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Does cartilage *ERα* overexpression correlate with osteoarthritic chondrosenescence? Indications from *Labisia pumila* OA mitigation

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Chondrosenescence (chondrocyte senescence) and subchondral bone deterioration in osteoarthritic rats were analyzed after treatment with the estrogenic herb *Labisia pumila* (LP) or diclofenac. Osteoarthritis (OA) was induced in bilaterally ovariectomized (OVX) rats by injecting mono-iodoacetate into the right knee joints. Rats were grouped (n = 8) into non-treated OVX+OA control, OVX+OA + diclofenac (5 mg/kg) (positive control), OVX+OA + LP leaf extract (150 and 300 mg/kg) and healthy sham control. After 8 weeks' treatment, their conditions were evaluated via serum biomarkers, knee joint histology, bone histomorphometry, protein and mRNA expressions. The LP significantly reduced cartilage erosion, femur bone surface alteration, bone loss and porosity and increased trabecular bone thickness better than diclofenac and the non-treated OA. The cartilage catabolic markers' (matrix metalloproteinase (*MMP*)-*13*, *RUNX2*, *COL10α*, *ERα*, *CASP3* and *HIF-2α*) mRNA expressions were down-regulated and serum bone formation marker, PINP, was increased by LP in a dose-dependent manner. The LP (containing myricetin and gallic acid) showed protection against chondrosenescence, chondrocyte death, hypoxia-induced cartilage catabolism and subchondral bone deterioration. The bone and cartilage protective effects were by suppressing proteases (collagen break-down), bone resorption and upregulating subchondral bone restoration. The cartilage *ERα* over-expression showed a strong positive correlation with *MMP-13*, *COL10α1*, histological, micro-computed tomography evidence for cartilage degradation and chondrosenescence.

Keywords. *ERα*; diclofenac; *Labisia pumila*; osteoarthritis; subchondral bone

1. Introduction

Articular cartilage degradation, synovitis, osteophyte formation and sclerosis are characteristic of osteoarthritis (OA) (Martel-Pelletier *et al.* 2016), which causes major disability worldwide in the postmenopausal and elderly population (Australian Institute of Health and Welfare 2017). Non-steroidal anti-inflammatory drugs (NSAIDs) and analgesics are usually prescribed to manage the pain, but it does not stop the OA progression. Prolonged NSAID use can trigger gastrointestinal and cardiovascular adverse effects (Rainsford 2006), more research studies are needed to stop or reverse the OA development. Catabolic stresses such as reactive oxygen species, inflammatory cytokines either due to mechanical stimuli or aging, estrogen-deficiency or advance glycation end can degrade and alter articular cartilage by upsetting normal chondrocyte (the only cell present in the cartilage) homeostasis, recapitulates molecular program and induces aging chondrocyte phenotypes (Aigner *et al.* 2007).

The subchondral bone changes during OA may occur before cartilage degradation, although contrary results have been reported (van der Kraan and van den Berg 2007; Burr and Gallant 2012). The thinning of subchondral bone plate

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and perforation of trabecular bone were reported in OA with bone changes significantly worse in postmenopausal subjects (Intema *et al.* 2010; Burr and Gallant 2012). However, treatment with a selective estrogen receptor (ER) modulator significantly ameliorated cartilage erosion and turnover in postmenopausal OA rats (Christgau *et al.* 2004). Estrogen enhances glycosaminoglycan synthesis, protects collagen from degradation by tumor necrosis factor-α and inhibits bone resorption (Martín-Millán and Castãneda 2013; Maneix *et al.* 2014).

Labisia pumila (LP) is a tropical plant traditionally used as herbal medicine for women to facilitate childbirth and post-partum care (Burkill 1935). LP possesses anti-inflammatory, antioxidative and bone-protective properties against osteoporosis (Fathilah *et al.* 2012; Karimi *et al.* 2013). This study demonstrated the therapeutic use of LP compared with diclofenac to delay osteoarthritic chondrocyte maturation and subchondral bone alteration in mono-iodoacetate OA-induced ovariectomized (OVX) OA rat models. This study also proposes that cartilage *ER*α over-expression may be another potential biomarker for chondrosenescence and cartilage degradation.

2. Materials and methods

2.1 *Plant material extract and high-performance liquid chromatography (HPLC) profiling*

Dried LP leaves (HERBagus Trading, Penang, Malaysia) were ground and macerated in 50% ethanol (v/v) at a 1:10 ratio (w/v) at room temperature for 72 h with mechanical shaking. The filtrates were concentrated by using a rotary evaporator and dried in an oven. The extracts were stored at 4°C until further use.

Ultra-HPLC (UHPLC) analysis of the extract was performed using an Ultimate 3000 UHPLC system (Thermo Fisher, USA) with C18 column, 5 μm, 250 mm × 4.6 mm (Luna Phenomenex, USA) maintained at 30°C and a DAD detector (Thermo Fisher, USA) set at 264 nm. The mobile phase consisted of (A) 0.1% formic acid in deionized water and (B) acetonitrile. The gradient was: 90% A at 0–10 min, 90–40% A at 10–30 min, 40% A at 30–40 min, 40–90% A at 40–50 min and 90% A at 50–60 min. The injection volume was 10 μL and the flow rate was 0.25 mL/min. The compound identification was based on the individual standards' peak retention times; myricetin (Sigma-Aldrich, USA, PubChem CID: 5281672) and gallic acid (Santa Cruz Biotechnology, USA, PubChem CID: 370).

