA) at 200 rpm and filtered through Whatman No. 1 filter paper. The water extracts were frozen at -70°C and lyophilized and the dried extracts stored at -20°C.

4.2. Porcine Articular Chondrocyte Culture

Articular cartilage samples were obtained from twelve pigs knee joints slaughtered for human consumption (aged:3 months) in a local abattoir (Pretoria, South Africa). Ethical approval was

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obtained from the Tshwane university of Technology Animals Research Ethics Committee Ref#:(AREC2020/11/001).

Primary chondrocytes were isolated from porcine articular cartilage, as described by Daniel et al. [41]. Briefly, porcine articular cartilage slices were digested with 0.25% collagenase (Abcam, MA, USA) at 37°C for 2 h. The cells were suspended in DMEM containing 10% foetal bovine serum (FBS),100 units/ml penicillin, and 100 mg/ml streptomycin, and cultured at 37°C with 5% CO_2.

After 24 h, the medium was removed and the cells added to the treatments as follows: i) negative control, ii) positive control (10 ng/ml IL-1β), iii) BG extracts (12.5, 50 and 200 µg/ml) + IL-1β (10 ng/ml), iv) quercetin antioxidant (12.5, 50 and 200 µg/ml) + IL-1 β (10 ng/ml). The quercetin was used as a reference antioxidant compound. After 72 h, the supernatants of the chondrocyte cell cultures were removed and used for the following assays.

4.3. Free Radical Scavenging Activities

The 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) has been widely used to evaluate the free radical scavenging activity of natural antioxidants, as described by Brand-Williams et al. [42].

4.4. Effect of Quercetin and BG Crude Extract on ROS Production

Reactive oxygen species production was estimated using the fluorescent probe 2′-7′-dichlorodihydrofluorescin diacetate (DCFH-DA) purchased from Molecular Probes, Eugene, OR, USA. DCFH-DA diffuses through the cell membrane and is enzymatically hydrolysed by intracellular esterases to the non-fluorescent DCFH. Briefly, 5 mM DCFH-DA was added to the different treatments with cultured chondrocyte cells and kept in a humidified atmosphere (5% CO_2, 37°C) for 30 min, washed in PBS, trypsinised, suspended in 1 ml PBS, centrifuged at 800 g for 10 min, and finally resuspended in 2 ml PBS. Fluorescence was measured as I. F. /mg protein, using a spectrofluorometer (Perkin-Elmer) λ excitation = 485 nm, λ emission = 525 nm. The amount of protein/sample was determined, as described by Bradford [43]

4.5. Nitrite Production by Chondrocytes Cultured with IL-1 β, Quercetin, and BG Crude Extract

Nitrite was determined by adding 100 µl Griess reagent (1% sulphanilamide and 0.1% naphthyl-ethylenediamine dihydrochloride in 5% of hydrochloric acid) to 100 µl sample [44]. The optical density at λ = 570 nm was measured using a microtiter plate reader. Nitrite concentrations were calculated by comparison with respective optical densities of standard solutions of sodium nitrite prepared in medium.

4.6. Glycosaminoglycan Secretion by Chondrocytes Cultured with IL-1β, Quercetin and BG Crude Extract

The level of GAGs was measured by spectrophotometry with a solution of 1.9-dimethylmethylene blue at λ = 535 nm [45]. The amount of glycosaminoglycans was calculated from a standard curve obtained for shark chondroitin sulphate.

4.7. Prostaglandins E_2 (PGE_2) Production by Chondrocytes Incubated with Cytokine IL-1β, Quercetin and BG Crude Extract

PGE_2 was determined in the culture supernatant by enzyme immunoassay (EIA) system using a commercially available immunoassay kit (Amersham-Pharmacia, UK) according to the manufacturer's instructions. The detection limit was 1 ng/ml. The values were expressed as ng/ml PGE_2 released.

4.8. Statistical Analysis

Statistica software was used to perform a 1-Way analysis of variance, and where there were significant differences, the means were separated using the Duncan's multiple range test at $p \leq 0.05$. All the results were expressed as Mean ± S.E. for the experiments performed in triplicates.

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Data Availability Statement: All data generated are illustrated within the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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