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Review

Avocado/Soybean Unsaponifiables (Piacledine®300) show beneficial effect on the metabolism of osteoarthritic cartilage, synovium and subchondral bone: An overview of the mechanisms

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Abstract: *Objectives*: The aim of this narrative review of the literature was to synthesize and comment the mechanisms of action of avocado/soybean unsaponifiable mixture (ASU-E, Piascledine®300) on articular tissues involved in the OA pathogenesis. *Materials and methods:* The search was performed in Pubmed and Scopus between January 1981 and December 2016. Keywords used were—any field—(Cartilage OR Bone OR Synovium) AND Avocado AND Soybean. 32 articles out-off 35 found have been considered. The review has included eleven *in vitro* and animal studies investigating Avocado Soybean Unsaponifiables (ASU) from Laboratoires Expanscience (Piascledine®300) used separately or in combination. Only research articles published in English and French have been taken into account. *Results:* ASU-E stimulated proteoglycans synthesis in chondrocytes cultures and counteracted the effects of IL-1 on metalloproteases and inflammatory mediators. Some of these effects were associated with inhibition of NF-κB nuclear translocation and stimulation of TGF-synthesis. ASU-E also positively modulated the altered phenotype of OA subchondral bone osteoblasts and reduced the production of collagenases by synovial cells. *Conclusions:* ASU-E has positive effects on the metabolic changes of synovium, subchondral bone and cartilage which are the main tissues involved in the pathophysiology of OA. These findings contribute to explain the beneficial effects of ASU-E in clinical trials.

Keywords: cartilage; bone; synovium; osteoarthritis; avocado; soybean

1. Introduction

Osteoarthritis (OA) is the most common form of arthritis affecting millions of people over the world and generating important costs for the social insurance. Recently, the OsteoArthritis Research Society International (OARSI) has defined OA as disorder involving movable joints characterized by cell stress and extracellular matrix degradation initiated by micro- and macro-injury that activates maladaptive repair responses including pro-inflammatory pathways of innate immunity. The disease manifests first as a molecular derangement (abnormal joint tissue metabolism) followed by anatomic, and/or physiologic derangements (characterized by cartilage degradation, bone remodeling, osteophyte formation, joint inflammation and loss of normal joint function), that can culminate in illness [1]. The main hallmarks of the disease are a progressive degradation of cartilage and meniscus, an inflammation of the synovial membrane and an abnormal subchondral bone remodeling leading to sclerosis. The most structural changes observed in cartilage are fibrillations, fissurations and at the late stage bone exposure. Chondrocytes form clusters and they undergo a hypertrophic differentiation associated with the mineralization of the extracellular matrix and angiogenesis [2]. OA synovial membrane displays a spectrum of changes including a marked hyperplasia of the lining layer, with a dense cellular infiltrate composed largely of lymphocytes and monocytes, through to a synovial membrane which is thickened by fibrotic tissue [3,4]. Overloaded subchondral bone is thickened as a result of an abnormal bone cells metabolism [5–7]. Further, microfractures and newly formed vessels constitute exchange routes between bone and cartilage [8]. Schematically, OA pathogenesis can be organised around three physiopathological vicious circles named « cartilage-cartilage », « bone-cartilage » and « synovium-cartilage » loops (Figure 1). Abnormal mechanical strains induced cartilage lesions and subsequently chondrocytes activation. Activated chondrocytes release locally high levels of Reactive Oxygen/Nitrogen Species (ROS/RNOS), Matrix MetalloProteinases (MMP) (*i.e.* collagenases, aggrecanases) and cytokines (*i.e.* interleukin (IL)-1,-6,-8 and Tumor Necrosis Factor (TNF)). Among these cytokines, IL-1 plays a critical role by activating NF-κB (Nuclear Factor kappa B) signaling pathway [9]. This cytokine, in association with others, stimulates, as an autocrine and paracrine manner, chondrocytes to produce more MMP and free radicals responsible for extracellular matrix degradation. This is the so called « cartilage-cartilage » vicious circle. Chondrocytes also communicate with subchondral bone cells *via* mediators like RANKL (Receptor Activator of Nuclear factor Kappa-B Ligand), a factor stimulating osteoclasts bone resorption [10]. Mechanical strains also stimulate osteoblasts to produce locally IL-6, but also growth factors like the Vascular Endothelial Growth Factor (VEGF) [11]. VEGF stimulates angiogenesis while IL-6, *via* channels and microcracks may stimulate overlying chondrocytes to produce matrix metalloproteinases [12]. By this way is created a vicious circle between subchondral bone and cartilage. The third pathogenic loop is between cartilage and synovial membrane. Osteochondral fragments, products of matrix degradation but also pro-inflammatory mediators (*i.e.* prostaglandin (PG) E2, nitric oxide (NO) or cytokines) trigger synovial membrane inflammation and activate synovial cells to produce catabolic and inflammatory mediators that directly degrade cartilage or stimulate chondrocytes to produce catabolic factors [13].

