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The applications of buckminsterfullerene C_{60} and derivatives in orthopaedic research

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Abstract

Buckminsterfullerene C_{60} and derivatives have been extensively explored in biomedical research due to their unique structure and unparalleled physicochemical properties. C_{60} is characterized as a ''free radical sponge'' with an anti-oxidant efficacy several hundred-fold higher than conventional anti-oxidants. Also, the C_{60} core has a strong electron-attracting ability and numerous functional compounds with widely different properties can be added to this fullerene cage. This review focused on the applications of C_{60} and derivatives in orthopaedic research, such as the treatment of cartilage degeneration, bone destruction, intervertebral disc degeneration (IVDD), vertebral bone marrow disorder, radiculopathy, etc., as well as their toxicity *in vitro* and *in vivo*. We suggest that C_{60} and derivatives, especially the C_{60} cores coupled with functional groups presenting new biological and pharmacological activities, are advantageous in orthopaedic research and will be promising in clinical performance for musculoskeletal disorders treatment; however, the pharmacokinetics and toxicology of these agents as local/systemic administration need to be carefully determined.

Keywords

Anti-oxidant, double-action compound, fullerene, orthopaedic applications, reactive oxygen species

History

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Introduction

Fullerenes (C_x), the third carbon allotrope, are similar in structure to graphene but rolled up to form hollow spheres with closed structure (1). In fullerene family, buckminsterfullerene C_{60} is the most abundant representative which was first discovered by Kroto et al. in 1985 through graphite vaporization under laser irradiation (2). Since its detection and bulk production, C_{60} has elicited intense interest on scientific scene due to its unique structure and features, which culminated in the 1996 Nobel Prize for Chemistry awarded to Kroto, Curl and Smalley for their seminal discovery. C_{60} is a remarkably stable compound composed of 60 carbon atoms arranged in a ''soccer cage'', with a diameter of 0.72 nm (Figure 1) (3). Its highly delocalized π double bond system contributes to an unusual redox chemistry. Thus, C_{60} has been characterized as a ''free radical sponge'' with an anti-oxidant efficacy several hundred-fold higher than conventional anti-oxidants (4). Also, C_{60} consists entirely of sp^2-hybridized carbons which render it a strong electron-attracting ability (5). Therefore, numerous functional compounds with widely different properties can be added to the fullerene cage. For example, the pristine C_{60} is highly hydrophobic. Covalent attachment of hydroxyl (–OH), amino ($–NH_2$) or carboxyl (–COOH) groups, enables it to be water-soluble (6) and facilitates its in-depth biomedical applications *in vitro* and *in vivo*. Herein, we will briefly review the unique features of C_{60} and derivatives, as well as the current advances of their biomedical applications, especially in orthopaedic research.

Oxidative stress in pathology and redox properties of C_{60} and derivatives

The endogenous production of reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical and hydrogen peroxide, is a consequence of basal cellular respiration, processed from mitochondrial oxidative phosphorylation (7). At a moderate level, ROS are recognized to be physically involved in cell signaling and required for biochemical energetics of life. When ROS overwhelm the cellular anti-oxidant defense system, oxidative stress would occur and cause damage to cellular proteins, lipids and nucleic acids (8), potentially implicated in the pathogenesis of atherosclerosis (9), neurodegeneration (10), cancer (11) and musculoskeletal disorders (12,13). Therefore, it is of therapeutic value to relieve the oxidative stress by removing excess ROS with extrinsic anti-oxidants, toward the goal to remedy the stressful pathological conditions.

