Effect of Purple Corn (Zea mays L.) Extracts on Cartilage Degradation Mediated by Advanced Glycation End Products

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ABSTRACT

Osteoarthritis (OA) is degenerated disease significantly impacts the quality of life of the elderly. The prominent feature is the destructive of articular cartilage which mediated by the inflammatory cytokines. Recently, advanced glycation end products (AGEs) which are highly found in diabetes-related matrix protein modification in cartilage. The accumulation of AGEs in cartilage matrix is associated with the development of OA. Pharmacological treatments of OA cause many adverse effects thus alternative medicine has been interested. Anthocyanins have been demonstrated many health benefits such as anti-diabetic and anti-inflammatory. In this study, we investigated the chondroprotective effects of anthocyanins from purple corn extracts. The HPLC analysis found the difference amount of anthocyanins in purple corn extracts. We found that purple corn extracts effectively blocked the glycosaminoglycans release from the cartilage explants. Therefore, the effects of anthocyanins and inflammatory mechanism need to be further investigated.

Keywords: Osteoarthritis, Extracellular matrix, Advanced glycation end products
Introduction

Noncommunicable diseases (NCDs) are mostly caused from unsuitable lifestyle factors such as eating, sleeping, relaxing, exercising including to other activities. World health organization (WHO) also reported that NCDs population also tends to increase in the future and have a higher rate of sickness and death than other diseases on world population. In Thailand during the year 2017, NCDs population’s death rates still are up to 70 percentage when compared to past years (World Health Organization [WHO], 2017). There are many diseases in NCDs such as Diabetes mellitus (DM), Hypertension, Coronary artery disease, Stroke, Chronic kidney disease, Dyslipidemia, Cancer, Cirrhosis and Alzheimer’s disease (Thai Health Promotion Foundation [ThaiHealth], 2017). However, DM is one of the most common NCDs which results in high prevalence of diabetes. Diabetes population also have an increased risk of developing to health problems such as diabetic retinopathy, cardiovascular disease, lower limb amputation, diabetic neuropathy, kidney failure (WHO, 2014). Recently, there are many evidences demonstrated that hyperglycemia is one of the main causes of diabetes mediated osteoarthritis (Mendes et al., 2015).

Previous study showed an association between diabetes and development of osteoarthritis (OA) by comparing among DM patients and non-DM patients. They showed that the development and severity of OA were found in DM patients and the risks associated with joint replacement surgery was higher than non-DM patients (Louati et al., 2015). Hyperglycemia condition which is common feature of DM can be caused of the major health complications. Aldehyde group of glucose can form covalent adducts with proteins, lipids, and nucleic acids through a non-enzymatic process or glycation that led to an elevated formation of irreversible advanced glycation end products (AGEs). AGEs play an important role in the pathogenesis of diabetic complications contributing to OA and highly express in plasma, synovial fluid and cartilage tissue (Saudek, Kay, 2003).

The slow turnover or long lifetime of collagen in cartilage leads to the AGEs accumulation. The increased AGEs rate in cartilage collagen were higher than skin collagen (Verzijl et al., 2000) and changed properties of collagen such as loss of solubility and flexibility bring about to the increase of mechanical stress (Singh et al., 2014; Eymard et al., 2015). Moreover, the interaction of receptor for advanced glycation end products (RAGE) with AGEs can induce inflammation via a various number of signaling cascades, including the MAPKs, JAK/STAT pathway and NADPH oxidase (Ott et al., 2014). AGEs-mediated signaling via RAGE activate transcription factors, such as nuclear factor (NF-kB) or IFN-stimulated response elements (ISRE) followed by an increased expression of pro-inflammatory cytokines (Ott et al., 2014).

The proinflammatory cytokines such as interleukin-1beta (IL-1β) and tumor necrosis factor (TNF-α) (Hofmann et al., 2002) induce chondrocytes to secrete matrix metalloproteinases (MMPs) which further degrade cartilage extracellular matrix and contributing to OA (Syggelos et al., 2013; Miyata et al., 1998).