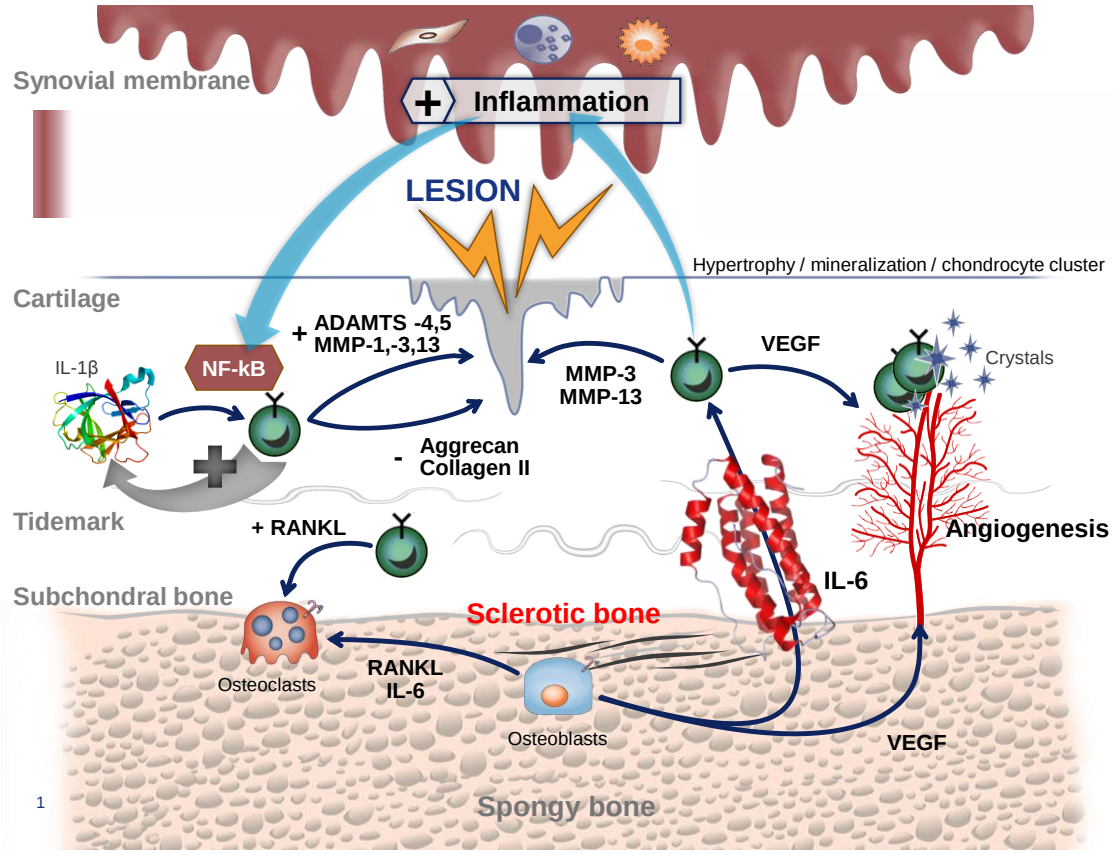

Figure 1. Representation of the OA physiopathology. MMP: matrix Metalloproteases; ADAMTS: A desintegrin and metalloproteinase with thrombospondin motifs; IL: Interleukin; PGE2: prostaglandin E2; RANKL: Receptor Activator of Nuclear factor Kappa-B Ligand; VEGF: Vascular Endothelial Growth Factor; NO: Nitric oxide.

Therefore, physician and other health care providers need of efficient therapeutic modalities that target bone, cartilage and synovium cells. Further, recent advances in the understanding of the disease revealed an important interconnection between co-morbidities, like metabolic syndrome and cardiovascular diseases [14,15], and OA, indicating that OA management requires safe treatment with no deleterious impact on these co-morbidities. Pharmacological treatment is yet dominated by Non Steroidal Anti-Inflammatory (NSAIDS) drugs and acetaminophen despite their risk of severe adverse effects especially when administered in the long-term and mainly in elderly people with co-morbidities [16]. In addition, the efficacy of acetaminophen in osteoarthritis is highly challenged [16,17]. Beside these conventional treatment, there is a class of products called SYmptomatic Slow Acting Drugs in OsteoArthritis (SYSADOA). Avocado Soybean Unsaponifiables (ASU) mixture are members of this class. Currently, the only ASU mixture investigated in well-conduct Randomized Controlled Trials (RCT) is made up of unsaponifiables fractions one-third avocado oil and two-third soybean oil. This ASU mixture is commercialized as a drug in many countries and called Piascledine®300 (Laboratoires Expanscience, Courbevoie, France). In this paper, Piacledine®300 will be named ASU-E. At this time, they are four well-conducted RCT, all supported by the manufacturer, demonstrating a beneficial symptomatic effect of ASU-E in the treatment of hip or knee OA [18–21]. From Maheu et al. study [20], Piascledine®300 was demonstrated to both significantly improve function as measured by Lequesne Functional Index

(LFI), from the first month of treatment and pain, as measured by global spontaneous pain, from the second month of treatment, versus placebo and over 6 months. In addition, the success rate of the treatment as defined by the rate of patients with an improvement of LFI ≥ 30% and a reduction in VAS pain ≥ 50%, was significantly higher in the Piascledine®300 versus placebo group at 6 months ($p < 0.01$). A meta-analysis including the randomized, double-blind, placebo-controlled trials concluded that patient may recommended to give Piascledine®300 a chance for 3 months as its effect size on pain was measured at 0.39 (95% CI: 0.01–0.76) and that on LFI was 0.45 (95% CI: 0.21–0.70) [22]. In the Blotman study [21], indirect proof of symptomatic efficacy of Piascledine®300 was obtained as the intake of rescue NSAIDs was significantly decreased versus placebo group ($p < 0.001$). More recently, a large RCT including 399 patients with hip OA has demonstrated that 3-year treatment with ASU-E reduced the percentage of radiological progressors evaluated by measuring joint space width on radiograph, suggesting a structure-modifying effect in hip OA [23]. ASU-E is recommended by the European League Against Rheumatology (EULAR) for the management of knee and hip OA symptoms [24]. Recently, OARSI guidelines have qualified ASU-E as a treatment with an "uncertain appropriateness" for knee OA management. According to the OARSI experts the term « uncertain » is not considered as a negative recommendation which could preclude the use of ASU-E. Rather, it requires a role for physician-patient interaction in determining whether such treatments may have merit in the context of their risk-benefit profile and the individual characteristics, co-morbidities and preference of the individual patient [25].