2.2 *Animal study*

Forty female Sprague–Dawley rats (10–12 weeks old) purchased from the Faculty of Veterinary Medicine, Universiti Putra Malaysia were acclimatized for a week at room temperature under 12-h light–dark cycles and allowed free access to water and rat chow (Gold Coin, Malaysia). The animal study protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (UPM/IACUC/AUP-R050/2015). Estrogen-deficiency was induced by bilateral ovariectomy on anesthetized rats under sterile conditions (Høegh-Andersen *et al.* 2004). Sham procedures were performed on the healthy controls. OA was induced by monosodium iodoacetate (MIA) injection (Sigma-Aldrich, USA) (60 mg/mL) into the right knee using a 26 G sterile needle, 2 weeks after ovariectomy. The healthy rats received normal saline instead of MIA. The rats were divided into five groups ($n = 8$); sham (healthy), OVX+OA (non-treated), DIC (OVX+OA treated with diclofenac 5 mg/kg body weight), LP150 (OVX+OA treated with LP 150 mg/kg body weight) and LP300 (OVX+OA treated with LP 300 mg/kg body weight). The doses were based on a previous toxicity study (Singh *et al.* 2009). The dried extracts were diluted in distilled water and administered orally daily for 8 weeks, starting 2 weeks after MIA induction. Diclofenac was used as the positive control as it is the NSAID commonly prescribed for OA. The femur and tibia bones, as well as blood serum were collected and kept at −80°C until further analysis.

2.3 *Histology*

The femur bones of OA rats were fixed in 10% formalin neutral buffered solution (Sigma-Aldrich) for 2 weeks, subsequently decalcified with 10% formic acid for 1 week, dehydrated with gradient ethanol using an automated tissue processor, embedded in paraffin, sectioned into 6 μm thickness and stained with safranin O (SO) (ScienCell Research Laboratories staining kit, California). The histopathological changes were analyzed single-blindly using the OARSI score (Pritzker *et al.* 2006). Each section was graded; 0 (cartilage morphology intact), 1 (surface intact but with changes in chondrocyte and cartilage morphology), 2 (surface discontinuity), 3 (vertical fissures), 4 (erosion), 5 (denudation) and 6 (deformation) and staged; 0 (no OA activity), 1 (<10%), 2 (10–25%), 3 (25–50%) and 4 (>50%). The grade was scored based on the mean cartilage damage depth while the stage was scored according to the mean percentage of area damage. A final score was then calculated (grade × stage) to minimize the bias.

2.4 *Bone microarchitecture*

Right femur and tibia bones were scanned using a SkyScan 1076 micro-CT scanner (SkyScan, Kontich, Belgium) at 72 kV voltage, 130 μA tube current and 18 μm pixel size with a 0.5° rotation step of 360°. The images were reconstructed using SkyScan NRecon software (version 1.6.3.3). Regions of interest at the subchondral and metaphyseal

trabecular regions were analyzed using SkyScan CTAnalyser software (version 1.11.0.0) to calculate bone morphometric parameters which are bone volume over total volume (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and total porosity (po(tot)). Bone mineral density (BMD) was quantified by calibrating computed tomography (CT) images with calcium hydroxyapatite phantom SP-4002 (SkyScan, Kontich, Belgium).

2.5 *Serum biochemical parameters*

Serum matrix metalloproteinase-13 (*MMP*-13) and pro-collagen type-I N terminal (PINP) levels were measured by enzyme-linked immunosorbent assay kits according to the manufacturer's instructions (Elabscience Biotechnology Co., China).

2.6 *Gene-expression analysis (quantitative polymerase chain reaction (qPCR))*

Total RNA was isolated from the right tibia with a Qiazol reagent and RNeasy lipid tissue mini kit (Qiagen, Germany). The RNA samples were reverse transcribed with a RT2 First Strand kit (Qiagen, Germany) and the gene of interest was quantified by a Custom RT2 Profiler PCR Array using RT2 SYBR Green qPCR Mastermix (Qiagen, Germany). Thermal cycling and fluorescence detection were performed using a CFX96 Touch qPCR System (Bio-Rad, USA). The relative mRNA levels were calculated using the $2^{-\Delta CT}$ method, which were normalized to GAPDH (NM_017008).

The primer sequence for designing the plate for gene-expression analysis and the gene accession numbers (gene name; accession number) are provided in supplementary table 1.