In the present, there is no complete treatments of OA but only to manage symptoms or change physical activity behavior to decrease the joint movement. Because of the adverse effects of the pharmacological treatment, the natural products are interested for alternative therapeutic agents against OA. We are interested in purple corn (Zea mays L.) that had less starch and a lower glycemic index than their white counter parts. This plant is considered to be less toxic and good for DM patients and dieters (Lao, Giusti, 2016). Apart from hypoglycemic agent, purple corn also contains...
the phenolic phytochemicals such as phenolic acids and flavonoids that are an anti-inflammatory agents. The remarkable of flavonoid groups are anthocyanins that are water soluble pigments responsible for the purple, blue and red color of plant tissues (Kamiloglu et al., 2015) such as cyanidin-3-glucoside (C3G), pelargonidin-3-glucoside (Pg-3-glc), peonidin-3-O-glucoside (P3G) and malvidin-3-glucoside (M3G).

Previous studied showed the effects of anthocyanins in anti-inflammatory of arthritis rat model and IL-1β-induced bovine nasal explants, there was a decrease in the rate of degradation of both proteoglycan and type II collagen (Jean et al., 2012). Delphinidin, an aglycone anthocyanin, inhibited IL-1β-induced expression of COX-2 and production of PGE2 in human chondrocytes by preventing NF-kB phosphorylation (Haseeb et al., 2013). Besides Sumac leaves containing anthocyanins decreased the production of key free radical molecules released during degenerative OA such as nitric oxide (NO), reactive oxygen species (ROS), glycosaminoglycans (GAGs), and prostaglandins (PGE 2) in IL-1β-induced human chondrocyte (Panico et al., 2009). Malvidin decreased expression levels of pro-inflammatory cytokines such as TNF-α and IL-1β and MMPs in OA rats via the inhibition of NF-κB (Dai et al., 2017).

The accumulated studies already suggest an anti-cartilage degradation, MMPs expression and anti-inflammation of anthocyanins both on animal model and proinflammatory cytokines induced chondrocytes model but the effects of anthocyanins on AGEs-induced OA still have not been extensively studied. Thus, in this study we are interested to investigate the chondroprotective effects of purple corn extracts on AGEs induced cartilage.

Objective of the study

The objective of this study was to investigate the effect of purple corn extracts on AGEs-induced porcine cartilage degradation.

Materials and methods

Purple corn extracts preparation

Purple corn (Zea mays L.) were planted and harvested in 2017 from Amphoe Sanpatong, Chiang Mai, Thailand. Purple corn were extracted by 0.1 N HCL and methanol (20:80) at RT for 24 hours, purple corn to methanol ratio (P/E, 1:10) and then evaporated in vacuo at 40-45°C and freeze-dried to obtain dry extracts.

High performance liquid chromatography (HPLC) analysis

Anthocyanin contents in methanolic purple corn extract were analyzed by using an Agilent 1260 series HPLC (Agilent Technologies Ltd.), equipped with a binary pumping system. Modified from Chromadex method, samples (10 µl) were injected into a Zorbax Eclipse Plus C18 column (4.6×100 mm, particle size 3.5 m). The mobile phase consisted of water/formic acid (90:10, v/v) (A) and acetonitrile (B). The gradient program was accomplished at 35 °C: initial time, 4% solution B; 8 min, 15% solution B; 24 min, 80% solution B; 30 min, 4% solution B at a flow rate 1.0 ml/min. Major individual compounds of extracts were analyzed at a wavelength of 530 nm. The anthocyanin standard compounds, Cyaniding-3-O-glucoside (C3G), Peonidin-3-O-glucoside (P3G), Pelargonidin-3-glucoside (Pg-3-glc) and Malvidin-3-glucoside (M3G) were purchased from Sigma. The peak area of the extracts were calculated using calibration curve which constructed by injecting the reference standards concentration range 0-50 µg/ml.
Porcine cartilage explant preparation

Porcine articular cartilage from the metacarpophalangeal joints of 6-8 month old of pigs was dissected into 25 cm³ discs. Then the cartilages were incubated in DMEM containing penicillin/streptomycin 5% fetal calf serum (FCS) for 30 min (at 37°C, 5% CO₂). The cartilages were incubated in refresh medium 24 h for sterile checking. After that, cartilages (30-35 mg/well) were cultured in 24 well-plates in DMEM for 24 h (at 37°C, 5% CO₂).

Optimization of AGEs concentrations

Cartilage discs were treated with 6.25-50 µg/ml of AGEs (EMD Millipore. Corp., Billerica, MA, USA). Culture media were collected and replaced on day 0, 7, 14, 21, 28 and 35 for measurement the release of hyaluronic acid (HA) and glycosaminoglycans (s-GAG). Then cartilage tissues on day 35 were digested with 10 units papain for analysis of the uronic acid remaining.

