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A CRITICAL LINK BETWEEN ADVANCED GLYCATION END PRODUCTS, OSTEOPOROSIS AND TYPE 2 DIABETES MELLITUS

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ABSTRACT

Osteoporosis, a bone disease where bone density and quality are reduced is commonly the consequence of estrogen deficiency. However, various studies could show an increased number of fractures among type 2 diabetes mellitus (T2DM) patients although no or very little loss of bone mineral density was observed. Therefore, decreased bone quality has been suggested as a potential reason for this phenome. Among the potential factors causing a decreased bone quality, accumulation of advanced glycation products (AGEs) has been identified. However, the primary molecular linkage between T2DM, AGE and osteoporosis still need to be discussed. The aim of this paper is to review the potential molecular links between the actions of AGEs and the pathogenesis of osteoporosis in patients with T2DM. PubMed and Google Scholar were searched for original and review articles using the following keywords: advanced glycation products (AGE), hyperglycaemia, diabetes type 2 *mellitus*, osteoblasts, osteoclasts and osteoporosis. Hyperglycaemia results in excessive accumulation of AGEs with a subsequent increase in reactive oxidative species production plus oxidative damage which then activates the bone inflammatory response. Additionally, AGEs act by interfering with osteoblasts proliferation, differentiation, plus impairing the production of collagen and osteocalcin. AGEs downregulate bone metabolism, bone density and induce apoptosis of osteoblasts. Another effect of AGEs on bone is that it can induce the upregulation of AGEs-receptors expression. The interaction between AGEs and their receptors result in the generation of reactive oxygen species and inflammatory process that involves the osteoblasts and osteoclasts, which results in osteoporosis. AGEs cause down-regulation of both osteocalcin and alkaline phosphatase. In conclusion, accumulation of AGEs in bone results in an impairment of bone mineralization, decrease in extracellular matrix quality and apoptosis of the osteoblasts, as well as an enhanced osteoclastogenesis that contributes to the deterioration of bone quality.

1.0 Introduction

Type 2 diabetes mellitus (T2DM), one of the four subtypes of diabetes, is a common metabolic disorder seen in older adults but with increasing numbers now also seen in much younger patients. The prevalence of T2DM is increasing at a worrying rate, for example, South East Asia has according to a recent WHO publication an estimated 96 million people with diabetes whereby 90% of them are having T2DM. [1] A study by Wan Nazaimoon *et al*. showed that the overall prevalence of diabetes in Malaysia is about 22.6% whereby the global prevalence is at 8.5% (2014). [2] This rapid increase in diabetes especially T2DM seems to be associated with the current worldwide obesity epidemic accompanied by a lack of physical activities. A recent newspaper report showed that Malaysia has the highest number of obese people in Southeast Asia with more than 2.6 million adults and more than 477,000 children below the age of 18 years.[3]

Continuous or even brief episodes of hyperglycaemia are associated with malfunctions at the cellular, subcellular or even the molecular level. Therefore, hyperglycaemia is considered to be the main cause of diabetes-related complications like neuropathy, nephropathy, retinopathy or cardiovascular complication like left ventricular hypertrophy.

The causative connection between diabetes and bone fragility, mainly the increased fracture risk, is not widely accepted although various studies could show an increased fracture risk in patients with T2DM, hyperglycaemia or diabetic complications.[4-8] The main argument against such causative connection is the fact that patients with T2DM commonly have a higher bone mineral density (BMD) than nondiabetic ones[9] and the 10-year fracture-probability calculation using the Fracture Risk Assessment Tool FRAX® is very low. On the other hand, FRAX® is known to underestimate the fracture risk in patients with T2DM[10] as it doesn't consider bone architecture or general bone quality. What is "bone quality"? The term itself is not clearly defined. According to Seeman[11] bone quality is the material and structural basis for bone strength while Licata[12] offers a more comprehensive definition as he includes in his definition all the factors that determine how the bones are resisting fracture. These factors include microarchitecture, accumulated microscopic damage, quality of collagen, the size of mineral crystals and the rate of bone turnover.

