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Lycium barbarum polysaccharides and ferroptosis: jumping into the era of novel regulated cell death

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Lycium barbarum polysaccharides (LBP) are the key bioactive components of *Lycium barbarum* (also named *Gouqizi* or *Goji* berry or wolfberry), a widely used Traditional Chinese herb for more than 2000 years. Believed to balance "*yin*" and "*yang*" within the body, *Lycium barbarum* is consumed for general health benefits. Besides, it also "nourishes" the eyes, kidneys, lungs, and liver. Indeed, *Lycium barbarum* has been considered as a "superfruit", an inexpensive supplement for many oxidative stress-related diseases. It is currently included in the Pharmacopoeia of the People's Republic of China.

Over the years, there have been numerous investigations using modern methodologies and technologies on *Lycium barbarum*. In particular, research has focused on LBP, the bioactive constituents of *Lycium barbarum*. Besides acidic heteropolysaccharides and polypeptides, LBP also contains 6 monosaccharides (arabinose, galactose, glucose, mannose, rhamnose, and xylose) as well as galacturonic acid and 18 amino acids. All these together in LBP bring about the broad range of pharmacological activities exhibited by *Lycium barbarum*, which include anti-aging, anti-cancer, anti-fibrotic, anti-inflammatory, anti-oxidative, immunomodulatory, and neuroprotective effects.

LBP is well known to be an anti-oxidant. It salvages cells from oxidative stress-induced cell death, many of which include chondrocytes, lens epithelial cells, myocardiocytes, neurons, retinal ganglion cells, retinal pigment epithelial cells, and trabecular meshwork cells as well as cell lines such as PC-12 and SH-SY5Y cells. Apart from reducing cell death as a result of oxidative stress, LBP also rescues cells by its anti-apoptotic properties in bone marrow mononuclear cells, corneal epithelial cells, gastric mucosal cells, hepatocytes, Leydig cells, photoreceptor cells, etc. On the other hand, LBP can also be pro-apoptotic; it induces cancer cell apoptosis by activation of pro-apoptotic caspases in bladder, breast, cervical, liver, and prostate cancer cells to name a few. Despite the conflicting roles in apoptosis, all evidence points to the involvement of LBP in regulated cell death.

Regulated cell death (RCD) (Galluzzi et al., 2018) is a molecularly controlled critical process in both plant and animal growth and development. It also allows the elimination of cells in response to stress and serves a fundamental role in the regulation of homeostasis and diseases. The two classical forms of RCD are apoptosis and autophagy.

The term apoptosis was first proposed by Kerr, Wyllie and Currie in 1972 (Kerr et al., 1972). It is the first-discovered form of RCD, observed during embryonic development, normal cell turnover in healthy adult tissue, and atrophy upon hormone withdrawal (Kerr et al., 1972). It is an active regulated caspase-mediated event with the following characteristic morphological changes: cytoplasmic and nuclear condensation, chromatin cleavage, formation of apoptotic bodies, and intact plasma membrane. Caspases are cysteine proteases expressed as inactive precursor enzymes. Based on their physiologic roles and substrate specificities, caspases are classified into two main groups, those involved in apoptosis (caspase-2, -3, -6, -7, -8, -9, and -10) and those related to caspase-1 (caspase-1, -4, -5, -13, and -14, as well as murine caspase-11 and -12). The first group is involved in cytokine processing while the latter takes part in proinflammatory cell death. Therefore, inflammation is not elicited during the process of apoptotic cell death. Apoptosis can be triggered by numerous death-inducing stimuli. When dysregulated, it can lead to diseases that range from cancer (uncontrolled cell proliferation) to neurodegeneration (unanticipated cell death). Extensive research on mechanisms and functions of apoptosis over the past 50 years has yielded invaluable now-textbook information on mechanisms and functions of apoptosis.

Autophagy is another form of classical RCD. The term autophagy was first used by Deter and de Duve (1967). There are three types of autophagy: macro-autophagy, micro-autophagy, and chaperone-mediated autophagy (Glick et al., 2010). Over the past 15 years, macro-autophagy (or autophagy) reclaimed research interests due to its proposed role in disease. It is a lysosome-mediated degradative pathway, active at basal levels but highly induced in response to external stressful stimuli such as starvation or hypoxia to enhance survival. Here, cytoplasmic materials are sequestered into autophagosomes, which then fuse with lysosomes to form autolysosomes where contents are degraded. Vacuolization and slight chromatin condensation are observed, after which autophagic cells are phagocytosed. Again, similar to apoptosis, the sequence of morphological and biochemical changes in autophagy is highly regulated. Given both the physiological and pathophysiological roles of autophagy, the disruption of autophagy can lead to diseases.

As in apoptosis, LBP's role in autophagy is conflicting. LBP modulates autophagy; it has been tested in many cell types and experimental paradigms. These include HeLa cells, Leydig cells, MC3T3-E1 cells, macrophage, microglia, PC12 cells, retinal ganglion cells, spinal cord neurons, SH-SY5Y cells, primary cultured hippocampal neurons, and human cutaneous squamous cell carcinoma A431 cells. LBP has also been shown to be beneficial in various experimental models such as colorectal cancer, diabetic peripheral neuropathy, diabetic testicular dysfunction, ethanol- or drug-induced gastric ulcer, hyperglycemia-aggravated ischemic brain injury, male infertility caused by obesity, non-alcoholic steatohepatitis, obstructive sleep apnea, and partial optic nerve injury among others. In these studies, both induction and inhibition of autophagy by LBP are observed and reported. Indeed, autophagy remains controversial from the perspectives of cancer biologists. Yet, autophagy is more generally recognized to be a beneficial event as it promotes cell survival. There is no doubt that LBP is generally beneficial and protective in disease progression by modulating autophagy.