The novel free radical scavenging property of C_{60} is attributed to its unique hollow spherical structure featured with 30 conjugated carbon–carbon double bonds and low lying lowest unoccupied molecular orbital (14). It was

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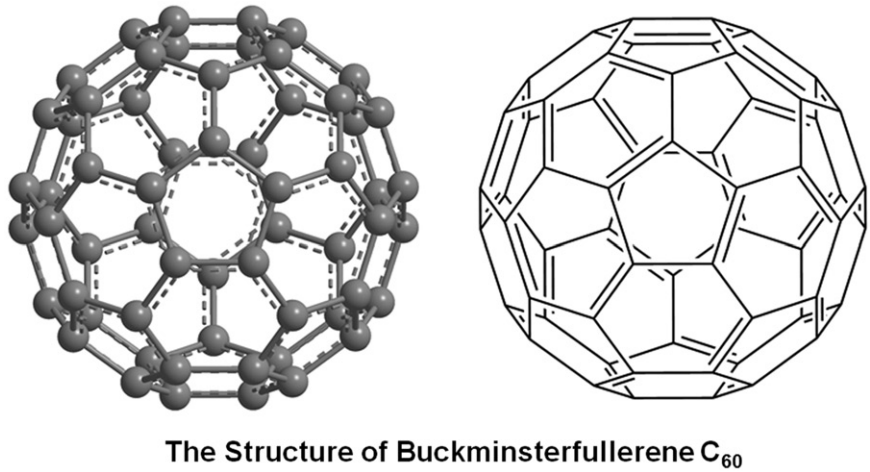
Figure 1. The structure of buckminsterfullerene C_{60}.



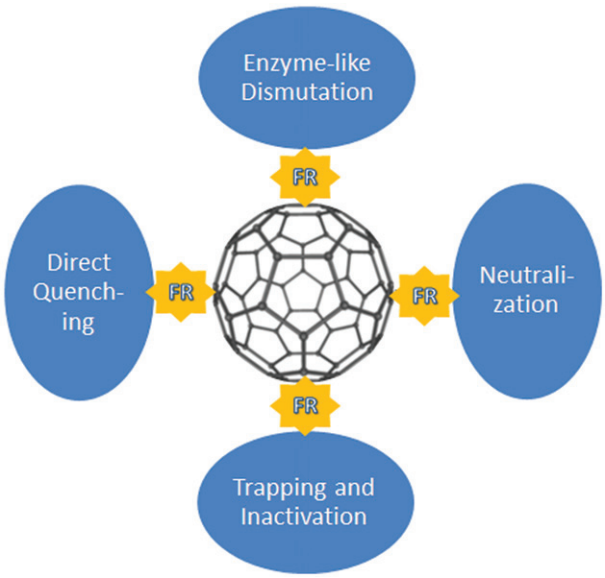


Figure 2. Possible mechanisms under which C_{60} acts as an efficient free radical scavenger. C_{60} removes free radicals potentially by direct quenching of NO, neutralization of singlet oxygen, enzyme-like dismutation of superoxide radicals, as well as trapping and inactivation of hydroxyl radicals.

reported that one C_{60} molecule can readily react with at least 15 benzyl radicals or 34 methyl radicals to form stable radical or non-radical adducts (4). Furthermore, as the quenching process is catalytic, C_{60} can react with many superoxides without being consumed. Thus, C_{60} has been considered as a ''free radical sponge'' and described to be the most efficient radical scavenger (4). Figure 2 shows the possible mechanisms under which C_{60} acts as an efficient free radical scavenger (15–18). To improve its hydrophilicity and increase its versatility, extensive studies have been focusing on the development of water-soluble C_{60} derivatives and the attachment of functional groups presenting new biological and pharmacological activities to the C_{60} anti-oxidant core.