At the cellular level, various studies could show that high glucose levels interfere with osteoblast functions including bone mineralization and even response to vitamin D therapy.[13-17] The damaging effect of high blood glucose levels has been attributed to the formation of glycated proteins and lipids known as advanced glycation end products (AGEs). In general, the formation of AGEs in the body is a natural, slow but persistent process. Beginning from the embryonic life, AGEs are accumulating with time and therefore are considered as a part of the ageing process. However, in patients with T2DM, a significant boost of AGEs levels is observed due to the consistent availability of high blood glucose levels in uncontrolled or undiagnosed patients. This in turn significantly contributes to the development of diabetic macro- and microvascular complications.[18-19]

2.0 The Biochemistry of Advanced Glycation End Products

The formation of AGEs is a natural chemical process or more specifically a non-enzymatic glycation between an aldehyde group of a free monosaccharide with a ε-amino group of an exposed amino acid residue of a protein. The same process leads to the browning of food during frying and is called Maillard reaction, in reference to the French chemist Louis-Camille Maillard who first described this process.

With regards to protein glycation, the most commonly exposed amino acids on the surface of proteins are lysine, hydroxyl lysine or arginine as they are found on haemoglobin, albumin, osteocalcin, collagen and other proteins.[18-20] The reaction between the aldehyde group of glucose and the ε-amino group, for example of lysine, leads to the formation of glucosyl-lysine which then undergoes additional reactions to form a Schiff base. The formed Schiff base is an unstable intermediate and therefore undergoes rearrangement into what is called an Amadori product (Figure 1) or ketosamine. The Amadori product has various potential fates but here we concentrate only on two of them: a) it is oxidized and forms AGE or b) it undergoes dehydration and de-amination to produce reactive carbonyl intermediates also known as α-dicarbonyls or oxoaldehydes. Examples of these reactive carbonyl intermediates are 3-deoxyglucosone (3-DG) and methylglyoxal (MG). These reactive carbonyl compounds (RCC) can react with proteins, lipids and DNA to form more AGEs. Depending on the level of AGEs in the cell, the cell can enter a stage of intracellular oxidative stress also called carbonyl stress.[21]

AGEs are irreversible molecules once synthesized[22]. Taking AGEs formed from collagen as an example, these AGEs include crosslinked and non-crosslinked modifications of the collagen fibres and these endproducts tend to accumulate in tendons, skin, cartilage and bone.[23-24]

AGE-induced crosslinking of collagen fibres is considered to be deteriorative for the mechanical properties of bone and an increase in their levels seems to be an indicator for impaired bone quality in diabetes (both type 1 and 2) as well as osteoporosis and ageing. A detailed review describing both enzymatic and non-enzymatic (AGE-induced) crosslinking of

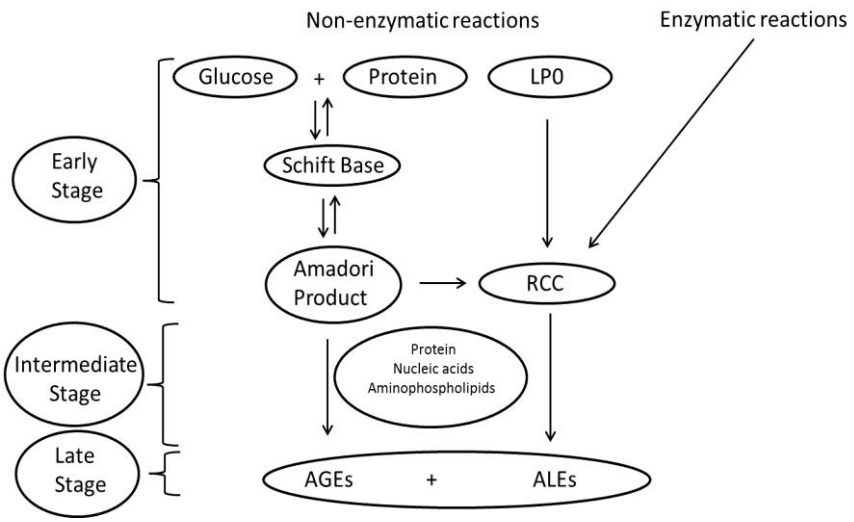

Fig. 1: Formation of Advanced Glycation Endproducts modified according to Singh *et al.*[21] and Semchyshyn and Lushchak.[34]

collagen fibres and their effect on bone quality can be found by Saito & Marumo.[25]