Effect of purple corn methanolic extracts on AGE induced cartilage degradation

Cartilage discs were co-treated with 25 µg/ml of AGEs and various concentration of crude extracts (6.25-25 µg/ml). Culture media were collected and replaced on day 0, 7, 14, 21, 28 and 35 for measurement the HA and s-GAG release. Then cartilage tissues on day 35 were digested with 10 units/ml of papain before analysis of the uronic acid remaining.

Measurement of s-GAG levels

Release of s-GAG in the conditioned culture medium from all culture conditions was measured by the dimethyl-methylene blue (DMMB) assay. Briefly, 200 µl of DMBM were added to 50 µl of Standard chondroitin sulfate-C (0-40 µg/ml) or culture medium. The complex of DMBM and s-GAG was measured by microtiter plate reader (MULTISKAN Ex, ThermoScientific) at 520 nm. And the amounts of s-GAG were determined from the standard curve as well as shown as % s-GAG release was calculated by using the formula: % s-GAG release = ((s-GAG of day 7, 14, 21, 28 or 35 – s-GAG of day 0)/ s-GAG of day 0)*100.

Measurement of HA levels

Release of HA from cartilage explant culture media was determined by the competitive enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Maxisorp, Nunc) were coated with 100 µg/ml umbilical cord HA (100 µl/well) in the coating buffer at 4°C overnight. And 1% (w/v) BSA in PBS was blocked for 1 hour. After washing, 100 µl of the mixture, either sample or standard competitor (HA Healon®: range 0-10,000 ng/ml) in biotinated hyaluronan binding proteins (B-HABPs) (1:100), was added. After incubation for 1 hour at room temperature, plates were washed and then peroxidase-mouse monoclonal anti-biotin (100 µl/well; 1:2000) was added and incubated for 1 hour at room temperature. Then the plates were washed again as well as the peroxidase substrate (100 µl/well) was added and incubated at 37°C for 5-10 minutes to allow the color to develop. The reaction was stopped with 50 µl/well of 4 M H2SO4 and the absorbance ratio at 492/690 nm was measured using the Titertek Multiskan M340 multiplate reader. %HA release was calculated from this formula: % HA release = (((HA of day 7, 14, 21, 28 or 35 – HA of day 0)/HA of day 0)*100.
**Measurement of uronic acid remaining in cartilage explants**

After papain-digestion of the cartilage discs, the uronic acid levels remaining in explants were measured by m-hydroxydiphenyl in a colorimetric assay. And 300 µl of concentrated sulfuric acid-borate reagent were added to standard glucuronic acid lactone (0-40 µg/ml) or the diluted sample and incubated for 15 mins at 100 °C. After that, they were cooled down in ice, added 12 µl of carbazole solution and incubated for 15 mins at 100°C. Uronic acid remaining was measured at 540 nm by microplate reader. Then the UA level was determined by a standard curve. % uronic acid remaining was calculated from this formula: % uronic acid remaining = (uronic acid content in cartilage (g) of day 35*dilution factor*dry weight (g)/uronic acid content in cartilage of control)*100.

**Histological analysis by Hematoxilin and eosin (H&E) and Safranin-O staining**

4% paraformadehyde fixed cartilage samples were embedded in wax and then cut into 5 ml thick sections perpendicular to the articular cartilage surface. The sections were evaluated for tissue morphology and GAGs accumulation by staining with hematoxylin-eosin (H&E) and Safranin-O, respectively.

**Results**

**Anthocyanins compositions in purple corn extract (Zea mays L.)**

The HPLC fingerprint of anthocyanins in purple corn extract showed in figure 1. The chromatogram showed that Cyanidin-3-glucoside (C3G), Pelargonidin-3-glucoside (Pg-3-glc), Peonidin-3-O-glucoside (P3G) and Malvidin-3-glucoside (M3G). Interestingly, C3G is the major anthocyanins found in purple corn. In addition, purple corn extract also have two unknown compound.

![Figure 1](image-url)  
**Figure 1** HPLC chromatogram showed the anthocyanins contents in purple corn. The anthocyanins standards used to define such as C3G, Pg-3-glc, P3G and M3G.