General applications of C_{60} and derivatives in biomedical research

C_{60} and derivatives were found to be beneficial in many biomedical applications due to the unique anti-oxidant features and various functionalizations of the C_{60} core: (1) Inflammation suppression: Fullerol, a polyhydroxylated derivative of C_{60}, and C_{60} hybrids bearing xanthine or thalidomide moiety suppressed nitric oxide (NO) and tumor necrosis factor-α (TNF-α) production of macrophages under lipopolysaccharide (LPS) activation and attenuated neutrophilic lung inflammation induced by quartz via eliminating oxidative stress as well as through the therapeutic actions of the linked drug groups (19–21). (2) Neuroprotection: C_{60}, fullerol and carboxyfullerene prevented neurons from excitotoxic and apoptotic injuries *in vitro* and *in vivo*, prevented transgenic neurodegenerative disorder as well as ischemia or iron-induced oxidative injuries in brain tissue, by scavenging free radicals (22–26). (3) Protection of visceral organs from oxidative injuries: C_{60}, fullerol and hexa(sulfobutyl)fullerene protected visceral organs, such as liver, heart, lung, kidney, etc., from oxidative injuries via scavenging free radicals (27–29). (4) Inhibition of cellular apoptosis: Functionalized C_{60} derivatives, such as glutathione C_{60}, carboxyfullerene, hexa(sulfobutyl)fullerene, C_3-fullero-tris-methanodicarboxylic acid, efficiently inhibited cellular apoptosis by suppressing oxidative stress (30–34). (5) Radioprotection: Fullerol and dendro[C_{60}]fullerene showed radioprotection of cells, tissues, and organs from free radical damage generated by ionizing radiation (35–38). (6) Inhibition of enzyme activities: Fullerol, trimalonic acid C_{60}, tris-malonyl-C_{60} and $C_{60}O_5(OH)_{18}$ inhibited activities of *Thermus aquaticus* (Taq) DNA polymerase, NO synthase and monooxygenase, due to the high hydrophobicity and electrophilicity of the C_{60} core, as well as through the incorporation of C_{60} nanoparticles into the catalytic pockets and the actions of the conjugated enzyme inhibitory groups via surface modification (39–41). (7) Photodynamic therapy: C_{60}, C_{60} linked with pyrrolidinium groups, C_{60} modified with l-phenylalanine, folic acid and l-arginine, and hexakis C_{60} showed inactivating effects on tumor (42–44), microbial (45,46) and viruses (47,48), by generating ROS upon illumination through photodynamic therapy strategy, working as photosensitizers. (8) Drug and gene delivery: Surface modifications of the C_{60} core to be conjugated with drugs, such as C_{60}-paclitaxel and C_{60}-PEI-FA/DTX, or functionalizations with DNA-binding groups, such as amino (seri, C_3)-C_{60} adducts and tetra-amino C_{60}, were explored and proved

to be promising for drug and gene delivery (49–52). (9) Cellular imaging and biodistribution detection: Gadolinium (Ga), holmium (^{166}Ho) and technetium (^{99m}Tc) ions were trapped in fullerene cages and investigated in the applications of cellular imaging and biodistribution detection (53–56). These findings exhibited promising features of C_{60} and derivatives and warrant their further beneficial applications in orthopaedic research.

Applications of C_{60} and derivatives in orthopaedic research

C_{60} and derivatives in cartilage degeneration treatment

How to improve the recruitment of chondrocytes derived from progenitor cells is always a big challenge in cartilage degeneration therapy regarding the loss of functional chondrocytes. Tsuchiya et al. (57) were the first group to explore the influence of C_{60} on cellular chondrogenic differentiation. In a micromass culture model *in vitro*, they found that water-soluble C_{60} strongly promoted the chondrogenesis of primary embryonic limb bud cells in proportion to the compound concentrations with alcian blue staining. Since it was reported that the enzymes responsible for chondroitin sulfate synthesis are concentrated in both rough and smooth endoplasmic reticulum (58), and C_{60} was documented to be incorporated into lipid membranes (59), also, highly charged polyanionic substances markedly stimulated the synthesis of proteoglycan (60), the authors proposed two possible mechanisms for their findings: in cellular reticulum, (1) C_{60} works as polyanionic substance featured with the π-electron-rich surface in itself; (2) C_{60} concentrates the polyanionic substances such as chondroitin sulfate and the polyanionic substances promote the synthesis of proteoglycan. However, the authors did not discuss the anti-oxidant property of C_{60} which may potentially function as another mechanism to enhance cellular chondrogenic differentiation.