The effect of AGEs in the mentioned tissues depends on which proteins also have been glycated. As lysine and arginine are residues that are often found in functional groups e.g. the catalytic groups of enzymes, the formation of AGEs can lead to the deactivation or loss of enzyme function.

3.0 Enzymatic Defence System Against Accumulation of AGEs

In healthy and young individuals, the accumulation of AGEs in the circulation, tissues or in the cytoplasm of cells is prevented through a group of enzymes that removes excessive amounts of glycating agents and repairs the glycated proteins. Glyoxalase I, aldehyde reductase and aldehyde dehydrogenase remove RCCs e.g. MG, 3-deoxyglucosone etc., while amadoriase and fructosamine 3-phosphokinase remove AGEs.[26-30]

Glyoxalase I and II are forming a detoxification system, also called the glyoxalase system, which is found in the cytoplasm of all cells where it suppresses the formation of MG- and glyoxal derived AGEs. Glyoxalase I and II are glutathione-dependent enzymes, therefore, the glyoxalase system loses its efficiency in situations where glutathione is depleted e.g. oxidative stress or when excessive methylglyoxal is produced like under hyperglycaemic conditions. Little is known about the effectiveness of the glyoxalase system in bone. However, a study by Suh *et al*. showed that MG induces oxidative stress and mitochondrial dysfunction in osteoblastic MC3T3-E1 cells leading to apoptosis.[31]

Glycated proteins, on the other hand, are removed via proteolysis and the resulting peptides and AGE-coupled peptides are cleared by the kidneys and excreted in the urine. However, glycated extracellular matrix proteins are highly resistant to proteolysis and therefore tend to accumulate. Additionally, to ageing, AGEs tend to accumulate under extensive oxidative stress like in smokers or prolonged hyperglycemia.[32]

4.0 Effect of Advanced Glycation Endproducts on Bone

Using a simplified model, bone can be divided into the cellular part consisting of mesenchymal progenitor cells, osteoblasts, osteocytes, osteoclasts and the extracellular matrix. In order to evaluate the effect of AGEs on bones, both components need to be considered. Additionally, a differentiation between AGEs and RCCs might be useful. As described above, an AGE is formed through a non-enzymatic reaction between an aldehyde group like e.g. in glucose and a ε-amino group of an amino acid residue on the surface of a protein. They also can be formed through the reaction of RCCs with proteins, nucleic acids and aminophospholipids. RCCs as well as AGEs, found in the body, can originate from an endogenous or exogenous source. Endogenous production is basically due to enzymatic activities of metabolic pathways involving carbohydrates, lipids etc. Examples would be glycolysis and the polyol pathway. Exogenous sources, on the other hand, can be food intake, medication or environmental contaminants. The latter two sources are especially true for the uptake of RCCs while AGEs are predominantly taken up with food.