One strategy to prevent or to treat osteoarthritis would consist in compound acting on the changes occurring in the three major tissues involved in OA pathogenesis: cartilage, synovial membrane and bone. Piascledine®300 (ASU-E) is a potential candidate for such an approach. This paper reviews the biological effects of ASU-E on these three tissues with the aim to explain how this product works in human (Table 1).

Table 1. Summary of the in vitro and animal studies evaluating ASU-E effects.

Reference	Product	Dose and duration (ID)	Model	Results
Chondrocytes in culture				
Mauviel A *et al.*, 1989 [31]	ASU-E	1 or 10 μg/ml ID: 24h or 5–7 days	Primary rabbit articular chondrocytes in monolayer with or without IL-1	Decreases PGE_2 with or without IL-1 Increases total collagens after 7 days of incubation No effect on cell proliferation
Harmand MF, 1990 [33]	ASU-E	10 μg/ml ID: 4h, 6, 9, 12 days	Primary human normal or OA hip cartilage	Increases GAG synthesis.
Mauviel A, Loyau G, Pujol JP 1991 [32]	ASU-E	10 μg/ml ID: 48h Pre ID: 5 days prior IL-1 48h	Primary rabbit articular chondrocytes with or without IL-1 Pre-incubation 5 days with ASU-E prior IL-1 addition for 48h	Totally blocks IL-1-stimulating effect on collagenolytic activity when incubated simultaneously with IL-1 Partially counteracts IL-1- stimulating effect on collagenolytic activity when ASU-E was added 5 days before IL-1
Henrotin Y *et al.*, 1998 [34]	ASU-E A2S1 A2S2 A-E S-E	ASU-E 10 μg/ml A-E: 3.3, 6.6, 10 μg/ml S-E:3.3, 6.6, 10 μg/ml ID: 72 h	Human primary OA chondrocytes in monolayer with or without IL-1	A-E and S-E inhibits spontaneous IL-8 and PGE_2 production A-E, but not S-E, inhibits spontaneous IL-6 production ASU-E inhibits IL-6, IL-8, PGE2 and MMP-3 spontaneous production. ASU-E is more effective than A-E or S-E added separately. ASU-E partially reverses IL-1 stimulating effect on IL-6, IL-8, PGE_2 and MMP-3 production.

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Reference	Product	Dose and duration (ID)	Model	Results
Boumediene K *et al.*, 1999 [37]	ASU-E	5, 10, 25 μg/ml ID: 12–24 to 48h	Primary calf articular chondrocytes in monolayer	Stimulates the expression of TGF-1, TGF-2 and PAI. No effect on TGF-RI and RII.
Henrotin Y *et al.*, 2003 [35]	ASU-E A-E S-E	ASU-E: 0.625 to 40 μg/ml A-E:3.3 and 10 μg/ml S-E: 6.6 and 10 μg/ml ID: 3 to 12 days	Human primary knee OA chondrocytes in alginate beads with or without IL-1 3 days exposure to IL-1 before treatment with ASU-E	Increases AGG synthesis and accumulation in alginate beads. Partial recovery of AGG production after IL-1 treatment. Inhibits basal MMP-3, IL-6, IL-8, PGE2, MIP-1 production. Partially counteracts the IL-1-effect on MMP-3 and PGE2 production.
Gabay O *et al.*, 2008 [36]	ASU-E	10 μg/ml	Mouse or human chondrocytes stimulated with IL-1 Cartilage submitted to a compressive mechanical stress (MS)	Decrease of MMP-3 and -13 expression and PGE_2 synthesis Inhibition of the degradation of IκB and suppression of NF-κB translocation. Inhibition of Erk ½ but no effect on the other IL-1-induced MAPK.
Osteoblasts in culture				
Andriamanalijaona *et al*., 2006 [48]	ASU-E	10 μg/ml ID 48h ASU-E + IL-1 or 24h IL-1 followed by 24h ASU-E or 24h ASU-E followed IL-1	Human alveolar bone explant or human alveolar bone cells in monolayer with or without IL-1.	In basal condition, ASU-E had no effect on TGF-1 gene expression. Pre-treatment with ASU-E or ASU-E added with IL-1β counteracts the inhibitory effect of IL-1 on TGF-1 gene expression. In basal condition, ASU-E stimulated protein synthesis. ASU-E added simultaneously with IL-1 counteracts the inhibitory effect of IL-1 on TGF-1 protein production.

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Reference	Product	Dose and duration (ID)	Model	Results
Henrotin Y *et al*., 2006 [45]	ASU-E	10 μg/ml ID: 72h	Human osteoblasts coming from the sclerotic or non-sclerotic subchondral bone of cadavers with idiopathic OA.	Inhibits IL-1 by non-sclerotic osteoblasts, but has no effect on OC, AP, TGF-1 and IL-6. Decreases ALP, OC and TGF-1 synthesis by sclerotic osteoblasts, but has no effect on PTHrp, IL-6 and IL-1.
Osteoblasts/chondrocytes co-culture				
Henrotin Y *et al*., 2006 [45]	ASU-E	10 μg/ml ID: 72h	Human OA chondrocytes co-cultured or not with osteoblasts (obtained from sclerotic (SC) or non-sclerotic (NSC) zones of OA subchondral plate	Prevention of the inhibitory effects of SC osteoblasts on matrix components by pre-treatment of SC osteoblasts with ASU-E. Increase of type II collagen mRNA level in co-culture with ASU-E pre-treated SC osteoblasts. No modification of MMP, TIMP-1, TGF-1, TGF-3 or iNOS gene expression and COX-2 mRNA levels in chondrocytes when co-cultured with ASU-E pre-treated SC osteoblasts.
Synovial cells in culture				
Mauviel *et al*., 1989 [31]	ASU-E	0.1, 1 and 10 μg/ml ID: 24h or 14 days	Rheumatoid human synovial cells in monolayer with or without IL-1	Partially reverses the inhibitory effect of IL-1 on collagen synthesis after 24 h. Slightly increases type I and type III collagen synthesis after 14 days of incubation. Inhibits basal and IL-1 stimulated PGE_2 production.
Mauviel, Loyau, Pujol, 1991 [32]	ASU-E	10 μg/ml ID: 48h Pre ID: 5 days prior IL-1 48h	Rheumatoid human synovial cells in monolayer with or without IL-1β	Increases collagenolytic activity in basal condition. Partially blocks IL-1-stimulating effect on collagenolytic activity when incubated simultaneously with IL-1β. Totally counteracts IL-1- stimulating effect on collagenolytic activity when ASU is added 5 days before IL-1