The further application of C_{60} to prevent cartilage degeneration was explored by a Japanese group (12). In the *in vitro* study, it was shown that under interleukin-1 β (IL-1 β) or H_2O_2 induction, water-soluble C_{60} down-regulated cellular production of matrix-degrading enzymes in chondrocytes from patients with osteoarthritis. At the same time, C_{60} dramatically enhanced the biosynthesis of proteoglycan and collagen type II, as well as decreased cellular apoptosis and senescence under catabolic stress. In the *in vivo* study, they found that intra-articular administration of C_{60} prevented the progression of cartilage degeneration in an osteoarthritis rabbit model with a dose-dependent manner. The protective effects of C_{60} were potentially attributed to its free radical scavenging property. As has been documented the induced production of ROS is associated with inflammation in varieties of cells (61–63). ROS can also lead to a proinflammatory state and an imbalance of catabolic and anabolic activities in articular cartilage. Since under certain pathological conditions, endogenous anti-oxidants are not sufficient to inactivate excess free radicals (64), it is a novel strategy to suppress the inflammation with administration of extrinsic anti-oxidants, such as C_{60}. Also, the authors suggested that the injected C_{60} worked as a ''molecular bearing'' with superlubricity (65) to coat, lubricate and protect the joint cartilage function. Therefore, administration of C_{60} might have a distinct therapeutic value as a strategy to prevent cartilage degeneration.

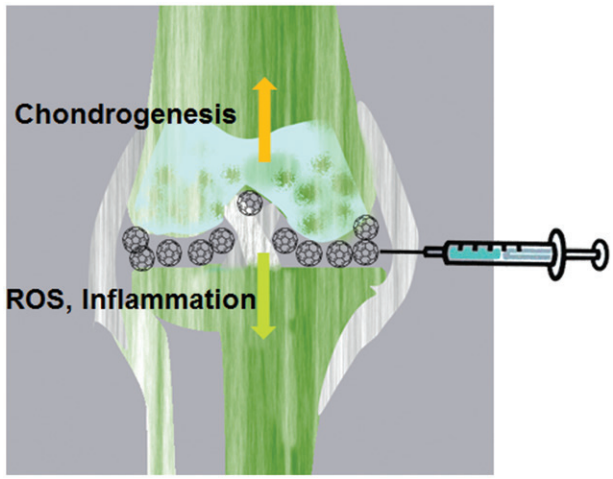


Figure 3. The strategy to treat cartilage degeneration with C_{60} and derivatives. C_{60} and derivatives enhance cellular chondrogenesis working as polyanionic substances and polyanionic substance concentrators, suppress cartilage inflammation by scavenging free radicals, and promote joint lubricity as a ''molecular bearing''.

The strategy to treat cartilage degeneration with C_{60} and derivatives is shown in Figure 3.

C_{60} and derivatives in bone destruction therapy

The use of glucosteroid has been shown to have some side effects on osteonecrosis and bone loss in which progression oxidative stress is implicated. Inhibiting the stress may be a promising strategy to solve this issue. It was shown that administration of an anti-oxidant vitamin E decreased the incidence of corticosteroid-induced osteonecrosis in a rabbit model (66). Liu et al. (67) went further in this field with the application of a more stable anti-oxidant fullerol. Their *in vitro* study showed that fullerol nanoparticles inhibited adipogenesis and simultaneously enhanced osteogenesis in a bone marrow mesenchymal stem cell line under dexamethasone induction. Further *in vivo* studies are needed to confirm the osteogenesis-enhancing potentials of fullerol as a therapeutic agent for glucosteroid-induced osteonecrosis treatment.