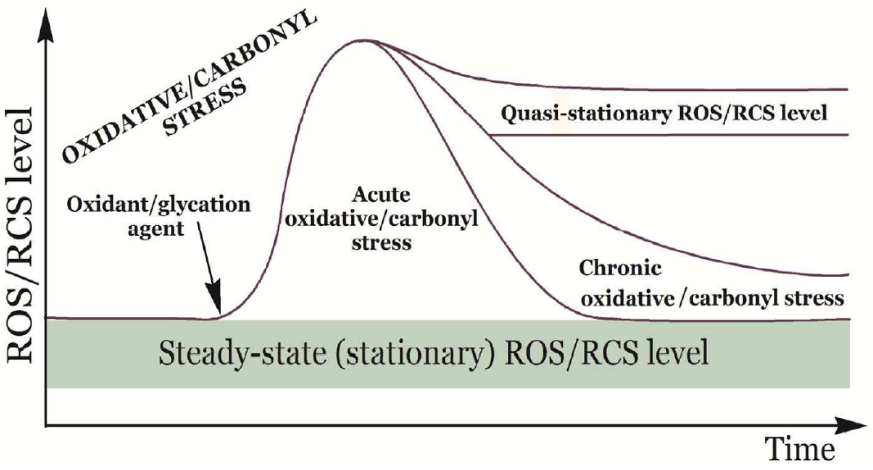

Fig.2: Adjustment of RCCs steady-state after an acute episode of carbonyl/oxidative stress (adopted from Semchyshyn and Lushchak[34])

How much each of the mentioned sources contributes to the level of AGEs in general and in bone, in particular, is difficult to estimate as so far only pentosidine, and N-carboxymethyl-lysine (CML) are measured as biomarkers for AGEs accumulation and it is not possible to differentiate their origin. Encouraging studies are trying to link serum levels of pentosidine and CML in T2DM patients to the severity of degenerative diseases like retinopathy.[33]

5.0 Reactive Carbonyl Species Induced Carbonyl Stress and Its Effect on Bone

Carbonyl stress, as defined by Semchyshyn and Lushchak[34] is an acute or chronic increase in steady-state levels of RCCs, AGEs and/or advanced lipoxidation end products (ALEs) either due to increased production/uptake or decreased elimination which disturbs cellular metabolism and leads to cellular damage.

For example episodes of hyperglycaemia as seen in T2DM, lead to increased NADH and FADH levels followed by an increase of the proton gradient beyond a particular threshold. At this point, complex III of the electron transport chain will stop functioning.[35] This, in turn, results in excessive production of reactive oxygen species (ROS) from mitochondria by activating Poly [ADP-ribose] polymerase 1 (PARP1).[36] In turn, PARP1 induces ADP-ribosylation of Glyceraldehyde-3-Phosphate Dehydrogenase (GADPH) leading to its inhibition together with the accumulation of glycolysis metabolites e.g. methylglyoxal (MG). MG or other RCCs will lead to a further production of AGEs. [37]

From the example above, it is clear that ROS, RCCs and AGEs production are interconnected. The general understanding at the moment is that RCCs behave biologically similar to ROS, especially the uncharged molecules in each group. However, differences in half-life, stability and the ability to cross cell membranes may need to be considered.

Additionally, the "steady state" for RCCs is dynamic and can re-establish itself for example after an episode of hyperglycaemia, however, at a higher level. Therefore, RCCs steady-state levels of diabetic patients tend to be higher than for normal patients (Figure 2).

What this means for bone is not fully clear as there are only very few papers looking at the *in vitro* effect of carbonyl stress in general. The few papers that looked at the effect of certain RCCs like methylglyoxal (MG) did use various types of osteoblasts cells. In general terms, it is known that the presence of increased amount of RCCs leads to increased glycation of proteins, aminophospholipids and nucleotides and therefore increasing the number of cellular AGEs and ALEs levels. MG is known to react predominantly with arginine which can be found in the active centre of many enzymes in general and antioxidant enzymes in particular and therefore an increase in ROS levels is expected.

Chan *et al*. showed for the first time that incubating human osteoblast cells with MG triggers apoptosis due to oxidative stress, c-Jun N-terminal kinase activation and mitochondrial membrane potential changes.[38] A similar experiment using hFOB 1.19 osteoblast cells showed a significant increase of cellular ROS levels (unpublished data) which correlated with an increase in cell death and increased expression of caspase 3

indicating apoptosis as a mechanism of death.[39] We also observed a decrease in alkaline phosphatase (ALP) and osteopontin protein levels, indicating inhibition of osteoblast cell differentiation and potentially mineralisation (unpublished data).