Besides apoptosis and autophagy, recent advances have identified novel RCD pathways; one of them is pyroptosis. Pyroptosis is newly discovered in 2001 (Cookson and Brennan, 2001) and is related to the formation of inflammasomes, multi-protein complexes of the innate immune system. The canonical type of pyroptosis is initiated by caspase-1 while human caspase-4, -5, or mouse caspase-11 launch the non-canonical inflammasome pathway. Both types of pyroptosis involve the cleavage of Gasdermin D to form the active pore-forming nitrogen-terminal domain of Gasdermin D, leading to cell membrane perforation, cell rupture, and cell lysis. More importantly, activated caspase-1 by the nitrogen-terminal domain of Gasdermin D cleaves the precursors of interleukin (IL)-1β and IL-18 to their respective active forms, IL-1β and IL-18, which are released out of the cell and elicit an inflammatory response. When pyroptosis happens, there are cell

swelling, membrane blebbing, cell rupture, and pore formation with pyroptotic bodies.

LBP is recently shown to play a direct role in suppressing pyroptosis. It inhibits NLRP3 inflammasome in hyperoxia-induced acute lung injury and $A\beta_{1-40}$ oligomers-induced retinal pigment epithelium damage (Hong et al., 2019; Yang et al., 2020). Despite the limited number of publications, this is new evidence to support the beneficial biological properties of LBP. This opened up a new area where more information on LBP's mechanisms of actions can be obtained.

Ferroptosis is the latest novel RCD pathway that has attracted overwhelmingly exponential attention recently; over 1000 papers have been published in the past 5 years. The term ferroptosis was first coined in 2012. As its name implied, ferroptosis is iron-dependent. It is characterized by intracellular iron (Fe^{2+}) overload and as a result of the Fenton reaction, accumulation of reactive oxygen species (Stockwell et al., 2020; Jiang et al., 2021; Tang et al., 2021). There is also simultaneous extensive lipid peroxidation. Necrosis-like morphological changes such as cytoplasmic swelling and reduced mitochondrial crista are observed. Ferroptosis involves cysteine and glutathione metabolism as well as glutathione-dependent phospholipid peroxidase GPX4, the central inhibitor of ferroptosis. Through the two major pathways, the extrinsic or transporter-dependent pathway and the intrinsic or enzyme-regulated pathway, ferroptosis when dysregulated impacts on various pathologies and diseases such as cancer, neurodegenerative diseases, stroke, nonalcoholic steatohepatitis, inflammatory bowel disease, chronic obstructive pulmonary disease, acute kidney injury, testicular dysfunction, heart problems, and ionizing radiation damage.

Despite the recent advances in ferroptosis research, there have not been any systematic reports associating LBP and ferroptosis except a few publications with indirect evidence. LBP has been shown to modulate the expression of GPX4 and GSH as well as lipid peroxidation (**Additional Table 1**). In cryopreserved murine two-cell embryos, LBP restored mitochondrial function by enhancing GPX4 expression while down-regulating the generation of reactive oxygen species (Yang et al., 2019). In a carbon tetrachloride-induced acute liver injury model, the significant increase in malondialdehyde indicated an activation of lipid peroxidation, which was effectively suppressed by LBP treatment (Xiao et al., 2012). Similar effects of LBP were also reported in aged mice (Li et al., 2007) and hepatic cadmium-induced oxidative stress in rats (Varoni et al., 2017). These studies together suggest a relatively strong association between LBP and ferroptosis; yet, whether and how the iron level was altered have not been reported. Since ferroptosis contributes to the death of retinal pigment epithelium and photoreceptors as well as various ischemic organ injuries, investigation on LBP's role in ferroptosis is warranted in light of its effects in "nourishing" the eyes, kidney, lungs, and liver.

We are entering a new era of ferroptosis and RCD research. A great deal of discoveries and breakthroughs on ferroptosis biology are waiting to be made and the field is expanding. To follow these footsteps and to synchronize with research tracks, there is a wealth of opportunities for Traditional Chinese herbs by intervening in ferroptosis-related pathways. In the near future, it is foreseeable that numerous animal models and pharmacological tools of ferroptosis will be employed. Here, Jiang et al. (2021) cautioned on important experimental issues and challenges that have to be considered. Nonetheless, research on LBP and ferroptosis will shed more light on LBP's mechanism of action in health and disease and in the development of effective therapies, supporting *Lycium barbarum*'s role as a "superfruit".

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Additional file:
Additional Table 1: *Indirect evidence suggesting an association between LBP and ferroptosis.*

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Additional Table 1 Indirect evidence suggesting an association between LBP and ferroptosis

Experimental paradigm	Effect on Iron level	Effect on ferroptosis-associated protein expression	Effect on oxidative stress	Effect on Lipid peroxidation	Effect on cell/mitochondria morphology	References
Age-related oxidative stress in aged mice	N/A	Decreased GSH-Px level	Increased SOD level and total antioxidant capacity	Decreased MDA level	N/A	Li et al., 2007
Carbon tetrachloride-induced acute liver injury	N/A	Increased GPX expression	Decreased and COX-2 level and increased Cu/Zn SOD expression	Decreased MDA level	Improved cell morphology	Xiao et al., 2012
Hepatic cadmium-induced oxidative stress in rats	N/A	Increased GSH	Increased SOD level	Decreased MDA level	N/A	Varoni et al., 2017
Previously-cryopreserved murine two-cell embryos	N/A	Increased GPX4 expression	Decreased ROS level and increased SOD1 expression	N/A	Improved cell morphology	Yang et al., 2019

COX-2: Cyclooxygenase-2; GPX: glutathione peroxidase; MDA: malondialdehyde; N/A: not applicable; ROS: reactive oxygen species; SOD: superoxidase dismutase.