**Table 1** Identification of major anthocyanins detected in the purple corn extracts.

<table>
<thead>
<tr>
<th>Percentage of anthocyanin amount (compared with compounds in crude extracts)</th>
<th>Total compounds in crude extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3G</td>
<td>Pg-3-glc</td>
</tr>
<tr>
<td>32 %</td>
<td>28.9 %</td>
</tr>
</tbody>
</table>
Effect of AGEs-induced porcine cartilage degradation

To optimize the AGE concentration which induce cartilage degradation, the porcine cartilage discs were treated with the various concentrations of AGEs (6.25-50 µg/ml) for 35 days. Cultures media was collected at day 0, 7, 14, 21, 28 and 35 for measuring the HA and s-GAG level. AGEs significantly increased the release of HA and s-GAG from the cartilage explants when compared to control (Figure 2A and 2B). The concentration of AGEs which induce the highest of HA and s-GAG releasing level were 25 µg/ml.

Figure 2 The effect of AGEs on the release of HA and s-GAG in culture media treated with AGEs (6.25-50 µg/ml) for 35 days. The culture media at day 0, 7, 14, 21, 28 and 35 from each group were collected and the levels of HA (A) and s-GAG (B) in media were measured. Each value is expressed as the mean ± S.D. * p<0.05 compared with control, ** p<0.01 compared with control.

Cartilage discs on day 35 of treatment were digested with 10 unit papain. Uronic acid content in AGES was decreased when compared to the control which responses the induction of the cartilage glycosaminoglycans degradation as shown in Figure 3

Figure 3 Uronic acid remaining in porcine cartilage tissue after treated with AGEs (6.25-50 µg/ml) for 35 days, cartilage were digested with 10 units papain and analyze for uronic acid remaining. Each value is expressed as the mean ± S.D. * p<0.05 compared with compared with control.

Porcine cartilage tissue on day 35 were sectioned and stained by Hematoxylin Eosin (H&E) and Safranin-O staining for cell morphology and s-GAGs accumulation, respectively.
The evaluation of chondrocyte morphology using H&E staining demonstrated that the treatment of chondrocytes with AGEs (6.25-50 µg/ml) did not affect the chondrocyte morphology and extracellular matrix architecture when compared to untreated groups as shown in Figure 4.

![Figure 4 H&E staining of porcine cartilage sections on day 35.](image)

Safranin-O staining showed s-GAGs accumulation in cartilage tissue after culture for 35 days. We found that the safranin-O staining had the lowest intensity in 25 and 50 µg/ml of AGEs group (Figure 5D and 5E) when was compared the untreated group.

![Figure 5 Safranin-O staining of porcine cartilage sections on day 35.](image)

**Effect of Purple corn extracts on AGEs-induced porcine cartilage degradation**

To investigate the effect of purple corn extract on AGEs-induced porcine cartilage degradation, the porcine cartilage degradation were induced with 25 µg/ml AGEs and co-treatment with purple corn extracts (6.25-25 µg/ml) for 35 days. Cultures media were collected at day 0, 7, 14, 21, 28 and 35 for measuring the HA and s-GAG level. We found that the variation of HA and s-GAG level in the condition media treated with AGEs were significantly raised as shown in Figure 6. The highest amount of HA and s-GAG graph are on day 28 and 7, respectively. When co-treatment with purple corn extracts (6.25-25 µg/ml) in the presence of AGEs, the results showed that doses of crude extracts (6.25-25 µg/ml) significantly decreased HA and s-GAG release in cultured media since day 7 to day 35 when compared to the AGEs treated group.
Figure 6 The effect of purple corn extracts on the release of HA and s-GAG in culture media treated with AGEs and co-treated with crude extracts (6.25-25 µg/ml) for 35 days. The culture media at day 0, 7, 14, 21, 28 and 35 from each group were collected and the levels of HA (A) and s-GAG (B) in media were measured. Each value is expressed as the mean ± S.D. # p<0.05 compared with control, ## p<0.01 compared with control, * p<0.05 compared with AGEs treated alone.

Cartilage discs on day 35 of treatment were digested with 10 unit papain. Uronic acid content in AGEs was decreased when compared to the control. After co-treatment with crude extracts (6.25-25 µg/ml), the results showed that uronic acid remaining in porcine cartilage tissue were increased when compared with AGEs treated alone as shown in Figure 7.