The hyper-resorption of bone by osteoclasts is another reason for bone destruction. Yudoh et al. (13) found that water-soluble C_{60} prohibited the differentiation of precursor cells into osteoclasts and osteoclastic resorption *in vitro* through inhibition of receptor activator of NFκB (RANK)-RANK ligand (RANKL) signaling pathway by direct removal of ROS as well as suppressing the production of proinflammatory cytokines. Furthermore, in their adjuvant-induced arthritic bone resorption rat model, intra-articular injection of C_{60} significantly inhibited local inflammation and joint destruction. Therefore, C_{60} showed another novel feature from the other side to prevent bone destruction by osteoclastic suppression and inflammation inhibition.

Figure 4 shows the strategy to treat bone destruction with C_{60} and derivatives by both promoting osteogenesis and suppressing osteoclastogenesis.

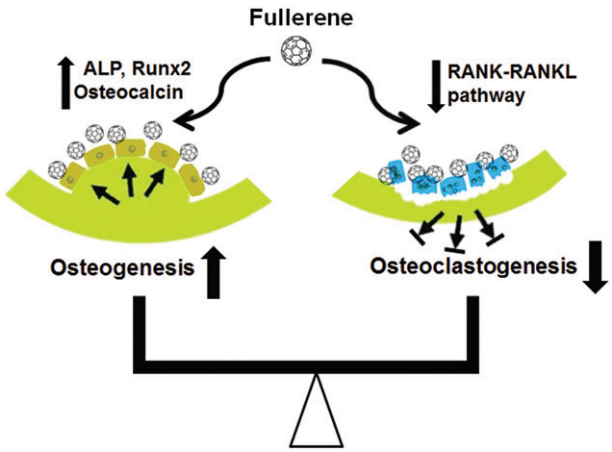


Figure 4. The strategy to treat bone destruction with C_{60} and derivatives. C_{60} and derivatives enhance osteogenesis by increasing osteogenic gene expression, and suppress osteoclastogenesis through inhibition of receptor activator of NFκB (RANK)-RANK ligand (RANKL) signaling pathway by direct removal of ROS as well as suppressing the production of proinflammatory cytokines.

Osteoporosis is a systemic skeletal disorder characterized by reduced bone mineral density, microarchitectural deterioration of the skeleton and increased risk of fracture (68). The traditional agents for osteoporosis treatment, such as bisphosphonate drugs and fluorine anion (N_aF), are either not efficiently absorbed in the gastrointestinal tract or fairly toxic, if orally administered (6). Thus, the vectored pharmaceuticals targeted at destructive bone tissue may be promising in this field. As has been reported, functionalized with diphosphonate groups or amide bisphosphonate and multiple hydroxyl groups, the C_{60} derivatives $C_{60}[C(PO_3H_2)_2]_2$ (69) and $C_{60}(OH)_{16}AMBP$ (70) conferred a strong affinity to the calcium phosphate mineral hydroxyapatite of bone. Therefore, the C_{60}-based bone tissue targeted compound cores demonstrated a promising prospect to be conjugated with traditional bone promotion agents for osteoporosis treatment.

C_{60} and derivatives in IVDD therapy

Symptomatic IVDD is strongly implicated as a cause of low back pain (71,72), which is one of the most common clinical conditions associated with musculoskeletal disorders, resulting in tremendous socioeconomic burden (73,74). There is growing evidence indicating that mitochondrial-derived ROS play a causal role in driving changes linked to IVDD. The oxidative stress caused damage accumulates in degenerative discs. Free radical scavengers have been shown to play a potentially important role in preventing IVDD (75). Also, the degenerative discs exhibit higher proinflammatory cytokine levels (e.g. TNF-α, IL-1 β, IL-6, IL-8, IL-12, IL-17 and others) versus non-degenerative discs (76–78). Suppression of disc tissue inflammation is another strategy to prevent IVDD. C_{60} and derivatives may be recruited in this strategy due to their anti-oxidant and anti-inflammatory features. Recently, we performed a pilot study to investigate the therapeutic effects of fullerol on nucleus pulpsus (NP) cells under inflammatory induction and annulus fibrosus puncture-induced disc degeneration in a rabbit model (manuscript under review). We found that fullerol effectively reversed the matrix degradation of NP cells under either H_2O_2 or IL-1 β induction, and the intradiscal injection of fullerol prevented IVDD by increasing water and proteoglycan content as well as by inhibiting ectopic bone formation.