Additionally to the inhibition of antioxidant enzymes by RCCs, binding of AGE to its cell surface membrane receptor RAGE (receptor of advanced glycation endproducts) increases oxidative stress. The binding of AGEs to RAGE activates the following pathways: p38 MAP Kinase, nuclear factor kappa B (NF-κB), p21 Ras and Jak/STAT.[40-41] Due to the activation of the mentioned pathways, increased expression of cytokines such as interleukins 6 and 1, tumour necrosis factor α (TNFα), as well as growth factors and adhesion molecules have been measured.[42] In particular, the increased expression of TNFα leads to additional ROS production and thus creating a vicious cycle.

6.0 Osteoporosis in Diabetes Mellitus

Following the introduction of the term "bone quality", the determination of osteoporosis now not only depends on the determination of bone quantity and mineral density but also on the properties of the bone tissue.
Expression of RAGE is vital for bone metabolism. It was reported that RAGE overexpression inhibits osteoblasts proliferation *in vitro*. This inhibitory effect results from suppression of WNT, PI3K and ERK signalling. Previous reports showed that inhibition of cell growth in case of RAGE overexpression is partially secondary to the change in the WNT signalling pathway .[43-44]

As discussed above, T2DM reduces bone quality rather than BMD due to AGE-Collagen crosslinking which can be divided into enzymatic immature divalent cross-links, mature trivalent cross-links (important for the strength of bone matrix), and oxidation- or glycation- induced non-enzymatic cross-links i.e. AGEs. Both hyperglycaemia and oxidative stress reduce the enzymatic beneficial cross-link formation in favour of non-enzymatic AGE-induced ones that are weakening the bone matrix[45].

The glycation process may enhance fibronectin, type III collagen, α3(IV) collagen, type V collagen, type VI collagen synthesis in ECM via upregulation of transforming growth factor-β (TGF-β) intermediate.[46] Glycation of laminin and type I and type IV collagens, key molecules in the basement membrane, reduces adhesion to endothelial cells for both matrix glycoproteins.[21]

T2DM stimulates osteoclast while inhibits osteoblast function, leading to accelerated bone loss, osteopenia and subsequent osteoporosis. Hyperglycaemia prompts synthesis of receptor activator of nuclear factor-kappa B ligand (RANKL), macrophage colony-stimulating factor (MCSF) and tumour necrosis factor (TNF) which are osteoblast-derived activators for proliferation and differentiation of osteoclasts. On the other side, long-term exposure to hyperglycaemia inhibits osteoblast proliferation and function, through decreasing the expressions of transcription factor (Runx-2), osteocalcin and osteopontin. Mesenchymal stem cells differentiation into adipocytes is increased. A decrease in neovascularization may exacerbate bone loss. Additionally, AGEs accumulation causes the bone quality to be reduced, which may finally result in pathological fractures.[48]

7.0 Advanced Glycation End products and Bone Remodelling Process

As discussed earlier in diabetes, excess AGEs are stored in the bone and can play an important role in the pathogenesis of osteoporosis.[49]

Advanced glycation end products stimulate human mesenchymal stem cell apoptosis which would have negative consequences for the formation of adipose tissue, cartilage and bone.[50] It has recently been reported that AGEs interfere with osteoblast differentiation as well as with the production of collagen and osteocalcin (bone matrix proteins), leading to reduced bone quality.[51]

Alikhani *et al* (2007)[52] reported that CML-collagen, a predominant AGE in bone and serum of osteoporotic patients, stimulates calvarial periosteal cell apoptosis more than the unmodified collagen. It also stimulates apoptosis in primary cultures of human osteoblastic cells. Such apoptotic effect was found to be mediated through RAGE receptors. CML-collagen was also found to increase the activity of both p38 and c-Jun N-terminal kinase (JNK).[53]

The mechanism of induction of apoptosis of osteoblastic cells by AGEs was found to be through the activation of the MAP kinase signalling pathway.[52] So, this research comes to the conclusion that AGEs may contribute to a deficient bone formation .[52]