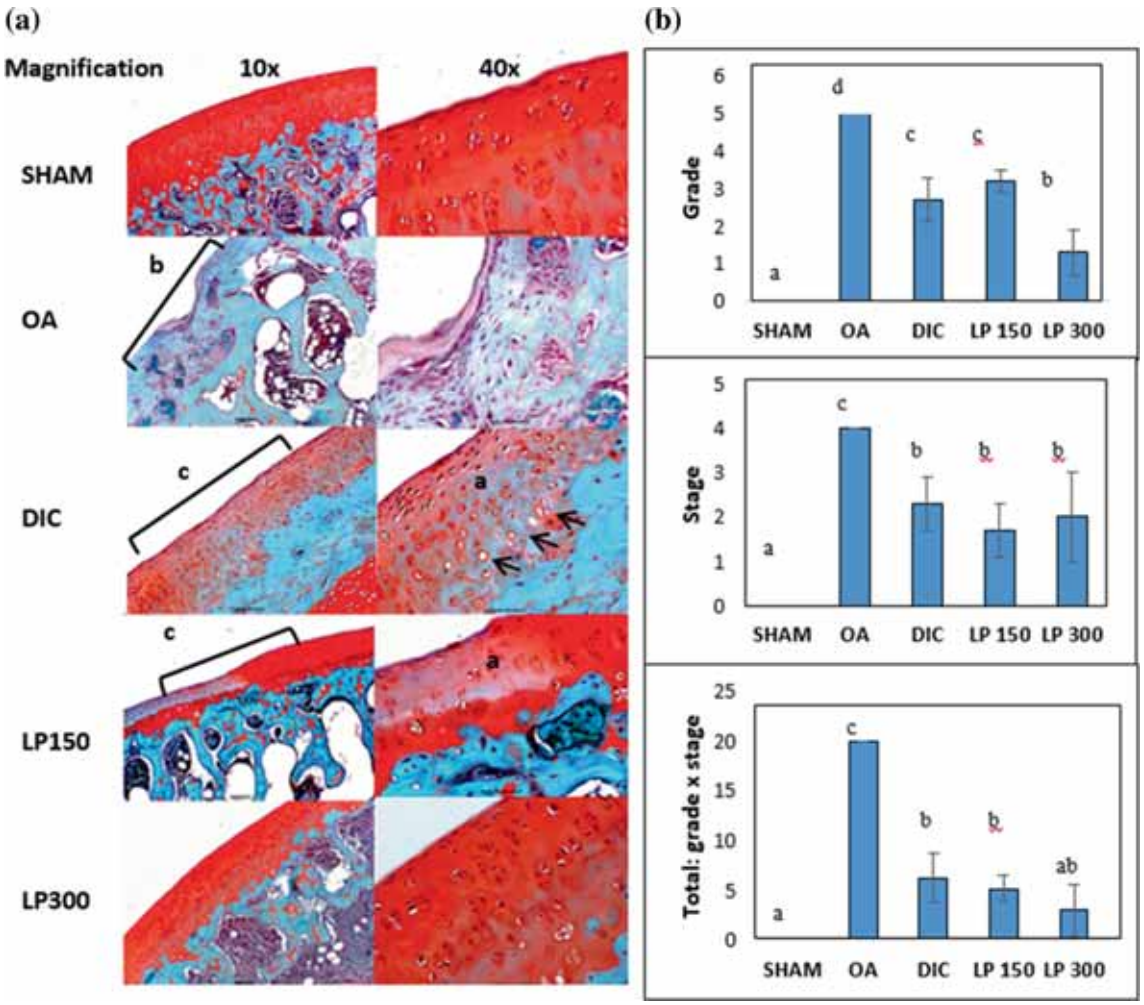

Figure 1. (**a**) Histology photomicrographs stained with SO after 8 weeks of treatment (magnification: 10× and 40×). Non-treated OA showed severe cartilage loss exposing subchondral bone surface (**b**). DIC- and LP150-treated rats showed intact cartilage with reduced SO intensity (**a**), superficial fibrillation (**c**) and chondrocyte death (arrows). LP300 showed smooth cartilage surface and similar chondrocyte orientation to the healthy sham. (**b**) The OA severity of non-treated rats was significantly reduced by DIC and LP, evaluated by the OARSI score. Results are presented as mean ± SD. OA, non-treated osteoarthritis; DIC, diclofenac 5 mg/kg; LP150, LP 150 mg/kg; LP300, LP 300 mg/kg.

2.7 *Statistical analysis*

All data were expressed as mean ± standard deviation (SD). Data were analyzed using one-way analysis of variance for statistical significances at $p<0.05$ followed by the Duncan's post-hoc test using SPSS 22.0 software. The correlation was analyzed using Pearson's correlation analysis.

3. Results

3.1 *HPLC profile*

The HPLC analysis of LP identified gallic acid (17.4 min) and myricetin (39.0 min) as the main compounds, by comparing the individual peak retention times with that of the pure standard (supplementary figure 1). The evaluation of the standard calibration curve showed that 1 g of LP leaf extract contained 2.93 mg gallic acid and 0.89 myricetin.

3.2 *Histological evaluation*

The fissures, extensive cartilage erosion with over 50% cartilage loss exposing calcified cartilage and subchondral bone surfaces in the non-treated OA-induced control rats (figure 1a) were mitigated by treatments with LP and DIC. The LP 300 mg/kg treatments produced the best results with a smooth articular cartilage surface, chondrocyte morphology and intense SO staining similar to those of the healthy sham control rats. The DIC-treated rats showed intact cartilage with less intense SO staining, condensed collagen fibers, superficial layer fibrillation and empty lacunae, while rats treated with LP150 showed minor superficial fibrillation and low intensity red staining in <25% of total cartilage area. Semi-quantitative OARSI scores indicated that all treatments significantly reduced the OA severity (figure 1b).