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Reference	Product	Dose and duration (ID)	Model	Results
Cinelli M *et al*., 2009 [44]	ASU-E	5, 10 and 20 μg/ml ID: 48h	Healthy or RA synoviocytes cultured in monolayer	Decreases VEGF production and increase TIMP-1 by RA human synovioctyes.
Animal studies				
Khayyal *et al*., 1998 [39]	ASU-E A-E S-E	ASU-E :39 mg/kg/day or 20 or 40 or 80 mg/kg/day A-E: 13mg/kg/day S-E:26 mg/kg/day ID: 2 weeks Control: saline/tween 80 solution	Femoral head cartilage of rat wrapped in piece of sterile cotton and implanted into subcutaneous tissue of mice	ASU-E, A-E and S-E reduce the loss of GAG and hydroxyproline from cartilage induced by granulomatous tissue. The greatest effect is observed with ASU-E. ASU-E effect on GAG and hydroproline content is dose-dependent. Treatment with ASU-E, A-E or S-E inhibits the formation of granulomatous tissue around the cartilage implant but the most market effect is exhibited by ASU-E.
Cake M *et al*., 2000 [43]		900mg/weekday Placebo: paraffin oil ID: 2 or 5 months	Mature merino wheters with OA induced by bilateral lateral menisectomy N = 48	Reduces loss of toluidine blue stain in tibial plateau than in placebo or non-operated controls. Increases uncalcified cartilage thickness in the middle zone of the lateral femoral condyle. Lower increase of subchondral bone plate thickness in the lateral tibial plateau.
Altinel L *et al*., 2007 [42]	ASU-E	300 mg every day or every 3 days Control: normal diet ID: 3 months	Male outbred sheepdogs N = 24	Increase of both TGF-1 and TGF-2 levels in knee joint fluid in treatment groups compared to controls

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Reference	Product	Dose and duration (ID)	Model	Results
Boileau C *et al*., 2009 [40]	ASU-E	10 mg/kg/day ID: 8 weeks	Experimental knee dog model (anterior cruciate ligament transection) N = 16	Decrease of the size of the macroscopic lesions (tibial plateaus) compared to control Decrease in the severity of cartilage lesions (tibial plateaus and femoral condyles) Decrease in the scores of all histological parameters (structural changes, cellularity, Safranin-O staining and pannus invasion on the femoral condyles) No difference on the tibial plateaus for Safranin-O and pannus invasion Reduction of iNOS and MMP-13 production in cartilage Reduction of the total histological changes and cellular infiltration in synovium Reduction of loss of subchondral bone volume and calcified cartilage thickness
Jaberi F *et al*., 2012 [41]	ASU-E	300 mg every day Placebo: empty capsule ID: 3 months	Male outbred dogs Full-thickness cartilage lesion in both femoral condyle Microfracture in the left knee N = 22	No significant differences in cartilage surface histology, cell distribution, and cell viability.

ASU-E: Avocado/Soybean Unsaponifiables from Expanscience; A-E: Avocado fraction from ASU-E; COX: cyclooxygenase; GAG: glycosaminoglycan; IL: interleukin; iNOS: inducible Nitric Oxide synthetase; MMP: Matrix Metalloproteinases; MAPK: Mitogen-activated protein kinases; MIP: Macrophage Inflammatory protein; NF: Nuclear factor; PAI: Plasminogen activator inhibitor; PGE_2: prostaglandin E2; PTH-rp: Parathyroid hormone related peptide; RA: Rheumatoid arthritis; S-E: Soybean fraction from ASU-E, TGF: Transforming growth factor; TIMP: Tissue Inhibitor of Metalloproteinases; VEGF: Vascular Endothelial Growth Factor.

2. Materials & methods

The search was performed in Pubmed and Scopus between January 1990 and November 2016. Keywords used were—any field—(Cartilage OR Bone OR Synovium) AND Avocado AND Soybean. 32 articles out-off 35 found have been considered. The review has considered eleven *in vitro* and animal studies investigating the biological activity of the ASU-E (ASU produced by Laboratoires Expanscience, Courbevoie, France). Only research articles published in english have been taken into account.