C_{60} and derivatives in vertebral bone marrow fatty degeneration and inflammatory edema treatment

As disc has a very close relationship with vertebral bone marrow and they are separated by a thin layer of endplate (79), IVDD is associated with the lesions of vertebral bone marrow as well. Fatty marrow replacement and inflammatory edema in IVDD can be detected by MRI with Modic Type II changes (80). Also, mature discs almost totally rely on diffusion of essential solutes through the marrow contact channels in the vertebral endplate for nutrition and metabolic exchange (79,81). The focal fatty marrow conversion from normal red hemopoietic bone marrow (82) might obstruct the nutrient transport from bone marrow to endplate. Moreover, the growth of fat cells and inflammatory edema in the rigid intraosseous compartment can increase pressure and compress vessels and further decrease blood flow (83,84). Therefore, we speculated that inhibition of inflammatory mediators and adipogenesis of vertebral bone marrow stromal cells (vBMSCs) may retard the progression of IVDD. Our study showed that fullerol suppressed ROS and inflammatory cytokine production under IL-1 β induction, inhibited the adipogenic differentiation of vBMSCs *in vitro* and, therefore, may prevent vertebral fatty marrow deposition and inflammatory responses during IVDD (85). Furthermore, it was reported that the intravenously injected water-soluble C_{60} could penetrate the blood–brain barrier (86). We thus hypothesize that fullerol may also pass through the marrow contact channels across the vertebral endplate to reach disc tissue. If it is proved to be true, the local injection of fullerol into vertebral bodies can be beneficial to both prevent vertebral bone marrow inflammation and fatty degeneration as well as directly prevent disc tissue degeneration. The *in vivo* study is currently underway in our lab.

C_{60} and derivatives in radiculopathy treatment

IVDD can also cause spinal nerve root inflammation which is in association with back pain. The ingrowth of nociceptive neural fiber into deeper parts of the degenerative disc is considered as one of the most widely accepted pathomechanisms related to chronic discogenic pain (87). As the IVDD proceeds, disc inflammation may promote axonal growth of afferent fibers from the dorsal root ganglia (DRG) (88) to innervate the disc by secreting proinflammatory mediators. The pain signal could be triggered as the neurons of the DRG transmit the inflammatory signal through the spinal cord to the pain centers of the brain (89). Thus, relieving the inflammatory tension of the DRG would be of great significance to treat low back pain caused by IVDD. In our *in vitro* study, we revealed that fullerol treatment suppressed the inflammatory responses of DRG and neuronal apoptosis by decreasing the level of ROS and potentially enhancing anti-oxidative enzyme expression (90). Also, Huang et al. (91)

prepared a new C_{60} hybrid bearing thalidomide as a potential double-action anti-inflammatory agent, and found it was capable of simultaneous inhibition of LPS-induced NO and TNF-α production. Thus, the C_{60} core coupled with functional groups shows novel features to prevent inflammation. Taken together, C_{60} and derivatives have great potentials to serve as ROS scavenger and inflammation reliever for radiculopathy and low back pain treatment.

Figure 5 shows the general strategy to treat IVDD, vertebral bone marrow lesion and radiculopathy, with C_{60} and derivatives, against low back pain.

To summarize, C_{60} and derivatives showed promising potentials for application in orthopaedic research (Table 1).