3.3 *Bone microarchitecture evaluation*

The micro-CT images revealed structural deterioration and bone surface alteration in the non-treated OVX+OA control rats (figure 2), which were mitigated by all the treatments. The LP300 and DIC rats had a smooth femoral bone surface almost similar to that of the healthy sham control rats. The bone changes occurred mainly on the metaphyseal trabecular rather than the epiphyseal region (figure 3) for both the femur and tibia. The LP300 rats significantly protected against the trabecular bone changes in the OVX+OA-induced rats especially for the BV/TV and po(tot) in the tibia epiphysis and the Tb.Sp in the tibia metaphysis. The non-treated OVX+OA control rats had significantly deteriorated bone volume fraction (BV/TV), po(tot), Tb.Sp, and BMD compared with the healthy sham control. Interestingly, the LP300 rats had significantly increased Tb.Th even though

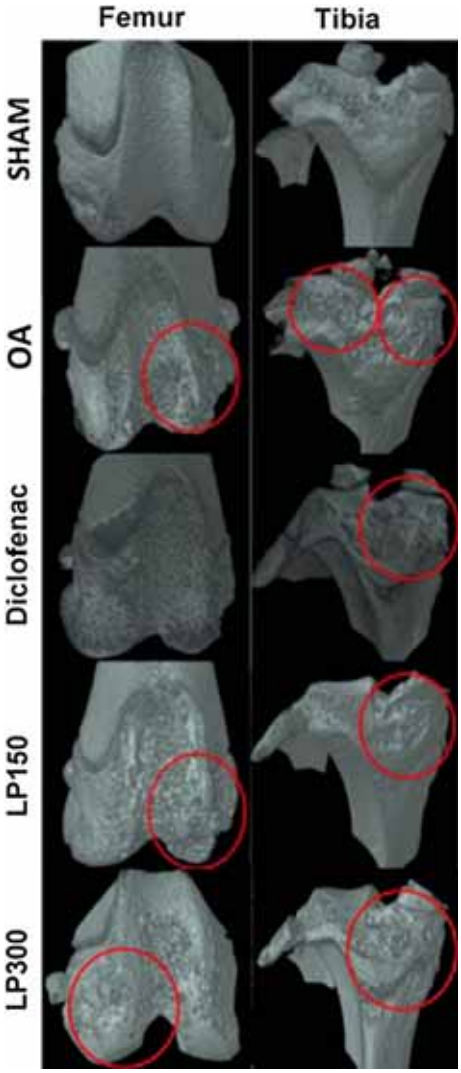

Figure 2. Three-dimensional images of subchondral bone surface of femur and tibia from micro-CT analysis. Sham exhibited subchondral bone contours with a smooth surface while extended subchondral bone lesions (circle) were observed in non-treated OA. Treatment with DIC and LP ameliorated the bone lesions compared with non-treated OA. OA, non-treated osteoarthritis; DIC, diclofenac 5 mg/kg; LP150, LP 150 mg/kg; LP300, LP 300 mg/kg.

the Tb.Th was not significantly altered in the non-treated OVX+OA control rats. The LP150 rats had significantly improved tibia metaphysis Tb.Sp, while the DIC rats showed no improvement from the non-treated OVX+OA control rats.

3.4 *Serum and gene biochemical parameters*

Serum PINP levels did not change significantly in the OA rats compared with healthy sham, but was significantly increased in all treated rats (figure 4a). OA significantly