2.1. ASU-E definition

Laboratoires Expanscience have developped and produced a pharmaceutical product, Piascledine®300, which is effective for the "symptomatic treatment of hip and knee osteoarthritis". The active principle of this pharmaceutical compound is called ASU Expanscience™ (ASU-E) and is composed of a unique mixture of soybean unsaponifiables and avocado specific unsaponifiables [27]. Unsaponifiables are a set of molecules, soluble in plant oils that are not transformed in soaps during oil hydrolysis using strong bases. This chemical reaction being called saponification. The specificities of the process yielding the ASU-E are protected by patents (patent US 314 118, 1994, patent PCT FR00/02 601, 2000; Patent PCT FR02/02715, 2002). The originality of Piascledine®300 is based on the A/S ratio (1 : 2) and its specific composition of both unsaponifiables, resulting from the proprietary process. The main fraction of avocado oil unsaponifiables (the H fraction), accounting for up to 50%, corresponds to a family of nine molecules out of which eight have been chemically characterized [28]. They are composed of a furyl nucleus, substituted in position 2 by an aliphatic, mono- or polyunsaturated chain. The chain length varies from 13 to 17 carbon atoms, always an odd number [29]. This fraction is highly specific for the avocado oil unsaponifiables contained in ASU-E and may be linked with its pharmacological activities. Squalen (2%), long-chain saturated hydrocarbons (up to 5%), polyols (up to 15%), tocopherols (trace amounts) and sterols (between 4 and 20%), mainly as -sitosterols, are the other components of the avocado unsaponifiables. The soybean oil unsaponifiables, almost totally identified, include saturated hydrocarbons (about 1%), squalen (about 4%), tocopherols (around 10%), sterols (between 40 and 65%), terpene alcohols (about 1–10%), aliphatic alcohols (less than 1%) and methyl sterols (less than 5%) [30]. In France, ASU-E is widely administered by oral route at a dose of 300 mg per day.

2.2. ASU-E on cartilage metabolism

2.2.1. In chondrocyte culture

ASU-E was first tested on rabbit articular chondrocyte cultured in monolayer. After 24 hours exposure, ASU-E (0.1 to 10 μg/ml) did not significantly modulate collagen synthesis. Conversely, long incubation periods (8 to 14 days) with ASU-E increased collagen synthesis [31]. Incubation of rabbit chondrocytes for 48 hours with 10 μg/ml of ASU-E slightly increased collagenase production [32]. This effect was no longer observed and remains unexplained. In *in vitro* experimentation, IL-1β was used to mimic the metabolic changes occurring *in vivo* in OA cartilage.

As expected, incubation of rabbit chondrocytes with IL-1β induced an important release of collagenase. ASU-E (10 μg/ml) totally abolishes its action. Moreover, incubation of rabbit chondrocytes for 5 days with ASU-E prior to a 48 h-exposure to IL-1 prevents partially the effect of IL-1.

In human chondrocytes, ASU-E (10 μg/ml) increased proteoglycans synthesis and accumulation in the extracellular matrix. This effect is significant after 6 days of incubation and is more marked in OA than normal chondrocytes [33]. In primary human OA chondrocytes, short-term (up to 72 hours) exposure of ASU-E (10 μg/ml) inhibited spontaneous and IL-1-induced synthesis of stromelysin-1 (MMP-3), IL-6, IL-8 and PGE_2 [34]. Thereafter, to investigate the long-term effect of ASU-E (12 days), human OA chondrocytes were cultured in three dimensional alginate beads [35]. In this tridimensional matrix, chondrocytes continue to express a cartilaginous phenotype for at least 12 days and newly synthesized extracellular matrix accumulate around spherical chondrocytes entrapped in alginate scaffold. One major finding of this experience was that 12 days of ASU-E exposure did not affect cell viability, confirming the absence of toxicity of this product. Further, ASU-E increased in a dose-dependent manner aggrecan synthesis quantified by a specific sandwich enzyme-linked immunosorbent assay (ELISA) using two monoclonal antibodies, one raised against the keratan sulfate region of aggrecan and another directed against the hyaluronic acid binding region of the molecule. In parallel, ASU-E also inhibited the synthesis of pro-inflammatory mediators including Macrophage Inflammatory Protein (MIP)-1, Prostaglandin (PG)E_2, Nitric Oxide (NO), IL-6, IL-8 and the production of MMP-3. In this experimentation, human chondrocytes were also pre-incubated with IL-1β for 3 days prior treatment with ASU-E. As anticipated, IL-1β pre-treatment inhibited aggrecan production. After 9 days of treatment with ASU-E, the production of aggrecan by chondrocytes was restored. In contrast, aggrecan production remained inhibited in chondrocytes culture untreated with ASU-E. This finding indicates that ASU-E may reverse the deleterious effect of IL-1β on the production of major cartilage matrix components. This study also demonstrated that ASU-E at the ratio A1S2 had a significantly more marked effect on IL-6, IL-8 and NO production than A or S added alone at the same concentration as in the A1S2 mixture. Together, these observations add support to the use of avocado/soybean mixture to treat OA.

This was confirmed by Gabay *et al.* [36] on costal mouse chondrocytes cultured in monolayer and stimulated by IL-1β. ASU-E (10 μg/ml) decreased IL-1β-stimulated MMP-3 and collagenase-3 (MMP-13) gene expression and PGE_2 production. In addition, this study identified the signaling pathways targeted by ASU-E. ASU-E inhibited Nuclear Factor (NF)-κB nuclear translocation and the phosphorylation of Extracellular signals Regulated Kinases (ERK) 1/2 pathway but had no effect on p38 and Jun-amino-terminal Kinase (JNK) pathways. Similarly, ASU-E decreased ERK1/2 phosphorylation induced by compression of mouse cartilage explants suggesting that ASU-E, not only counteracts the effects of chemical deleterious factors like IL-1, but also those of mechanical stress.

The positive effects ASU-E on cartilage metabolism were explained by the increase of Transforming Growth Factor (TGF) β isoforms 1 and 2 production. Indeed, ASU-E at the concentrations of 10 and 25 μg/ml increased the synthesis of both TGF isoforms by bovine chondrocytes in monolayer [37]. Interestingly, ASU-E appeared to synergize with TGF-β1 to stimulate the expression of TGF-β1, but not TGF-β2 gene. These growth factors are well-known for their capacity to stimulate the synthesis of extracellular matrix components and counteracts IL-1 effects. Therefore, it was speculated that ASU-E increases TGF-1/2 which in turn stimulate aggrecan production and counteracts IL-1 stimulating effects on MMP and inflammatory factors production [37].