As previously mentioned, collagen (a long-lived protein in the bone matrix) is the main target for AGEs formation. In particular, AGEs mediated collagen over-crosslinking can cause increased bone brittleness and loss of its flexibility and elasticity.[54]

Another report confirmed that CML-collagen, stimulates both p38 and JNK activity in osteoblasts.[55] p38 and JNK are involved in the upstream signalling of apoptosis.[56]

It was reported that osteoclastogenesis by RANKL mRNA up-regulation and the impairment of bone matrix mineralization through down-regulation of osteocalcin mRNA and ALP is enhanced by the activation of the AGE-RAGE pathway, specifically by RAGE mRNA up-regulation.[57]

It was illustrated that the accumulation of AGEs will significantly decrease ALP activity and type 1 collagen production, whereas it will increase ROS production, this will result in a weak osteoblastic bone formation.[58]

The same study reported that bisphosphonates are able to regress the harmful effects of AGEs. This valuable action of bisphosphonates is due to a suppression of intracellular ROS generation, through inhibition of geranyl-geranylation of Rac, a small G protein which is a critical element for the NADPH oxidase complex.[58]

The above study concluded that AGEs increase apoptosis rate and a decrease osteoblasts proliferation, both of these effects could be reversed by exposure to bisphosphonates.[58]

There is a rising proof that AGEs and their receptors cause oxidative stress generation and subsequently induce inflammatory responses in both osteoblasts and osteoclasts, so being involved in osteoporosis pathogenesis in diabetes.[59]

Similarly, AGEs present in collagen can inhibit osteoblasts phenotypic expression and induce the osteoclasts activity.[60] Also, AGEs modified proteins can cause alteration of bone remodelling.[61]

It has been identified that osteoporotic patients had significantly higher pentosidine serum levels in comparison to the non-osteoporotic group.[62] The serum pentosidine is correlated with increasing osteoclasts activity.[23]

The bone material properties, mineralization process and the micro damage are all influenced by collagen cross-link formation which directly affects bone mineralization process.[63]

As described by McCarthy *et al*, that the effect of AGEs in the bone extracellular matrix in term of osteoblastic cells proliferation and differentiation is dependent on the stage of osteoblastic differentiation.[53] Moreover, they showed that AGE-specific binding receptors are existing in cultured osteoblast-like cells and that those receptors are regulated by the osteoblastic developmental stage.[64]

Hein *et al* demonstrated that AGE modification is present in the osteoporotic bone.[65] They also showed a negative relationship between the staining intensity of AGEs in osteoporotic bone and the percentage of trabecular bone.[64]

Good bone material properties can be secured by proper enzymatic crosslink formation at the same time inhibition of AGEs cross-linking process which is regulated by the levels of glycation, level of oxidative stress, and the rate of bone turnover.[63]

8.0 Conclusions

Accumulation of AGEs in the bone extracellular matrix affects the bone remodelling process.

AGEs cause a disturbance in the proliferation, differentiation and function of different types of bone cells that are participating in the process of bone remodelling. The interaction between AGEs and their receptors results in the formation of ROS and inflammatory responses in both osteoblasts and osteoclasts that are involved in osteoporosis pathogenesis in diabetes. It can be concluded that this interaction serves as a fundamental link between diabetes and osteoporosis.

In conclusion, AGEs impair the osteogenesis process by stimulating osteoblasts apoptosis and decreasing osteoblasts proliferation. Glycation of bone proteins impacts both bone resorption and formation by affecting osteoclastic and osteoblastic cell proliferation and functions. Additionally, AGEs decrease ALP activity and type 1 collagen production that results in a weak osteoblastic bone production. Understanding AGEs synthesis, accumulation, biochemical and molecular characteristics plus the cellular receptors for AGE is indicated, also the AGE-induced effects on both extracellular and intracellular functions need to be more investigated, so effective therapies that can block excessive accumulation of AGEs in the bone and their interaction with RAGE receptors can be established as a platform for both prevention and even treatment of osteoporosis among diabetic patients.

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