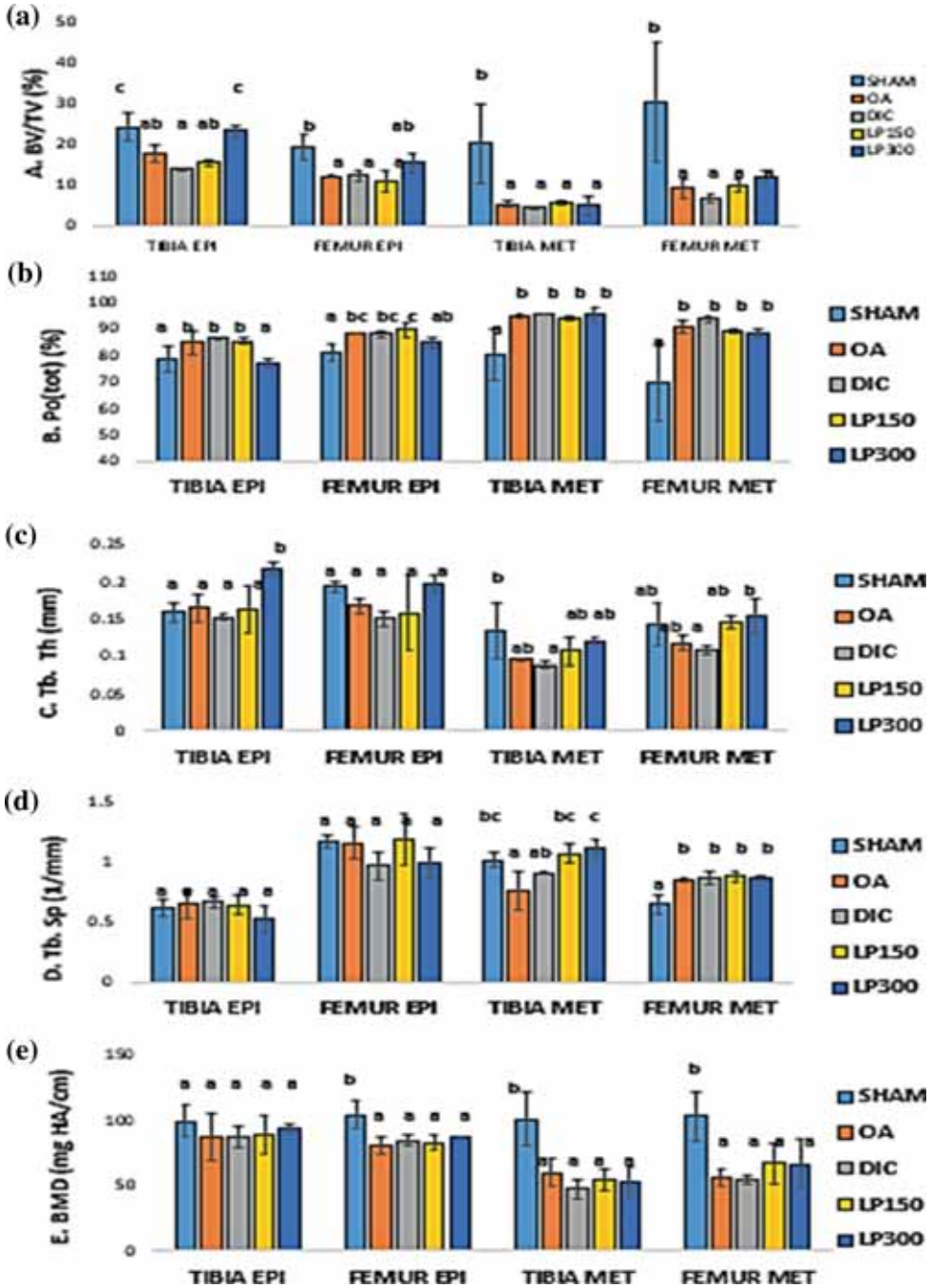

Figure 3. Bone microarchitecture parameters of the entire epiphyseal and metaphyseal trabecular in tibia and femur after 8 weeks of treatment: (**a**) BV/TV (bone volume fraction), (**b**) Tb.Th, (**c**) Tb.Sp, (**d**) total porosity and (**e**) BMD. Results are presented as mean ± SD. Means with different letters are significantly different. OA, non-treated osteoarthritis; DIC, diclofenac 5 mg/kg; LP150, LP 150 mg/kg; LP300, LP 300 mg/kg.

increased the *MMP*-13 protein levels, and the mRNA expressions of *MMP*-13, tissue inhibitor of metalloproteinase (*TIMP*)-1 and -3, *HIF*-2α, *COL10α1*, *RUNX2*, *ERα* and *CASP3* (figure 4). The LP300 significantly ameliorated all biomarker changes induced by OVX-OA (*MMP*-13, *TIMP*-3, *RUNX2*, *ERα*, *COL10α1* and *CASP3*), although the reductions were not significant enough for *HIF*-2α and *TIMP*-1. The LP150 showed significant reduction in all evaluated biomarkers except for *TIMP*-1 and -3 and *RUNX2*, indicating dose-dependency effects. DIC also significantly reduced all the biomarkers monitored except for *RUNX2* and *TIMP*-3. There were no significant differences in the *ACAN* mRNA level between all treated OA rats and OVX (shown in supplementary figure 2).