ASU-E (10 and 25 μg/ml) also enhanced the synthesis of Plasminogen Activator Inhibitor (PAI)-1 by bovine chondrocyte, an effect that participates in blocking the plasmin cascade that leads to metalloprotease activation. Given that TGF-β1 itself is known as a stimulant of PAI-1 expression in most cell types, including articular chondrocytes [38], it may be possible that part of the increased PAI-1 expression observed here could be due to a primary effect of ASU-E on TGF-1 and TGF 2 synthesis. This finding indicates that ASU-E, not only inhibits the synthesis of key MMPs involved in OA pathogenesis, but also regulates the activation process of these enzymes.

2.2.2. In animal model

The first study evaluating *in vivo* the possible "chondroprotective" effect of ASU-E was performed using a model in which rat articular cartilage wrapped in cotton was implanted subcutaneously in mice. The presence of cotton induces a granulomatous reaction which enhances the destruction of adjacent cartilage. Agents that tend to delay or prevent cartilage degradation might be suggested to have a "chondroprotective" action, which may or may not be linked to an associated anti-inflammatory effect on the granulomatous lesion itself [39]. In this model, unsaponifiables of avocado (13 mg/kg) and soybean (26 mg/kg) were tested individually or in combination at the ratio 1 : 2 (39 mg/kg) as is found in ASU-E. The products were administrated orally during 2 weeks. Compared to saline solution, A or S unsaponifiables administrated individually or in combination partially prevented the loss of hydroxyproline and glycosaminoglycans in cartilage. The combination was significantly more efficient on these parameters than avocado and soybean added separately. As hypothesized, this effect may results of an anti-inflammatory effect of ASU-E on granulomatous tissue. Indeed, ASU-E decreased the size and fluid content of the granuloma mass surrounding the cartilage, indicating a potent anti-inflammatory effect.

In dog, OA can be induced experimentally by the section of cruciate ligament. The section of ACL induced an instability responsible for abnormal strain at the cartilage surface, and consequently the degradation of this tissue. In this model, Boileau and colleagues [40] have compared the effects of ASU-E (10 mg/kg/day) vs placebo administrated orally during 8 weeks immediately after surgery on the cartilage and subchondral bone structural changes. By immunohistochemistry, they also investigated the effects of ASU-E on Nitric Oxide Synthetase (iNOS) and MMP-13 expression. iNOS synthesized high levels of NO in inflammatory condition and MMP-13 is a key MMP responsible for type II collagen degradation. Interestingly, ASU-E prevents the appearance of cartilage lesions. Histologically, the severity of cartilage lesion in both tibial plateaus and femoral condyles and the cellular infiltration in synovium was decreased. Treatment with ASU-E also reduced loss of subchondral bone volume and calcified cartilage thickness compared to placebo.

Another group of researchers has investigated in 22 outbred male dogs the effects of ASU-E on the healing of full-thickness cartilage lesion in the medial femoral condyle associated or not with subchondral bone microfracture. Microfracture is one of the most commonly used primary surgical interventions to promote cartilage healing by inducing the production of fibrocartilage tissue and by increasing the amount of type II collagen in the cartilage tissue. Dogs received ASU-E 300 mg/day or an empty capsule as placebo during 12 weeks. In this model, there were any additive effect of ASU-E to microfracture induced repair tissue as evaluated by ICRS visual histological assessment scale [41]. In healthy dogs, 3 months of ASU-E (300 mg/day or 300 mg every three days) treatment increased the levels of TGF-β1 and 2 in the knee synovial fluid when compared to the controls (normal diet) [42].

As the sheep's knee is the most similar to the human knee, an ovine model of OA is relevant to study drug efficacy. In sheep, lateral menisectomy induced gross pathological changes comparable with early human OA, with articular cartilage erosion in the lateral compartment and moderate market osteophytes. Histological lesions are limited to lateral compartment after 3 months, but extend to the entire joint after 6 months. Using this model, Cake et al. [43] has showed that ASU-E (900 mg/weekday) administrated orally during 6 months tended to decrease macroscopic and histological severity of OA lesion. However, this difference was not significant. In contrast, results obtained by computer-assisted histomorphometric analysis revealed subtle though statistically significant effects of the drug in maintaining articular cartilage proteoglycan content and decreasing subchondral bone sclerosis in lateral compartment [43].

2.3. ASU-E on Synovium inflammation

Three studies investigated the effect of ASU-E on human rheumatoid synovial cells [31,32,44]. In these experiments, rheumatoid synovial cells were enzymatically removed from samples obtained during hip operations and cultured in monolayer. ASU-E (0.1 to 10 μg/ml) did not affect the spontaneous production of collagens by synovial cells even after 14 days of incubation. As anticipated, IL-1 inhibited collagen synthesis by these cells. In the presence of IL-1, ASU-E (10 μg/ml) restored collagen synthesis to levels similar to those of the control cultures. At a concentration of 10 μg/ml, ASU-E reduced the amount of PGE_2 secreted by 77% relative to the control cultures and slightly inhibited the stimulatory effect of IL-1 (-20%). The unsaponifiables extracts therefore appear to work against the inhibitory effect exerted by IL-1 on collagen synthesis. The same group demonstrated that incubation of synovial cells for 48 h with 10 μg/ml of ASU-E increased collagenase activity measured in the culture supernatant. IL-1 (100 pg/ml) induced an important increase of collagenase activity. This IL-1-stimulating effect was partially inhibited by ASU-E when it is added simultaneously for 48 hours. Moreover, incubation of synovial cells for 5 days with ASU-E (10 μg/ml) prior to a 48 h-exposure to IL-1 prevented the effect of IL-1. These results suggest that ASU-E could, at least partially, inhibit the harmful effects of IL-1 by reducing the collagenolytic activity of synovial cells. Finally, Cinelli *et al.* [44] compared the effect of ASU-E on the production of Vascular Endothelial Growth factor (VEGF) and Tissue Inhibitor of MetalloProteases (TIMP)-1 by rheumatoid or healthy synoviocytes. In RA synoviocytes, ASU-E significantly reduced VEGF levels, in a dose-dependent manner, while in healthy synoviocytes only ASU-E 10 and 20 μg/ml , slightly but significantly, reduced VEGF levels with respect to the basal value. ASU-E induced a significant increase in TIMP-1 levels with respect to baseline values only in RA at 20 μg/ml [44].