Figure 7 Uronic acid remaining in porcine cartilage tissue after treated with AGEs (25 µg/ml) and crude extracts (6.25-25 µg/ml) for 35 days, cartilage were digested with 10 units papain and analyze for uronic acid remaining. Each value is expressed as the mean ± S.D. # p<0.05 compared with control, * p<0.05 compared with AGEs treated alone.
The evaluation of chondrocyte morphology using H&E staining demonstrated that the treatment of chondrocytes with AGEs (6.25-50 µg/ml) co-treated with crude extracts (6.25-25 µg/ml) did not affect the chondrocyte morphology and extracellular matrix architecture when compared to untreated groups as shown in Figure 8.

Figure 8 H&E staining of porcine cartilage sections on day 35.

Safranin-O staining showed s-GAGs accumulation in cartilage tissue after culture for 35 days. It was found that the safranin-O staining had the lowest intensity in the AGEs group (Figure 9B). The 25 µg/ml crude extracts-treated group showed the strongest intensity of safranin-O staining (figure 9E) as well as in the untreated group. Interestingly, treatment of the AGEs group with crude extracts (6.25-25 µg/ml) demonstrated a stronger intensity of safranin-O staining (in a dose-dependent manner) when compared with the AGEs group, which indicates a remaining of matrix glycosaminoglycans surrounding the chondrocyte lacunae.

Figure 9 Safranin-O staining of porcine cartilage sections on day 35.

Discussion

Previous studies, three major purple corn anthocyanins were reported that were Cyanidin-3-glucoside, Pelargonidin-3-glucoside, Peonidin-3-glucoside and their derivatives of each anthocyanin structure (Lao et al., 2016). In addition to previous report, we also found Malvidin-3-glucoside. Our study and previous study found that major purple corn anthocyanins were Cyanidin-3-(6"-malonylglucoside) and Cyanidin-3-glucoside, respectively. Many studies showed that anthocyanins play role in anti-inflammatory of arthritis rat model and IL-1β-induced bovine nasal
explants by decreasing in the rate of degradation of both proteoglycan and type II collagen (Jean et al., 2012; Haseeb et al., 2013; Panico et al., 2009; Dai et al., 2017).

Mainly cartilage degradation was caused by proinflammatory cytokines such as prostaglandin, interleukin-1beta (IL-1β), (IL-6) and (TNF-α) which signal chondrocytes to synthesize and secrete proteolytic enzymes and cause the cartilage degradation (Stevens et al., 2009). Nevertheless, many evidence demonstrated the association of AGEs and osteoarthritis development (Gkogkolou, Böhm, 2012; Rasheed et al., 2011; Huang et al., 2011; Nah et al., 2008).

However different AGEs condition that could induce cartilage degradation between this study and previous study (Ma et al., 2015) that were 25 and 100 µg/ml, respectively. As our results, treatment of cartilage disc with 25 µg/ml of AGEs significantly induced the degradation of porcine cartilage which demonstrated by the significantly increasing of glycosaminoglycans releasing from the cartilage tissue and decreasing of the uronic acid content in cartilage tissue.

By the way anthocyanins have role in decreasing cartilage degradation that was induced by other inducers but in AGEs-induced cartilage degradation still have not been extensively studied. Thus, the chondroprotective effects of anthocyanins purple corn extracts on AGEs induced cartilage degradation is the objective of this study. We investigate the effects of purple corn extract on AGEs induced porcine cartilage discs for 35 day culture. Interestingly, purple corn extract co-treatment could significantly reverse AGEs induced cartilage degradation when compared to the AGEs treatment alone.

Therefore, the molecular mechanism, we will further investigate the effects of anthocyanins on the expression of pro-inflammatory cytokines and proteinases as well as the signaling pathways on AGEs stimulated primary human articular chondrocyte.

Conclusion

The screening of the chondroprotective effects of purple corn extracts were assessed in AGEs-induced porcine cartilage explants. When co-treatment with AGEs, the purple corn extracts effectively blocked the glycosaminoglycans release from the cartilage explants and retained the uronic acid content in cartilage matrix when compared to the AGEs treatment alone. Our results suggested the chondroprotective effects of purple corn extracts on AGEs induced cartilage. The effects of anthocyanins and molecular mechanism on primary chondrocytes need to be further investigated.

References


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