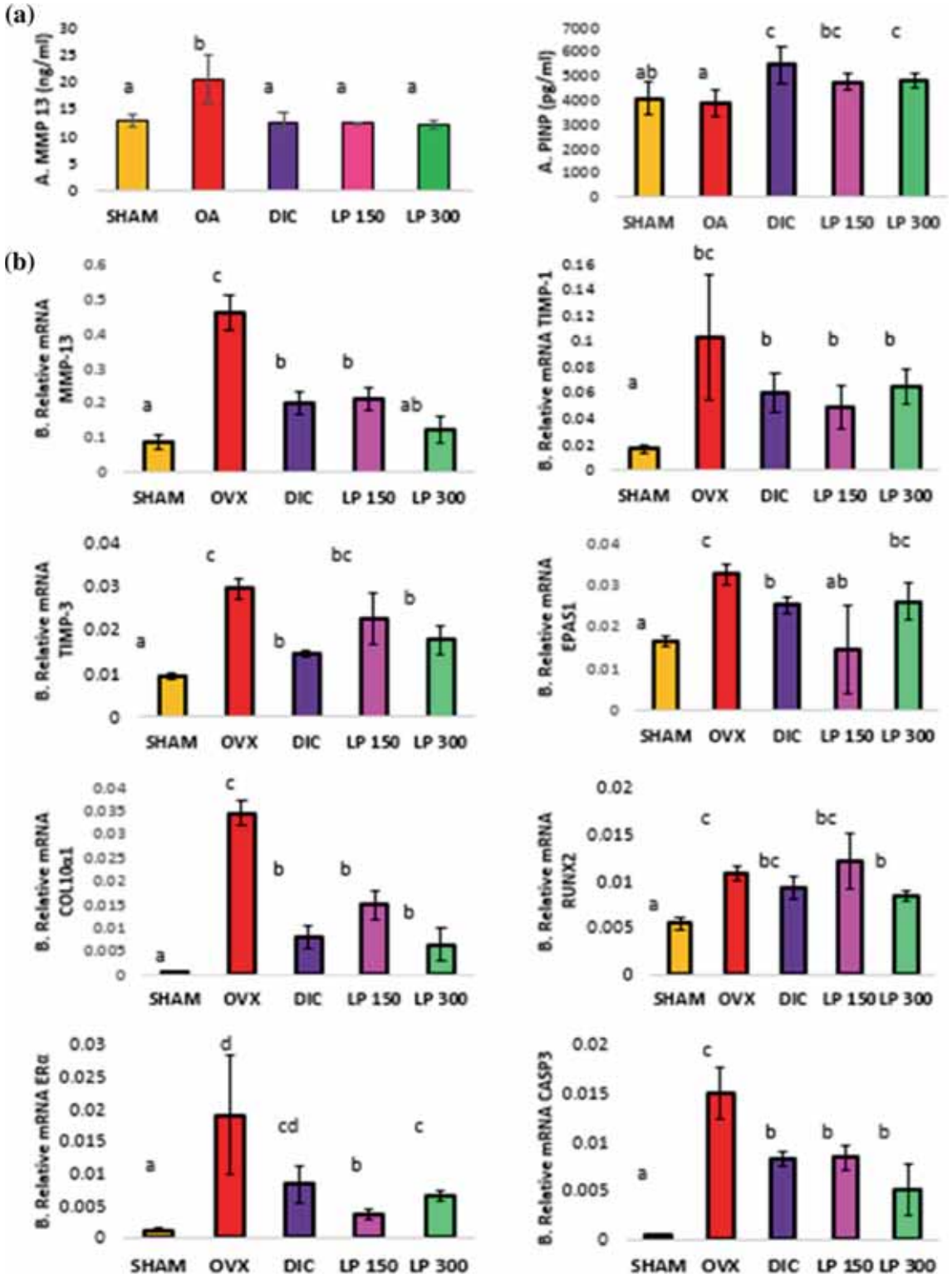

Figure 4. (**a**) Serum *MMP*-13 and PINP, (**b**) relative mRNA expressions of cartilage *MMP*-13, *TIMP*-1, *TIMP*-3, *HIF*-2α, *COL10α1*, *RUNX2*, *ERα* and *CASP3* after 8 weeks of treatment. Results are presented as mean ± SD. Means with different letters are significantly different ($p<0.05$, $n = 6$). OA, non-treated osteoarthritis; DIC, diclofenac 5 mg/kg; LP150, LP 150 mg/kg; LP300, LP 300 mg/kg.

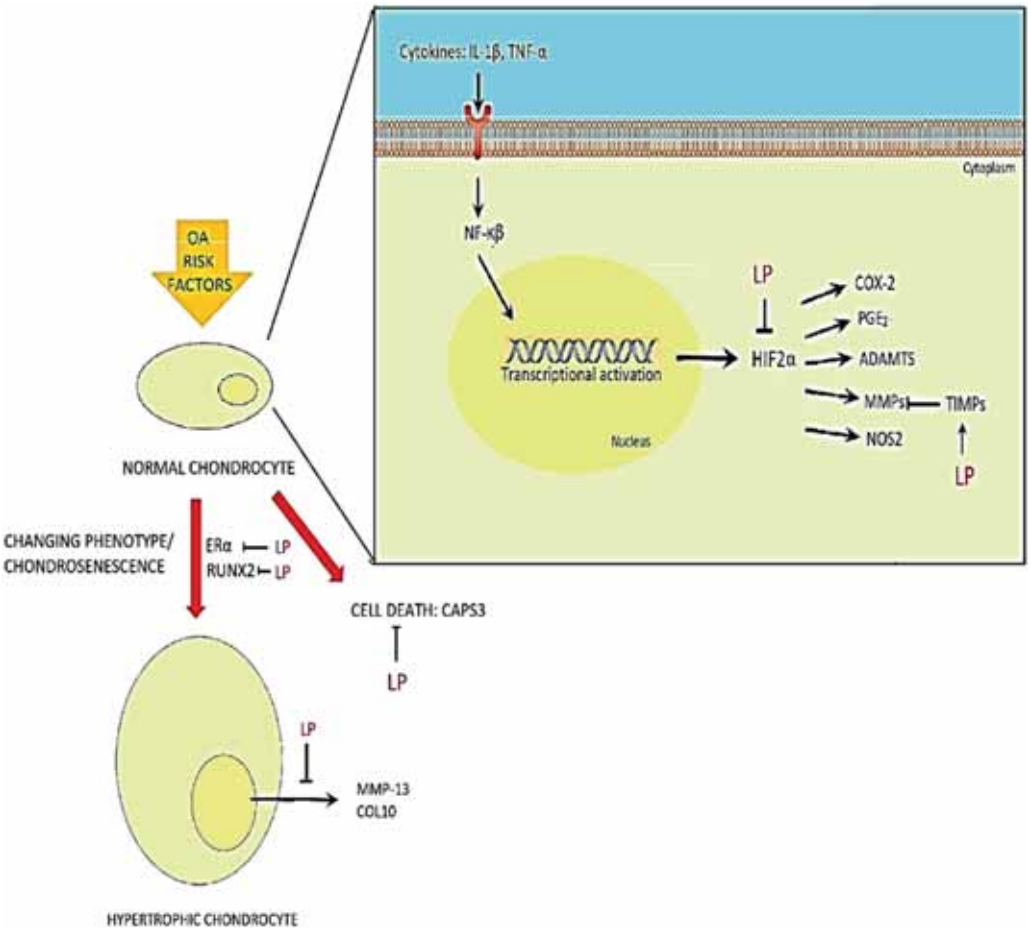

Figure 5. Proposed signaling pathway of LP in inhibiting chondrosenescence in postmenopausal osteoarthritis. LP inhibits the hypoxia biomarker *HIF*-2α, thus preventing production of cartilage catabolic markers COX-2, PGE_2, ADAMTS, *MMP*s and NOS2. The excessive catabolic activity induces stress and accelerates the aging-chondrocyte phenotype. LP prevents catabolic activity and controls chondrosenescence by reducing markers *ER*α, *RUNX2*, COL10 and *MMP*-13.