2.4. ASU-E on subchondral bone remodeling

OA subchondral bone osteoblasts expressed a particular phenotype that contributes to abnormal subchondral bone remodeling. In monolayer, OA osteoblasts coming from the thickening (called sclerotic) subchondral bone located just below the main cartilage lesion produced higher Alkaline Phosphatase activity (AP), VEGF, IL-6, IL-8, osteopontin (OPN), osteocalcin, TGF-β1 and type I collagen, but less ParaThyroid Hormone related peptide (PTHrp) than osteoblasts coming from the

non-thickening neighbouring area (called non-sclerotic, NSC) [7]. IL-1 production was similar in both cell type cultures.

Aside from inhibition of IL-1ß production, ASU-E (10 μg/ml) had no effect on NSC osteoblasts. In contrast, ASU-E significantly decreased AP, OC, and TGF-ß1 synthesis by SC osteoblasts ($p < 0.01$), but had no effect on PTHrP, IL-1ß, or IL-6 production [45]. Interestingly, IL-6 with its soluble receptor (IL-6R) enhanced VEGF and MMP-13 production by both NSC and SC osteoblasts [46]. ASU-E (10 μg/ml) drastically inhibited VEGF and MMP-13 production by both cell types treated with IL-6/IL-6R [46]. These effects of ASU-E on osteoblast could be related to the effects of this product on the synthesis of TGF-β. Indeed, ASU-E counteracts the inhibitory effect of IL-1 on TGF-β1 production by bone cells from alveolar maxillar bone, and synergized with IL-1 to stimulate TGF-β2 protein production [48].

2.5. ASU-E on Bone/cartilage crosstalk

The bone-cartilage crosstalk can be investigated by co-culturing human osteoblasts and chondrocytes. In this model, osteoblasts of the sclerotic or non-sclerotic area of OA subchondral bone were cultured in monolayer and OA chondrocytes in alginate beads. Osteoblasts and chondrocytes were separated by a porous membrane allowing passage of soluble mediators.

When they were co-cultured with sclerotic osteoblasts, chondrocytes produced significantly less aggrecan, whereas genes coding for MMP-3 and MMP-13 were upregulated [46]. The pretreatment of SC osteoblasts with ASU-E (10 μg/ml) fully prevented the inhibitory effects of SC osteoblasts on aggrecan and type II collagen gene expression, and even significantly increased type II collagen mRNA level over the control (chondrocytes alone) value. After 10 days of incubation, ASU-E prevented osteoblast-induced inhibition of aggrecan core protein production. In contrast, pretreatment of SC osteoblasts with ASU-E did not significantly modify the expression of MMP-3, MMP-13, TIMP-1, TGF-ß1, TGF-ß3, iNOS, or COX-2 gene by chondrocytes [45].

3. Discussion

OA is complex disease involving all articular tissues including, meniscus, ligaments, capsule cartilage, synovial membrane and subchondral bone. The ideal OA treatment should be active on all these tissues because they are all involved in OA physiopathology, signs and symptoms. ASU-E is largely used in the treatment of OA around the world. Their effects on symptoms and structural changes in OA articular tissues have been well documented. In chondrocytes culture, ASU-E increases AGG synthesis and accumulation in the extracellular matrix and inhibits the production of pro-inflammatory and pro-catabolic mediators [31–36]. These findings clearly indicate that ASU-E may act on different facets of OA chondrocyte metabolism and tends to normalize it. Further, ASU-E counteracts the deleterious effect of IL-1 on chondrocytes metabolism. Some of these effects have been described to be associated with inhibition of NF-κB nuclear translocation and increase production of transforming growth factors β1 and β2 in chondrocytes [37]. These data give a rationale to explain the structural effects observed in animals [39–43] and humans [23]. In animals, this effect is associated with a decrease of iNOS and MMP-13 and with an increase of TGF-1/2 levels in synovial fluid. This perfectly corroborates the *in vitro* study and indicates that

after oral intake, ASU-E ingredients or metabolites reach the joint and modulate chondrocyte metabolism (Figure 2).

SC subchondral bone is now recognized to play a major role in OA development and is considered as a potential therapeutic target. Subchondral bone sclerosis is suspected to be linked to cartilage degradation, not only by modifying the mechanical properties of subchondral bone [49], but also by releasing biochemical factors that affect cartilage metabolism [50]. Numerous studies have demonstrated that osteoblasts from sclerotic OA subchondral bone are phenotypically different from non-sclerotic osteoblasts [51–54]. In example, we have shown that osteoblasts from the sclerotic subchondral bone located just below the main cartilage lesion had increased AP and produced more IL-6, IL-8, osteopontin, osteocalcin, TGF-β1, and type I collagen than osteoblasts from the nonsclerotic adjacent area [7]. Since it has been demonstrated that osteoblasts from sclerotic subchondral showed an altered phenotype, they constitute a potential therapeutic target for drugs used in the treatment of OA. Interestingly, ASU-E inhibits AP and OC synthesis by SC osteoblasts, 2 markers of new bone formation, and decrease the synthesis of TGF-ß1 by osteoblasts, a growth factor postulated to be one of the key regulators of local bone formation [57]. Altogether, these observations indicate that ASU-E might be effective on SC subchondral osteoblasts and favor a return to bone homeostasis.