Toxicity of C_{60} and derivatives

The toxicity assessment of C_{60} and derivatives is an absolute and obvious prerequisite for their potential use in biomedicine, and a complete knowledge of the underlying mechanisms is necessary for designing an efficient therapeutic strategy for their alleviation. Although C_{60} is a ''free radical scavenger'', the delocalized π double bonds of the fullerene cage can absorb energy from light to efficiently produce an excited triplet state and, through energy and electron transfer to molecular oxygen, produce both singlet molecular oxygen and superoxide which may injure cells (92). The balance between ROS scavenging and generation mediates its cytoprotection or photo-cytotoxicity on cells. Also, C_{60} may cause damage to the plasma and nuclear membranes as well as cellular organelles due to the potentials to form aqueous aggregates of C_{60} nanoparticles (nC_{60}). Furthermore, the preparation methods for aqueous C_{60} may also affect its cytotoxicity caused by solvent residues (93). For example, ROS have been detected in aqueous $nC_{60(solvent/tetrahydrofuran (THF))}$ preparations (94,95), and Zhang et al. (96) demonstrated that $nC_{60(solvent/THF)}$ preparations contained oxidizing agents (THF degradation products) that explained ROS activity. Moreover, the introduced functional groups into the C_{60} molecule may also yield cytotoxicity.

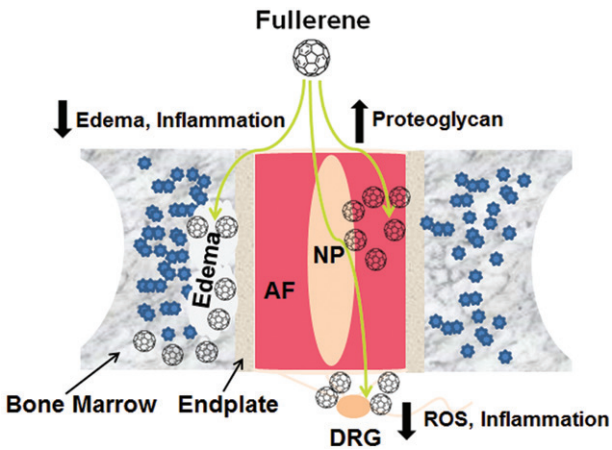


Figure 5. The general strategy to treat IVDD, vertebral bone marrow lesion and radiculopathy, with C_{60} and derivatives, against low back pain. The ''free radical sponge'' C_{60} and derivatives suppress disc tissue inflammation, promote disc tissue regeneration, prevent vertebral bone marrow edema and fatty degeneration and inhibit DRG inflammatory responses under high oxidative stress.

As has been reported, with 48 h exposure to C_{60}, the LC_{50} for human dermal fibroblasts is 20 μg/L (97), while it drops down to 2 μg/L for neuronal human astrocytes (95). It was documented that a maximal dose (100 μg/ml, about 88.7 μM) of fullerol-induced cytotoxic injury on human endothelial cells (98) and was cytotoxic to human lens epithelial cells at concentrations higher than 20 μM (92). While Sayes et al. (97) explored the differential cytotoxicity of water-soluble fullerenes and reported that $C_{60}(OH)_{24}$ with inhibitory property for aggregation significantly enhanced its biocompatibility toward both human dermal fibroblasts and liver carcinoma cells compared with C_{60}. In our group, we performed both lactate dehydrogenase and WST-1 assays to appraise fullerol's cytotoxicity on mouse vBMSCs and human NP cells. The results from the study indicated that 1 μM fullerol had little cytotoxicity for up to 7 days of *in vitro* culture, while 10 μM fullerol demonstrated statistically significant cytotoxicity. It shows that the toxicity of C_{60} and derivatives depends on the targeted cell and tissue types as well as the administered compound doses.