4. Discussion

Bilateral ovariectomy is more severe than normal menopause because there are no ovaries to continue producing small levels of hormones unlike natural menopause. Estrogens protect against pro-inflammatory cytokines, which causes chondrocyte death, induces bone resorption, and suppresses bone formation. Thus, ovariectomy causes rapid cartilage degradation and bone deterioration (Kameda *et al.* 1997; Martín-Millán and Castãneda 2013). MIA inhibits glyceraldehyde-3-phosphatase dehydrogenase to disrupt chondrocyte glycolysis, causing chondrocyte apoptosis, synovial inflammation and neovascularization, and subsequently cartilage degeneration and subchondral bone alteration (Guzman *et al.* 2003), which were apparent in the histological and micro-CT images here. Osteoporosis induced by bilateral ovariectomy triggers severe estrogen-deficiency, osteoporosis and osteoarthritis development (Høegh-Andersen *et al.* 2004), which was further aggravated by the intra-articular MIA injection to imitate human OA.

Micro-CT is the current state-of-the-art technology for assessing bone microarchitecture in rodents, with the benefit of being non-destructive, to enable the samples to be divided equally for the subsequent histological and gene-expression analysis. A strong correlation between micro-CT and bone histology histomorphometry exists in humans (Akhter *et al.* 2007) and animals (Bonnet *et al.* 2009). The micro-CT images showed severe bone damages in the metaphyseal (osteoporosis) and subchondral (articular contour distortion and congealing) regions of the OVX+OA rats.

LP has anti-inflammatory and antioxidant properties (Karimi *et al.* 2013) which may have helped in suppressing the OVX+OA-induced inflammation and oxidative stress, to protect the cartilage and subchondral bone against degradation. The LP contains mainly myricetin, gallic acid, flavonoids, polyphenols and triterpenes (supplementary figure 1) (Chua *et al.* 2011, 2012; Karimi *et al.* 2011). Myricetin and gallic acid helped in ameliorating cartilage degradation in rabbit and murine arthritis models (Wen *et al.* 2015; Yuan *et al.* 2015). The myricetin suppressed prostaglandin E2 (PGE_2), nitric oxide and MMP levels in human

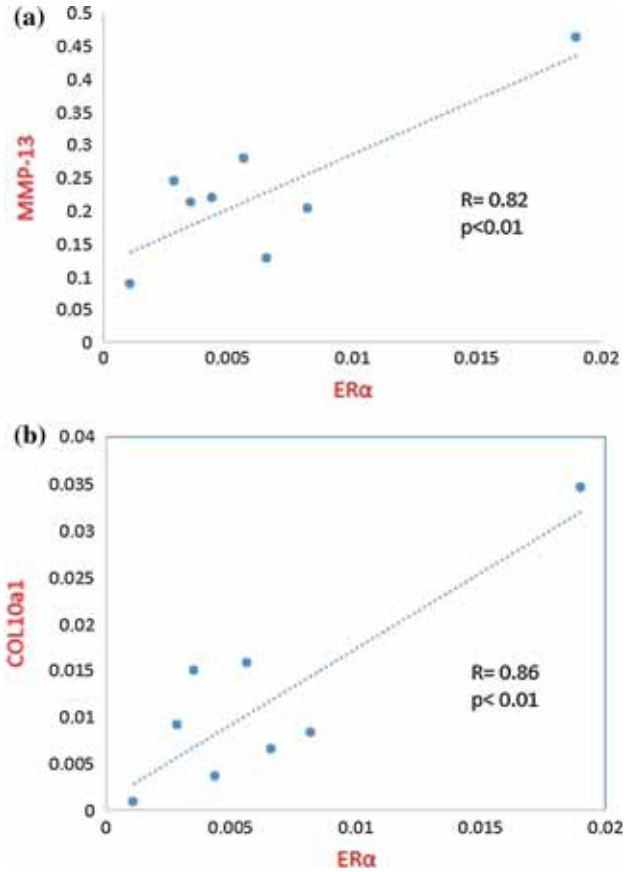

Figure 6. Correlation between (**a**) mRNA *ERα* and *MMP*-13 and (**b**) between mRNA *ERα* and *COL10α1*.

chondrosarcoma cells (Wang *et al.* 2016) and gallic acid reduced the nitric oxide, PGE_2 and interleukin-6 release from lipopolysaccharide-aggravated murine macrophages (BenSaad *et al.* 2017). The LP extract ameliorated cartilage degradation in OA-induced rats (Madzuki *et al.* 2018). Other flavonoids and polyphenols in the LP extract may also have contributed to the prevention of cartilage degradation via their reported antioxidant and anti-inflammatory properties (Chua *et al.* 2012).