Since the presence of tidemark microcracks that appear early in OA have been identified in OA cartilage, it has been suggested that soluble mediators produced by SC osteoblasts may modulate chondrocyte metabolism and contribute to cartilage degradation [12]. To verify this hypothesis, we developed an original model of culture in which SC osteoblasts and chondrocytes are cultured in the same environment, but do not come into contact. The originality of our model lies in the fact that we use osteoblasts from the sclerotic subchondral bone, and that OA chondrocytes are cultured in alginate beads. A key element in cartilage degradation is an increase in MMP activity. Interestingly, we observed that SC osteoblasts induced a strong elevation of MMP-3 and MMP13 synthesis by chondrocytes, whereas NSC osteoblasts or normal skin fibroblasts had no effect [45]. This observation indicates that osteoblast-induced cartilage degradation is related to its particular SC phenotype. In addition to this stimulating effect of SC osteoblasts on MMP synthesis, we also observed that AGG content in alginate beads decreased when chondrocytes were co-cultured with SC osteoblasts [45] Together, these findings indicate that SC phenotype of osteoblasts induces a marked dysregulation of chondrocyte metabolism, characterized by a decrease of matrix component synthesis and an increase of MMP production. This imbalance between anabolic and catabolic factors could lead to cartilage matrix depletion. ASU-E prevented the inhibitory effect of SC osteoblasts on aggrecan synthesis but had no significant effect on MMP, TIMP-1, COX-2, or iNOS expressions. This finding demonstrates that ASU-E may protect cartilage by acting at the subchondral bone level and suggests a new mechanism of action for this potential structure-modifying drug. As all data presented are specific to ASU-E, they are, obviously, neither applicable to nor valid for other ASU available on the market.

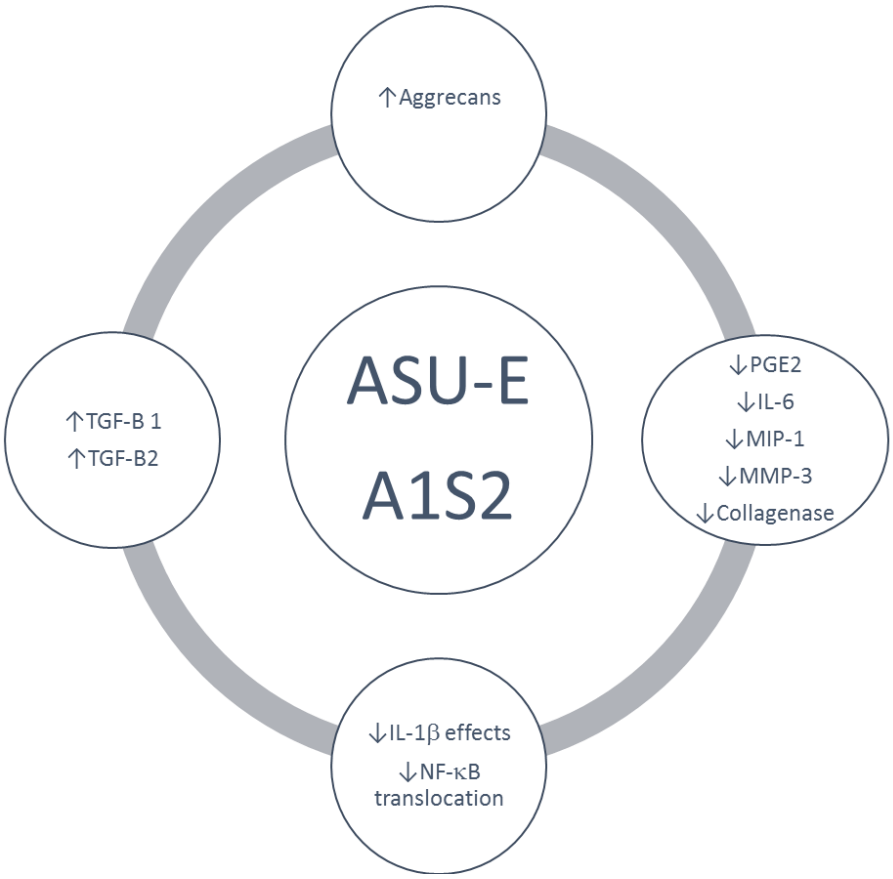

Figure 2. Representation of the effect of ASU-E (A1S2) on OA chondrocytes metabolism.

4. Conclusion

In conclusion, ASU-E has a unique composition obtained after unique processes. ASU-E was demonstrated to have positive effects on the metabolic changes occurring in three main tissues involved in OA pathophysiology. By this way, ASU-E has a global action on the joint and may interfere with the three pathological vicious circles binding cartilage, subchondral bone and synovial membrane. These experimental observations give a rational to explain the clinical efficacy of ASU-E. Some additional investigations would be helpful to better understand the role of ASU-E on synovial membrane and meniscus. More particularly, it would be interesting to investigate the effects of ASU-E on NF-κB and Wnt signaling pathways and on the related production of pro-inflammatory cytokines and metalloproteases by synoviocytes.

Conflict of interest

The author declares to have ponctual consulting fees from Laboratoires Expanscience.

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