Since C_{60} derivatives have been reported to be widely distributed in all tissues (56), the toxicokinetics of these compounds after *in vivo* administration deserve critical attention. It has been pointed out C_{60} and derivatives have long biological half-life within the exposed animals (5). The relatively long biological half-life raises concern about bioaccumulation and long-term effects. Mori et al. (99) revealed that orally administered C_{60} and C_{70} at a dose level

Table 1. Applications of C_{60} and derivatives in orthopaedic research.

Fullerene types	Biomedical actions	References
C_{60}	Chondrogenesis promotion via working as polyanionic substance or concentrating polyanionic substances; cartilage degeneration prevention via free radical scavenging potential and superlubricity property	(12,57)
Fullerol, C_{60}, $C_{60}[C(PO_3H_2)_2]_2$, $C_{60}(OH)_{16}$AMBP	Bone destruction treatment via osteogenesis enhancing, osteoclastic suppression and inflammation inhibition, due to ROS scavenging potential, or osteoporosis prevention by conjugating the fullerene core with traditional bone promotion agents through targeting therapy strategy	(13,67,69–70)
Fullerol	IVDD prevention via matrix degradation inhibition, water and proteoglycan content promotion, as well as ectopic bone formation prevention in disc tissue	Manuscript under review
Fullerol	Vertebral bone marrow lesion treatment via ROS and inflammatory cytokine suppression, as well as adipogenesis prevention of vBMSCs	(85)
Fullerol	Radiculopathy treatment via suppressing the inflammatory responses of DRG and neuronal apoptosis by decreasing the level of ROS and potentially enhancing anti-oxidative enzyme expression	(90)

of 2000 mg/kg did not cause toxicity to rats within 15-day observation. Genotoxicity was not found either. In experiments studying subchronic exposure to C_{60}, administration of the compounds for up to 24 weeks did not result in the formation of either benign or malignant skin tumors in mice (100). However, for critical safety sake, long-term evaluation of different animals administered with different doses is highly required. Furthermore, even though it was considered in general, the acute oral, dermal and airway toxicity is low (5), the administration routes should also be fully compared and appraised.

Future directions

Growth factors, such as bone morphogenetic protein 2 (101,102), 4 (103,104), 6 (105,106), growth and differentiation factor 5 (107,108), transforming growth factor-β (109–112), insulin-like growth factor 1 (113,114) and platelet-derived growth factor (115,116), have been extensively investigated and shown beneficial effects in orthopaedic research. However, their applications were significantly limited by the relatively short half-life of the factors due to the enzymatic degradation under a biologically environmental condition. As the therapeutic potentials of C_{60} have been expanded by linking with a variety of functional moieties such as peptides (117), oligonucleotides (118), porphyrins (119), flavonoids (120), etc., we hypothesized that linking C_{60} with growth factors is another way to develop its potential as a therapeutic agent against musculoskeletal disorders. Right now the biggest challenge is to screen out the most feasible hydrophilic linkers which connect growth factors and C_{60} core well and do not affect the beneficial effects of either side.

Nanotechnology, concerning C_{60} and derivatives, has shown promising prospect due to the unique features of these nanoparticles: the solubility and stability can be greatly enhanced; the drug delivery targets, as well as the efficacy and safety of the delivery strategy can be modified by coupling the core with functional groups; feasibilities of continuous and stable monitoring *in situ*; resistance to immunoreaction; non-biodegradability (121). However, hypersensitivity, unexpected changes in pharmacokinetic behaviors, possible reactions with tissues, as well as possible accumulation in the body, need to be extensively investigated.

Conclusions

C_{60} and derivatives have shown successful applications in intensive biomedical research due to their unparalleled physical and chemical properties. The current research hotspots are centered on compounds highly purifying, surface modification with functional groups, linking with desirable molecules for targeted or designated pharmacotherapy. For further orthopaedic research, especially *in vivo* applications, the pharmacokinetics and toxicology of these agents as local/systemic administration need to be carefully determined.

Declaration of interest

We thank the financial supports from NIH (R21AR57512) and NASS. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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