The suggested signaling pathway of LP is proposed in figure 5. Estrogen is known to ameliorate joint and bone disease and deletion of ERs in female mice caused cartilage damage, osteophytosis and joint subchondral bone changes (Martín-Millán and Castãneda 2013). However, the function of cartilage ER alpha (*ERα*) is still being debated. Arthritic mice treated with 17β-estradiol (E2) had significantly reduced cartilage erosion and degradation with or without joint *ERα* expression, demonstrating that joint *ERα* is not involved in the estrogenic protection mechanism against joint destruction but required only against synovitis (Engdahl *et al.* 2014). The ER co-localized with caveolin (membrane stress-protector proteins that potentiate senescence by inhibiting MAP kinase) extensively but not completely (Razandi *et al.* 2002; Baker and Tuan 2013), which suggests that *ER* hyper-expression may be related to chondrosenescence. In chondrocytes, both ERα and ERβ types are equally expressed (Ushiyama *et al.* 1999). High doses of estradiol treatment in adult mice attenuated growth plate chondrocyte proliferation in control mice causing reduced growth plate height, but not in cartilage-specific *ERα*-inactivated mice (Börjesson *et al.* 2010). It suggests that *ERα* accelerates the senescence and proliferative exhaustion of growth plate chondrocytes. The synovial estradiol level was strongly correlated with the OA severity in 21 patients (Tsai *et al.* 1992), with increased ER bindings in the medial compartment of the femoral condylar and tibia plateau cartilages, suggesting that excessive synovial estradiol and higher ER bindings may be involved in the knee OA development, especially in postmenopausal women. This study repeatedly ($n = 6$) showed increases in joint *ERα* expressions by 16-fold in the non-treated OVX+OA control rats that was significantly reduced with the LP and diclofenac treatments (figure 4). This study also showed a strong positive relation between the rats' cartilage *ERα* mRNA expression with *MMP*-13 ($R = 0.86$, $p<0.01$) and *COL10α1* ($R = 0.82$, $p<0.01$) in figure 6, which suggest that cartilage *ERα* expressions may be an indicator for chondrosenescence and cartilage status in these rats.

The changing chondrocyte phenotype in the OVX+OA rats showed similarities to the endochondral ossification process, where the resting healthy cartilage chondrocytes had transformed into hypertrophic chondrocytes and mature chondrocytes, expressing markers such as *COL10α* and *MMP*-13 (Reynard and Loughlin 2013). This OA chondrocyte senescence (chondrosenescence) caused a total chondrocyte function loss (Aigner *et al.* 2007). The *MMP*-13 acted in a feedback loop, to degrade the main cartilage building material, collagen (Neuhold *et al.* 2001).

OA often starts with joint defects caused by injury or overload, where the cartilage damage is noticeable at impact loads of 25 MPa (Repo and Finlay 1977). Even ostensible minimal injury can increase the joint degradation risk, together with other risk factors such as age, hormone deficiency, metabolic syndromes and genetics. These factors produce pro-inflammatory cytokines that play major roles in joint catabolism (Liu-Bryan and Terkeltaub 2015). Additionally, pro-inflammatory cytokines are able to induce *HIF*-2α (also designated EPAS1) production (Tanaka *et al.* 2002). *HIF*-2α directly regulates catabolic factors such as *MMP*-1, *MMP*-3, *MMP*-13, *MMP*-9, ADAMTS4, COX-2, PGE_2 and NOS2 (Yang *et al.* 2010). The LP suppressed catabolic enzymes and *HIF*-2α levels (Madzuki *et al.* 2018) to mitigate cartilage degradation. Moreover, *TIMP*-1 inhibits the production of *MMP*s, while *TIMP*-3 inhibits the production of both *MMP*s and ADAMTSs (Arpino *et al.* 2015). The high level of *TIMP*s in OA (Naito *et al.* 1999) reflects the increased levels of *MMP*s (Ishiguro *et al.* 1999).

Bone remodeling is increased in early OA by three–five fold with an average of over 3.50 μm/day compared with the normal rate of 0.70–1.00 μm/day, thus increasing

subchondral bone porosity, reduced bone density, causing transient bone loss, altered bone surface and thinning of the subchondral plate (Burr and Gallant 2012). Bones remain osteopenic during OA which weakens the bone structure (Epstein 2005). Treatment of OVX+OA rats with LP and diclofenac significantly inhibited bone volume (BV/TV) loss and trabecular porosity, while increasing the trabecular thickness. This is supported by the enhanced serum PINP by LP and diclofenac which indicated new bone formation.

The pro-inflammatory cytokines that increase with aging, induce the catabolic enzyme production to favor chondrocyte stress and cartilage degradation. In response to repair the damage, the chondrocytes hyper replicate and proliferate (Mobasheri *et al.* 2015). However, age-related mitochondrial dysfunction, oxidative stress and the altered cell signaling caused the chondrocytes to accelerate the cartilage matrix degradation, chondrocyte death and chondrosenescence (Loeser *et al.* 2016), which are displayed in OA. The chondrosenescence goes through a chondrogenic differentiation pathway to enter pre-hypertrophic, hypertrophic and maturation phase which is directly promoted by *RUNX2* (Reynard and Loughlin 2013). The *RUNX2* was down-regulated by LP and diclofenac, thus delaying chondrosenescence and subchondral bone alterations. Attenuating the articular chondrocytes *RUNX2* significantly helped in reducing the *COL10α1* (a specific hypertrophic chondrocyte marker) and *MMP*-13 (Liao *et al.* 2017). The *RUNX2* is involved in mineralizing cell differentiation and increased subchondral bone plate mineralization during OA bone remodeling (Zhang *et al.* 2006; Burr and Gallant 2012).

5. Conclusions

LP and diclofenac showed good protective effects against OA via retarding chondrosenescence, chondrocyte death, hypoxia-induced cartilage degrading enzymes and subchondral bone deterioration. The cartilage *ERα* over-expression showed a strong positive correlation with *MMP*-13, *COL10α1*, histological and micro-CT evidence for cartilage degradation and chondrosenescence.

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Compliance with ethical standards

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted; Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia approval (UPM/IACUC/AUP-R050/2015).

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